Treatment of type 2 diabetes with antioxidant vitamin C therapy

by

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**ABBREVIATIONS**

6,6-D$_2$ glucose – deuterated glucose tracer; AA – ascorbic acid; Akt – protein kinase B; aPKC – atypical protein kinase C; BH$_4$ – tetrahydrobiopterin; Cold-Ginf – “cold” glucose infusion; DAG – diacylglycerol; DCF- 2’, 7’-dichlorofluorescein; DCFH- 2’,7’- dichlorofluorescin; DHA – dehydroascorbic acid; DXA – dual energy X-ray absorptiometry; EGP – endogenous glucose production; ELISA – enzyme-linked immunosorbent assay; eNOS – endothelial nitric oxide synthase; FFA – free fatty acid; FFM – fat-free mass; GIR – glucose infusion rate; GLUT-1 – glucose transporter 1; GLUT-4 – glucose transporter 4; Gpx – glutathione peroxidase; GS – glycogen synthase; GSH – glutathione; GSSG – glutathione disulfide; H$_2$O$_2$ – hydrogen peroxide; HbA1c – glycated haemoglobin; HO$^\cdot$ – hydroxyl radical; HOMA – homeostasis model assessment; Hot-Ginf – “hot” glucose infusion; HPLC – high performance liquid chromatography; IMCL - intramyocellular lipid; iNOS – inducible nitric oxide synthase; IRS-1 – insulin receptor substrate 1; JNK - c-jun N-terminal kinase; MAPK – mitogen activated protein kinase; MCR – metabolic clearance rate of glucose; MPE – moles percent excess; NADPH – nicotinamide adenine dinucleotide phosphate; NO – nitric oxide; NOGM – non oxidative glucose metabolism; NOS – nitric oxide synthase; nNOS – neuronal nitric oxide synthase; O$_2^\cdot$ – superoxide anion; ONOO$^-\cdot$ - peroxynitrite; PGC-1α - peroxisome proliferator-activated receptor coactivator 1; PKC – protein kinase C; PLA – placebo; Ra – rate of glucose appearance; Rd – rate of glucose disposal; RIA – radioimmunassay; RNS – reactive nitrogen species; ROO$^\cdot$ - lipid peroxide radical; ROS – reactive oxygen species; SOD – superoxide dismutase; SVCT2 - sodium-dependent vitamin C transporter 2; T2D – type 2 diabetes; TAA – total ascorbic acid; UA – uric acid; WBGD – whole body glucose disposal; VC – vitamin C
ABSTRACT

Background

Oxidative stress has been implicated in the etiology of skeletal muscle insulin resistance in type 2 diabetes (T2D). Therapies that target skeletal muscle oxidative stress might therefore promote improvements in insulin action at this pivotal site of dysfunction in people with T2D. High dose antioxidant vitamin C supplementation has been shown in some previous studies to improve whole body glucose disposal and glucose control in people with T2D. However, mechanisms of action through which vitamin C improves insulin action have not been well elucidated in vivo. Furthermore, there is a paucity of data reporting on the bioavailability of high dose oral vitamin C in human skeletal muscle. Thus, there is a need to investigate the effects of high dose oral vitamin C supplementation on its transport into, and augmentation of levels in, skeletal muscle, as a basis to its potential antioxidant actions in skeletal muscle.

The aims of this thesis were therefore to firstly investigate the effects of chronic high dose vitamin C supplementation on skeletal muscle levels of vitamin C, protein expression of the vitamin C transporter sodium-dependent vitamin C transporter-2 (SVCT2), and oxidative stress in people with T2D. A further key aim was to generate data on the clinical efficacy of vitamin C supplementation as a means of improving skeletal muscle insulin sensitivity in people with T2D. It was hypothesized that in people with T2D long-term supplementation with high dose vitamin C supplementation would (a) increase skeletal muscle vitamin C concentration; (b) reduce SVCT2 protein abundance; (c) decrease markers of skeletal muscle oxidative stress; and (d) improve insulin-stimulated skeletal muscle glucose uptake.
Abstract

Methods

Study in people with T2D: Males (n=12) and females (n=1) with T2D (aged 57.9 ± 2.5 y [mean ± SEM] with BMI: 30.5 ± 0.8 kg/m²) commenced a randomized, placebo-controlled cross-over clinical trial involving four months of twice daily supplementation with vitamin C (VC; 2 x 500 mg ascorbic acid/day) or placebo (PLA). Participants demonstrated stable metabolic control (HbA1c: 7.6 ± 0.2 %) prior to the trial and were on oral anti-hyperglycaemic medications including Metformin, Sulfonylureas and/or DPP-4 inhibitors. Insulin sensitivity was assessed using a hyperinsulinaemic, euglycaemic clamp coupled with infusion of a stable glucose tracer (6, 6-D₂ glucose). Participants underwent muscle and blood sampling at pre- and post- supplementation timepoints.

Study in healthy individuals: Healthy males (n=8, aged 25.9 ± 2.3 y) completed a randomized placebo-controlled, cross-over trial involving supplementation with ascorbic acid (2 x 500 mg/day) or placebo over 42 days. Participants underwent muscle and blood sampling on days 0, 1, 7 and 42 during each treatment.

Skeletal muscle biopsies were measured for concentrations of vitamin C, SVCT2 protein expression and oxidative stress/redox markers that included \(2', 7'-\)dichlorofluorescin (DCFH) oxidation and superoxide dismutase (SOD) activity. In the study in people with T2D, additional oxidative stress markers that included the ratio of reduced glutathione to oxidized glutathione disulphide (GSH/GSSG) and \(F_2\)-Isoprostanes; as well as nitric oxide synthase (NOS) and dehydroascorbic acid (DHA) were also investigated in skeletal muscle samples.
Results

Study in people with T2D: Seven participants (6 males, 1 female, aged 59.4 ± 3.5 y) completed the trial in full compliance. VC supplementation significantly increased skeletal muscle total and reduced ascorbic acid content (VC: 22.3 ± 1.4 to 29.9 ± 1.9 μg/g vs. PLA: 23.1 ± 1.3 to 24.2 ± 1.1 μg/g, p=0.017 and VC: 6.5 ± 0.9 to 10.7 ± 1.4 μg/g vs. PLA: 6.0 ± 0.7 to 6.3 ± 1.1 μg/g, p=0.017, respectively). Insulin-stimulated rate of glucose disappearance (ΔRd) also improved significantly following VC (VC: 3.5 ± 4.1 to 14.0 ± 3.8 μmol/kg FFM/min vs. PLA: 5.7 ± 3.1 to 8.9 ± 3.2 μmol/kg FFM/min, p=0.009). Furthermore, VC supplementation reduced insulin-stimulated muscle DCFH oxidation (VC: 31.7 % decrease [mean per cent change from baseline] vs. PLA: 16.2 % increase, p=0.012). Neither basal measures of oxidative stress nor DHA concentration in skeletal muscle were significantly altered by supplementation. Contrary to the original hypothesis, VC supplementation increased protein abundance of SVCT2 (VC: 120.4 % increase vs. PLA: 11.0 % increase, p=0.008) in skeletal muscle. VC also increased protein abundance of eNOS (VC: 71.3 % increase vs. PLA: 12.6 % decrease, p=0.022), but decreased total SOD activity relative to placebo (VC: 10.1 % decrease vs. PLA: 11.6 % increase, p=0.006) in skeletal muscle. VC had no significant effect on total NOS activity, HbA1c or endogenous glucose production.

Study in healthy individuals: VC supplementation significantly increased skeletal muscle VC concentration after 7 days, which was maintained at 42 days (VC: 30.2 ± 2.4 to 38.8 ± 3.6 μg/g versus PLA: 30.9 ± 2.7 to 29.3 ± 1.8 μg/g, p=0.001). VC supplementation significantly increased skeletal muscle SVCT2 protein expression (main treatment effect p=0.006); although it did not alter levels of oxidative stress or redox markers in skeletal muscle.
Conclusion

In summary, chronic high dose VC supplementation significantly increased skeletal muscle vitamin C concentration and SVCT2 protein expression in both healthy individuals and people with T2D. Furthermore, chronic high dose vitamin C supplementation improved levels of insulin-stimulated glucose disposal and oxidative stress in skeletal muscle of people with T2D. A higher vitamin C concentration and lower levels of reactive species in skeletal muscle after supplementation implicate enhanced antioxidant scavenging by vitamin C in the improved skeletal muscle insulin sensitivity. Results of this thesis provide novel insights into effects of vitamin C supplementation in healthy and diabetic skeletal muscle in vivo. Moreover, findings of this thesis provide evidence for the clinical efficacy and safety of regular, high dose vitamin C supplementation as a potential adjunct therapy to improve skeletal muscle insulin resistance in people with T2D.
1.0. LITERATURE REVIEW

1.1. Type 2 diabetes

1.1.1. Epidemiology and background

The prevalence of diabetes has reached epidemic proportions worldwide (1), with estimates of global prevalence in adults predicted to increase from 382 million people in 2013 to 592 million people in 2035 (1). Of those individuals affected with diabetes, approximately 90-95 % have Type 2 diabetes (T2D) (2). T2D is associated with obesity (3) and is a major risk factor for coronary heart disease and stroke (4). People with T2D are also at an increased risk of blindness, kidney failure and limb amputation due to microvascular complications including retinopathy, nephropathy and neuropathies (4). The Australian Diabetes, Obesity and Lifestyle Study (AusDiab) reported that 7.4 % percent of Australian adults had known or newly diagnosed diabetes. Moreover, an additional 16.4 % of study participants were found to have impaired glucose metabolism as characterized by impaired fasting glucose or impaired glucose tolerance (5).

T2D and impaired glucose metabolism are diagnosed based on abnormally elevated blood glucose levels (Figure 1.1). A frank diagnosis of diabetes is made when a random plasma glucose level of ≥11.1 mmol/l or a fasting glucose level of ≥7 mmol/l accompanies obvious symptoms such as thirst or polyuria (6). Diagnosis of diabetes can also be confirmed via an oral 2h glucose tolerance test (OGTT) procedure (6). Hyperglycaemia in T2D is a result of insulin resistance in normally insulin-sensitive peripheral tissues (e.g. skeletal muscle, adipose tissue) combined with insufficient insulin secretion from pancreatic β-cells (6) and an impaired capacity of glucose to
regulate its own uptake and production (7). Additionally, insulin resistance in the liver can promote an abnormal production of glucose, further contributing to hyperglycaemia postprandially (8) and postabsorptively in poorly controlled type 2 diabetes (9).

**Figure 1.1.** Diagnosis of diabetes and impaired glucose metabolism

Diabetes is diagnosed when obvious symptoms exist in addition to a fasting plasma glucose (FPG) of $\geq 7$ mmol/l or a random plasma glucose (RPG) of $\geq 11.1$ mmol/l. If initial screening indicates a FPG between 5.5-6.9 mmol/l, an oral glucose tolerance test (OGTT) should be followed up to test for diabetes (glucose $\geq 11.1$ mmol/l). If the diagnosis is impaired fasting glucose (IFG) or impaired glucose tolerance (IGT), testing for diabetes using OGTT should be repeated annually (from (6)).
Insulin resistance in tissues could be evident several decades before the clinical manifestation of T2D (10). During the pre-diabetic stage, insulin-secreting pancreatic β-cells can compensate for the insulin resistance by increasing the production and secretion of insulin, thus enabling maintenance of glucose homeostasis (11). However, persistent hyperinsulinaemia eventually leads to β-cell failure, impaired insulin secretion, and clinical diabetes (11).

Causes of T2D are not completely understood (3). T2D is widely believed to be a multifactorial disease with genetic and environmental determinants (3). Concordance in monozygotic twins has been reported to be between 69-90 % in cross-sectional and prospective studies (12). Although genetic predisposition plays an important role in the etiology and manifestation of T2D, environmental factors might be required for the disease to become overt in genetically susceptible individuals (13). Large observational studies, metabolic studies and clinical trials strongly implicate poor diet, obesity and physical inactivity as contributing factors in the growing diabetes epidemic (2).

1.1.2. Treatment approaches for glucose control in T2D

Improving blood glucose control is the cornerstone of treatment in T2D in order to prevent the development and progression of complications and to improve quality of life (14). National evidence guidelines for treatment typically indicate a progression from lifestyle modifications (i.e. weight loss, exercise & diet control) to increasing pharmacological options based on individual circumstances (14) (Figure 1.2). Although weight loss and exercise regimens are modestly effective for weight loss and glucose control in T2D (15), they are limited by long-term adherence to programs
by patients (16). Commonly Metformin (an insulin sensitizer) and/or a sulfonylurea (an insulin secretagogue) are indicated as initial pharmacological treatments in T2D (14); however other individualized strategies incorporating other drugs with different mechanisms of action are often adopted.

**Figure 1.2.** An algorithm of treatment for improved glucose control in T2D

*Treatment of impaired glucose control in T2D often progresses from lifestyle modifications to Metformin treatment to more advanced individualized pharmacological approaches (from (14)).*
The use of pharmacological treatments in T2D has been associated with undesirable side effects including oedema and weight gain from use of thiazolodinediones, insulin and sulfonylureas (17, 18), hypoglycaemia from use of insulin and sulfonylureas (17); and gastrointestinal discomfort from use of GLP-1 agonists and acarbose (19, 20). Moreover use of some thiazolodinediones has been linked to an increased risk of cardiovascular disease, morbidity, heart failure and bone fractures in people with T2D (21-23). Considering the aforementioned side effects of current diabetes treatments, the pursuit of effective new treatment therapies with minimal side effects and a solid scientific basis is warranted.

1.1.3. Summary and significance #1

T2D is a major chronic disease with an incidence growing substantially in Australia and worldwide. Underlying its disease incidence is a strong genetic susceptibility coupled with modern environmental drivers of poor diet and physical inactivity. Treatments aimed at improving glucose control in people with T2D typically progress from lifestyle modifications to individualized pharmacological strategies. However traditional approaches involving lifestyle modifications and pharmacological treatments are limited by poor compliance and widespread side effects, respectively. This highlights the need for new effective therapies possessing sound scientific bases and safety profiles for use in the treatment of T2D.
1.2. Insulin resistance

1.2.1. The importance of skeletal muscle

Insulin resistance can be defined clinically as “the inability of a known quantity of exogenous or endogenous insulin to increase glucose uptake and utilization in an individual as much as it does in a normal population” (24). Skeletal muscle is quantitatively the most important tissue in peripheral insulin resistance, accounting for approximately 75-85% of whole-body insulin-stimulated glucose disposal during a hyperinsulinaemic, euglycaemic clamp (25-27). In T2D, impairments in skeletal muscle glucose disposal account for approximately 80% of the defective insulin mediated whole-body glucose disposal in vivo (28) (Figure 1.3).

![Figure 1.3. Defect of skeletal muscle glucose uptake in T2D](image)

Estimated contribution of skeletal muscle (shaded area of column) to whole body glucose disposal in controls (left) versus people with T2D (right) after physiological hyperinsulinaemia (From (28))
1.2.2. Insulin signaling in skeletal muscle glucose metabolism

Following the consumption of a carbohydrate-containing meal or exogenous glucose infusion, an increased blood glucose level signals the pancreas to increase insulin production, thus facilitating an increased delivery of insulin to peripheral tissues. In skeletal muscle cells, insulin binds to its receptor on the cell membrane, subsequently stimulating pathways of glucose transport, glucose oxidation and glycogen synthesis (Figure 1.4).

The binding of insulin to its receptor leads to a cascade of intracellular phosphorylation reactions on key tyrosine residues of signalling components. The phosphorylated insulin receptor recruits and phosphorylates insulin receptor substrate-1 (IRS-1), which in turn interacts with and activates the signalling molecule phosphatidylinositol -3- kinase (PI3-K) (29, 30). PI3-K catalyses the formation of phosphatidylinositol 3, 4, 5-triphosphate (PIP3) in the cell membrane, which acts as an allosteric regulator of phosphoinositide-dependent kinase 1 (PDK1) (31). PDK1 is required for the phosphorylation and activation of Akt, a phosphoinositide-dependent serine/threonine kinase pivotal in several of insulin’s actions (31). Specifically, Akt is activated by phosphorylation at Thr\(^{308}\) by PDK1 and at Ser\(^{473}\) (31) by mTOR (Mammalian target of Rapamycin) complexed with RICTOR (32). Akt phosphorylates and inhibits glycogen synthase kinase 3 (GSK3) which results in activation of glycogen synthase (GS) and glycogen synthesis (33). GS can also be activated allosterically by glucose-6-phosphate (G-6-P) as a result of a “feed-forward” response to increased glucose transport (34). More recently, it has been proposed that a PI3K-dependent muscle-specific protein phosphatase (PP1G.M) can directly dephosphorylate and activate GS in response to insulin (33, 35, 36) (Figure 1.4).
Insulin-mediated translocation of glucose transporter GLUT4 to the plasma membrane facilitates increased glucose transport into muscle, although pathways underlying this mechanism are complex and relatively poorly understood (37). Akt-dependent glucose uptake might be linked to the phosphorylation of the 160 kDa protein, AS160 (38). AS160 has several candidate Akt phosphorylation sites and a GAP domain selective for small GTP-binding Rab proteins which, when active, are required for GLUT4 translocation to occur (38). It has been proposed that under insulin-stimulated conditions, phosphorylation of AS160 renders the GAP domain inactive and relieves the inhibitory basal effect of AS160 on Rab proteins, consequently resulting in trafficking of GLUT4 to the cell membrane and glucose transport into muscle (37, 38). In addition, activation of PIP3 downstream effector atypical protein kinase C (aPKC) is also believed to be important in insulin-mediated glucose transport; although mechanisms of its involvement are unclear (39).
**Figure 1.4.** Skeletal muscle insulin-stimulated glucose transport and glycogen synthesis

The phosphorylated insulin receptor (IR) recruits and phosphorylates IRS-1, which in turn interacts with and activates the signalling molecule PI3-K. PI3-K catalyses the formation of PIP3 in the cell membrane, regulating the activity of PDK1. PDK1 is required in the phosphorylation and activation of Akt, which promotes inhibition of GSK3, activation of GS, and glycogen synthesis. GS can also be activated allosterically by G-6-P and possibly via a muscle-specific PI3-K dependent phosphatase. Insulin-mediated glucose transport via GLUT4 might be linked to phosphorylation of AS160 and activation of aPKC. Thicker arrows represent better defined pathways than thinner lines (adapted in part from (40)).
1.2.3. Impairments in skeletal muscle insulin signalling in T2D

Impaired skeletal muscle insulin-mediated glucose transport and glycogen synthesis are hallmark defects in T2D (33). Studies involving nuclear magnetic resonance have demonstrated that impaired glycogen synthesis in people with T2D is rate-limited by dysfunctional glucose transport into skeletal muscle (41). However, specific impairments within the insulin-signalling cascade underlying impaired glucose transport defects are not clear. Total basal GLUT4 protein levels do not appear to be altered in skeletal muscle of people with T2D (42-44); although insulin-mediated plasma membrane levels of GLUT-4 are reduced due to impaired membrane trafficking and translocation of GLUT4 (45-47). Impairments in insulin-mediated levels of activation, protein expression and/or gene expression pertaining to proximal insulin-signalling components such as IRS-1 and PI3-K have been reported in skeletal muscle of people with T2D (39, 48-50). While people with T2D appear to have impaired activation of aPKC (39, 51), impaired expression and/or activation of other key downstream components such as Akt are equivocal (33, 39, 49, 51-53). In addition, impaired glycogen synthesis in T2D has been associated with reduced activation of GS in some studies (54-58). Overall these findings suggest that the identity of the pivotal dysfunctional site(s) of the insulin signalling cascade in diabetic skeletal muscle glucose metabolism is uncertain.
1.2.4. Mechanisms of skeletal muscle insulin resistance in T2D

Increased lipid availability

Chronically elevated lipid levels might be a key underlying factor in skeletal muscle insulin resistance associated with T2D. Insulin resistance and T2D are associated with increased levels of circulating free fatty acids (FFA), intracellular long-chain acyl CoA esters and intramyocellular lipid (IMCL) stores (59-63). Moreover, increased IMCL levels in obesity and T2D were associated with increased transport of long chain fatty acids into skeletal muscle (62). The latter finding was observed despite an unaltered fat oxidation rate in these individuals, suggesting enhanced intramyocellular fat storage.

Chronic high fat feeding is known to cause skeletal muscle insulin resistance in experimental rodents (64). In addition, elevating levels of FFA via lipid infusion during hyperinsulinaemic, euglycaemic clamps induces insulin resistance in skeletal muscle of healthy people in as little as 3-6h (65, 66). In contrast, six months of calorie restriction with or without exercise was shown to decrease IMCL levels and increase insulin sensitivity in an overweight population (67).

Although increased levels of IMCL have been found in skeletal muscle of individuals with insulin resistance and T2D, increased IMCL levels have also been reported in highly trained endurance athletes who are highly insulin sensitive (68-70). Moreover, skeletal muscle oxidative capacity and mitochondrial function were found to be better predictors of insulin sensitivity than IMCL level in population groups ranging from well-trained athletes to individuals with T2D (71, 72). Thus, factors other than IMCL level might be important in promoting lipid-induced insulin resistance.
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The accumulation of lipid intermediates including ceramide and diacylglycerol (DAG) has been found to be associated with impaired skeletal muscle insulin signalling (59, 73) and insulin-stimulated glucose uptake (65). This inhibition of insulin signalling might occur via lipid intermediate-induced activation of protein kinase C (PKC) and PKC-θ, which can impair insulin signalling via serine/threonine phosphorylation of the insulin receptor and IRS-1 (65, 73). In addition, dietary fat-induced reactive oxygen species (ROS) production has been shown to activate the stress-sensitive molecules mitogen activated protein kinases (MAPK) (74), c-jun N-terminal kinase (JNK) (75), IκB kinase subunit β (IKK-β), NF-kappa B (NF-kB) transcription factor and PKC (76) in adipocytes and skeletal muscle. In particular, JNK can phosphorylate IRS-1 at serine residues, thus attenuating key metabolic pathways of the insulin signalling cascade in liver, adipose tissue and skeletal muscle (77).

Mitochondrial dysfunction

Mitochondrial biogenesis is a regulatory program involving inter-genomic control of mitochondrial turnover, functional content and number required for maintenance of various homeostatic demands (78). In skeletal muscle this involves intergenomic regulation by peroxisome proliferator-activated receptor coactivator 1 (PGC1α), nuclear respiratory factors 1 & 2 (NRF-1, NRF-2) and the mitochondrial transcription factor A. These molecular regulators facilitate the coordinate regulation of genes coding for complexes within the electron transport chain (78).

Mitochondrial dysfunction resulting from decreased mitochondrial biogenesis might impair normal lipid oxidation in skeletal muscle due to decreased mitochondrial content and functional capacity (79). With impaired lipid oxidation, dietary fat is
repartitioned away from oxidation to storage (62), and the production of toxic lipid intermediates is increased (80). The accumulation of ectopic IMCL and/or lipid intermediates such as DAG and ceramide might then increase activation of signalling molecules such as PKC and JNK, which can phosphorylate and inactivate important insulin signalling components and promote insulin resistance (78).

Genes regulating mitochondrial biogenesis including PGC1α and NRF-1; as well as uncoupling protein -3 (UCP-3; involved in protection of mitochondria from oxidative stress (81)) have been found to be downregulated in skeletal muscle of people with T2D (82-84). Moreover, treatment with the insulin sensitizer Rosligitazone or exercise training increases expression of these genes in association with improved insulin sensitivity in people with T2D (83, 85, 86). Insulin-stimulated ATP synthesis has also been found to be impaired in people with T2D (87, 88). Furthermore, gene and proteomic analyses in skeletal muscle of people with T2D revealed reduced expression levels of proteins involved in ATP synthesis and electron transport (82, 89, 90). Diminished in vivo mitochondrial function (using $^{31}$P-Magnetic resonance spectroscopy) and ex vivo mitochondrial respiration (using high-resolution respirometry) was also found in skeletal muscle of individuals with T2D (91-93).

Despite these findings of mitochondrial dysfunction, other studies have failed to report any alterations in mitochondrial content and/or function in people with T2D when compared to insulin-sensitive individuals (94-96). One possible confounding factor of studies is a failure to control for physical activity status of groups (97). It is well known that aerobic exercise training enhances mitochondrial biogenesis in skeletal muscle (98). Moreover, much evidence suggests that this adaptability is not impaired in individuals with T2D when matched for physical fitness, age and BMI (91, 92, 97, 99). Thus, due to the possibility of a reduced physical activity status of people
in T2D (100), it remains equivocal if mitochondrial biogenesis is intrinsically reduced in skeletal muscle in people with T2D. Furthermore, the likelihood of impaired lipid oxidatation arising from mitochondrial dysfunction has been challenged on grounds that the magnitude of reduced mitochondrial content reported in T2D (≈ 30 %) is not sufficient to impair fat oxidation, given the capacity of muscle to oxidize substrate far in excess of resting energy demands (101).

The role of mitochondrial dysfunction as a cause or effect in the pathophysiology of insulin resistance in skeletal muscle is a contentious issue. It was suggested in a recent review (78) that although genetic predisposition can promote an early manifestation of mitochondrial dysfunction in the course of insulin resistance, most evidence appears to indicate that mitochondrial dysfunction is a subsequent effect of early perturbations of insulin resistance such as nutrient excess, physical inactivity or increased ROS production (78, 102) (Figure 1.5).
**Figure 1.5.** Proposed role of the mitochondria in the development of insulin resistance

This schematic shows primary mitochondrial defects to the left of the hatched line in the center of the cell and the development of mitochondrial deficits in response to environmental cues and aging to the right of the hatched line. In primary disruption in the mitochondrial metabolic capacity, fat oxidation is diminished, fat intermediates accumulate and DAG, which appears to be the primary intermediate that then activates protein kinase C isoforms. These in turn phosphorylate and inactivate numerous kinase substrates in the insulin signaling pathway. The reduced insulin sensitivity exacerbates the metabolic perturbations by reducing glucose uptake and possibly by further down-regulation of the mitochondrial biogenesis program. The etiologies of primary mitochondrial defects are labeled 1–3. The etiologies of secondary disruption of mitochondrial dysfunction are labeled 4–7. High-fat diet can promote mitochondrial biogenesis; alternatively nutrient overload, which may include both glucose and fats, enhances both lipid intermediates that facilitate oxidative damage and impair insulin signaling. Furthermore, the nutrient overload presents excess reducing equivalents to the ETC that can result in increased ROS generation. The oxidative damage, in turn, disrupts the mitochondrial oxidative capacity, which then recapitulates the phenotype of primary mitochondrial deficits promoting insulin resistance (from (78)).
1.2.5. Summary and significance #2

Impaired insulin signalling in skeletal muscle is associated with insulin resistance in people with T2D. While impaired insulin-stimulated glucose transport and glycogen synthesis are key defects in skeletal muscle insulin resistance, it is unclear which (if any) specific insulin signalling component is pivotal in skeletal muscle insulin resistance in T2D. Furthermore, while increased lipid availability and mitochondrial dysfunction are associated with insulin resistance in skeletal muscle, their causative role in insulin resistance in T2D is equivocal.
1.3. Reactive oxygen species

1.3.1. Background to ROS

Reactive oxygen species (ROS; Table 1.1) are highly reactive chemical byproducts of metabolism and cellular processes in the body. ROS are produced at multiple cellular sites including in the mitochondria during oxidative phosphorylation (103), in the endoplasmic reticulum in response to protein folding stresses (104), in membrane-bound NADPH oxidases (105), through xanthine oxidase (XO) activation (106) and via phospholipase A2-dependent processes (107).

Table 1.1. Main reactive oxygen species

<table>
<thead>
<tr>
<th>Name</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Singlet Oxygen</td>
<td>(^1\text{O}_2)</td>
</tr>
<tr>
<td>Superoxide Radical</td>
<td>(\cdot\text{O}_2^-)</td>
</tr>
<tr>
<td>Hydroxyl Radical</td>
<td>('\text{OH})</td>
</tr>
<tr>
<td>Hydrogen Peroxide</td>
<td>(\text{H}_2\text{O}_2)</td>
</tr>
<tr>
<td>Peroxide Radical (lipid peroxide)</td>
<td>(\text{ROO}')</td>
</tr>
<tr>
<td>Aloxyl Radical</td>
<td>(\text{RO}')</td>
</tr>
<tr>
<td>Hypochlorous acid</td>
<td>(\text{HOCl})</td>
</tr>
</tbody>
</table>

(adapted from (10))

In basal conditions, ROS produced in mitochondria constitute a major source of cellular ROS (103). During oxidative phosphorylation, reducing equivalents (NADH, FADH\(_2\)) formed during energy metabolism provide electrons to be transferred along a series of electron carriers within the mitochondrial respiratory chain. The transfer of electrons generates increased membrane potential, increased oxygen consumption and ultimately the conversion of ADP to ATP (108). However, the process is not perfect and a small percentage (≈0.15%) of electrons leak from
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respiratory chain complexes (I & III), resulting in production of ROS including superoxide (\(\cdot \text{O}_2^-\)) (109). \(\cdot \text{O}_2^-\) is modestly reactive and can oxidize several biomolecules, notably dehydratases containing [4Fe-4s] clusters such as aconitase in the citric acid cycle (110) (Figure 1.6). \(\cdot \text{O}_2^-\) can also be converted into another reactive species, peroxynitrite (ONOO\(^-\)), which is highly reactive to DNA, proteins and lipids (111). However, \(\cdot \text{O}_2^-\) is relatively short-lived and is converted into the more stable hydrogen peroxide (\(\text{H}_2\text{O}_2\)) by endogenous antioxidant enzymes including manganese superoxide dismutase (MnSOD) and copper/zinc superoxide dismutase (Cu/ZnSOD). \(\text{H}_2\text{O}_2\) in turn can oxidize several biomolecules including protein kinases and phosphatases (112) and protein thiols (113). \(\text{H}_2\text{O}_2\) is decomposed to oxygen and water by cellular antioxidant enzymes including catalase, glutathione peroxidise (GPx) and thioredoxin peroxidase. However, in the presence of free metals such as iron and copper, \(\text{H}_2\text{O}_2\) can be converted into the highly reactive hydroxyl radical (\(\cdot \text{OH}\)) (111).

Interestingly, ROS have been implicated as both physiologically beneficial and pathologically harmful in the body. ROS have been shown to act as important intracellular signalling molecules for insulin signalling transduction in healthy tissues (114, 115). Additionally, exercise is known to increase levels of ROS in skeletal muscle and other tissues (116). ROS produced during exercise, which is primarily of non-mitochondrial origin (117), are also thought to play key roles in skeletal muscle adaptations associated with exercise training (118).

In contrast to this physiologically beneficial ROS, a chronic over-production of ROS systemically and in skeletal muscle promotes oxidative stress and might contribute to the pathogenesis of T2D (102, 119), cancer cachexia (120), and sarcopenia (121). Excess ROS produced during metabolic and cellular processes can oxidatively damage
macromolecules including DNA, lipids and proteins, modify cellular redox status, and alter cellular functions (10). Furthermore, by acting as signalling molecules (64), excess ROS might act as important causative secondary messengers in the impaired insulin signalling pathways associated with insulin resistance in T2D (108).

**Figure 1.6.** The cellular origins of ROS, their targets, and antioxidant systems

ETC, electron transfer chain; ER, endoplasmic reticulum; NADP(H), nicotinamide adenine dinucleotide phosphate; MnSOD, manganese superoxide dismutase; Cu/ZnSOD, copper/zinc superoxide dismutase; GPx, glutathione peroxidase; Prx, peroxiredoxin; Trx, thioredoxin (from (111)).
1.3.2. ROS: the good and the bad in skeletal muscle insulin sensitivity

Different experimental models have linked excess ROS production with the development of skeletal muscle insulin resistance. These models include (a) excess ROS production by mitochondria in experimental murine models of excess nutrient intake and T2D (64, 102, 122, 123); (b) excess ROS production by NADPH oxidases due to overactivity of the renin-angiotensin system and increased Angiotensin II levels (124); and more recently (c) excess ROS production by XO in experimental diabetes models (125).

In particular, the relationship between increased mitochondrial ROS and skeletal muscle insulin resistance has been well established in vitro and in vivo (64, 119, 122, 126). In humans it was demonstrated that both acute and chronic high fat dietary intakes can increase mitochondrial H$_2$O$_2$ production and oxidative stress in skeletal muscle of healthy, insulin-sensitive individuals (64). Moreover, obese, insulin-resistant humans have increased skeletal muscle mitochondrial H$_2$O$_2$ emission compared with lean insulin-sensitive individuals (64, 127). Hoehn et al. (126) demonstrated an increased mitochondrial ·O$_2^- \cdot$ production in palmitate-treated L6-myotubes in vitro and in high fat-fed experimental mice in vivo. Furthermore, approaches aimed at the selective quenching of mitochondrial ·O$_2^- \cdot$ including the genetic overexpression of MnSOD or supplementation with the mitochondrial ·O$_2^- \cdot$ targeted antioxidant Mn(III)–tetrakis (4-benzoic acid) porphyrin were shown to improve or prevent skeletal muscle insulin resistance in the high fat fed mice (126). In addition, Anderson et al. (64) employed the use of both a small antioxidant peptide (SS31) targeted to the mitochondrial inner membrane and the genetic overexpression of mitochondrial-targeted human catalase (MCAT) in experimental
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rats and mice respectively, fed high fat diets. All treatments resulted in a reduction of mitochondrial H$_2$O$_2$ emission by >50%. Moreover, chronic intake of SS31 or genetic over-expression of MCAT prevented the onset of high-fat-diet-induced insulin resistance in skeletal muscle in the experimental animals (64). Together, these and other studies (119) implicate excess mitochondrial ROS as both a causative factor in skeletal muscle insulin resistance and a key target for antioxidant-based prevention and treatment strategies.

In contrast to these findings, two recent studies (122, 123) found no benefit of mitochondrial-targeted antioxidant treatments in C57BL/6 mice for improving skeletal muscle insulin resistance despite marked reductions in mitochondrial ROS and improvements in skeletal muscle oxidative stress levels. These findings thus raise the possibility that targeting elevated mitochondrial ROS levels associated with skeletal muscle insulin resistance might not in fact be a fruitful treatment approach for improving insulin sensitivity in disease states (such as T2D) characterized by skeletal muscle insulin resistance.

Pathways through which ROS might impair insulin signalling have not been well defined, but ROS have been shown to activate stress-sensitive molecules including MAPK (74) and JNK (75). As previously discussed, JNK can phosphorylate the IRS subunit of the insulin signalling cascade at serine residues, thus attenuating key metabolic pathways of the insulin signalling cascade (77). Moreover, antioxidant supplementation in rats was shown to inhibit high-fat diet-induced activation of JNK and IRS-1 serine phosphorylation in skeletal muscle, thus preventing a reduction in insulin sensitivity (128). Mechanisms through which ROS activate JNK in tissues are unclear (129). However, it has been shown that ROS can activate JNK via oxidation and inactivation of specific JNK-inactivating phosphatases (129). Furthermore, it has
been shown that TNFα-induced mitochondrial ROS can activate JNK through activation of the apoptosis signal-regulating kinase (ASK-1) in human hepatoma cells (130).

In contrast to potentially harmful excess mitochondrial ROS production, the generation of ROS from non-mitochondrial sources has been shown to be important in relation to healthy insulin signalling in insulin sensitive tissues (105, 131). In particular, H₂O₂ production by plasma membrane bound NADPH oxidases has been shown to increase in response to insulin stimulation both in adipocytes and myotubes in vitro (105, 131). A recent study also implicated XO as a key ROS generator in response to insulin stimulation in skeletal muscle (106). Increased insulin-stimulated H₂O₂ in skeletal muscle might occur via a PI3-K and/or PKC induced calcium release mechanism of action in vitro (131). Moreover, H₂O₂ has been shown to oxidatively modify and inactivate key protein tyrosine phosphatases (PTEN & PTP1B) in adipocytes and myotubes, thus resulting in enhanced insulin signalling (105, 131). Although this physiological H₂O₂ appears to be primarily of NADPH oxidase (105, 131) or XO (106) origin, it was demonstrated that levels of H₂O₂, PI3-K/Akt pathway activation and insulin sensitivity were increased in skeletal muscle of high-fat-fed transgenic mice lacking the antioxidant enzyme GPx (GPx⁻/⁻) (115). Moreover, treatment of GPx⁻/⁻ mice with the antioxidant n-acetyl cysteine increased insulin resistance in skeletal muscle (115). GPx is expressed both in the mitochondria and cytosol (132), thus implying the possible involvement of mitochondrial ROS in maintenance of normal insulin sensitivity also. The seemingly paradoxical role of excess ROS in physiological versus pathological states in skeletal muscle could be a function of magnitude, duration and/or cellular origin of ROS produced (106).
1.3.3. Linking mitochondrial ROS to mitochondrial dysfunction in T2D

Excess mitochondrial ROS production in diabetic and nutrient overloaded insulin-resistant animal models has been linked to mitochondrial dysfunction in skeletal muscle (102, 122). Moreover, treatments with general or mitochondrial-specific antioxidants were shown to normalize altered mitochondrial content (as characterized by altered citrate synthase activity) in high-fat, insulin resistant C57BL/6 mice (102, 122, 123). In humans, mitochondria isolated from obese, insulin resistant individuals was found to possess decreased content (as characterized by reduced total protein and citrate synthase activity levels) compared with mitochondria from lean, insulin-sensitive individuals, despite a capacity for increased ROS production (127). It has been proposed that increased ROS-induced damage to mitochondrial DNA, lipids and proteins could impair mitochondrial biogenesis and mitochondrial DNA replication (102). In particular, damage to mitochondrial DNA could impair genes and proteins linked with energy metabolism (104). In turn, reduced mitochondrial content and altered lipid oxidation (133) could promote ectopic lipid deposition and propagation of a lipid-/lipid intermediate- induced decrease in skeletal muscle insulin sensitivity (134, 135). Presently the relationships between insulin resistance, excess mitochondrial ROS and mitochondrial dysfunction and their temporal order in the pathophysiology of skeletal muscle insulin resistance is uncertain (78, 122) and remain highly controversial (for more information readers are referred to recent arguments by Holloszy (136) and Goodpaster (135)).
1.3.4. Do people with T2D have increased levels of ROS in skeletal muscle?

Studies that have directly measured ROS production in human T2D skeletal muscle have been scant, limited methodologically by ex vivo measurement techniques, and have produced uncertain findings (97, 137, 138). Abdul-Ghani et al. (137) reported no difference in overall mitochondrial ROS production in skeletal muscle of people with T2D versus healthy controls, although the individuals with T2D had an increased mitochondrial H$_2$O$_2$ generation per unit of ATP production. Given that ATP production is driven by cellular energy demand in vivo, these findings suggest an increased in vivo ROS production in skeletal muscle of people with T2D (137). Another study (97) reported a tendency for increased mitochondrial ROS production in individuals with T2D when compared with control participants matched for age, BMI, physical fitness and mitochondrial content. In contrast to these studies, Minet & Gaster (138) found reduced absolute mitochondrial H$_2$O$_2$ production in primary myotubes from individuals with T2D and no difference in the ratio of mitochondrial H$_2$O$_2$ production per unit of ATP production when compared to control participants. However, as discussed by the authors, different findings in the latter in vitro study compared to the previous studies could relate to the absence of in vivo metabolic conditions relevant to ROS production in T2D (138). Overall, these studies might also be limited by the accuracy of assays used to assess ROS production from isolated mitochondria. In particular, commonly used redox-sensitive probes such as Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) could be cross-reactive with lipid hydroperoxides and/or other redox-related metabolites (108, 139) and are prone to artifactual ROS formation (140). Thus a degree of caution is required in the interpretation of these findings (108).
In addition to direct measurements of ROS production, indirect oxidative stress measurements reflective of excess ROS including protein carbonyls and 8-hydroxy-2’-deoxyguanosine were shown to be increased in skeletal muscle of people with T2D when compared to non-diabetics (141, 142). In contrast to these findings, Brinkmann et al. (143) reported reduced levels of F2-Isoprostanes in skeletal muscle of people with T2D when compared with age and BMI-matched controls. With respect to the latter finding, the authors speculated that decreased muscle F2-Isoprostanes in the people with T2D might have arisen as a compensatory response to insulin resistance; such that total mitochondrial activity and thus mitochondrial ROS formation were reduced (143).

Oxidative stress is defined broadly as a suprathreshold imbalance between the production and scavenging of ROS by the body’s antioxidant defence network, favouring ROS production (64). Thus, a limitation of using these oxidative stress markers as indicators of ROS production is that decreased antioxidant levels could theoretically promote oxidative stress without a concomitant increase in ROS production. Studies investigating levels of antioxidant enzymes in skeletal muscle of people with T2D have produced mixed findings when compared to the levels of insulin sensitive controls. Protein levels of MnSOD were found to be reduced in skeletal muscle of individuals with T2D in some studies (141, 144) but not others (143). On the other hand, Brinkmann et al. (143) reported increased levels of peroxiredoxins (PRDX2 & PRDX6) but unaltered levels of GPx in skeletal muscle of people with T2D using immunohistochemical methods. Exercise training can alter levels of antioxidant enzymes (145). Therefore a lack of control for physical activity levels could complicate findings of antioxidant enzyme levels in people with T2D. Collectively, these findings suggest that, compared with healthy controls, the skeletal
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muscle antioxidant status in people with T2D is unclear and further studies are required that closely match age, body mass and physical activity levels.

A lack of comprehensive data on the oxidative stress milieu in skeletal muscle of people with T2D coupled with the absence of currently available accurate *in vivo* measurement techniques for the probing of ROS (108) makes it difficult to establish a clear conclusion on alterations in levels of ROS in skeletal muscle of people with T2D.

Finally, a proposed integrated model linking mechanisms of skeletal muscle insulin resistance is presented in Figure 1.7. Here, ROS are implicated as important signalling factors in establishing skeletal muscle insulin resistance. Excess ROS production also links increased lipid availability with mitochondrial dysfunction associated with skeletal muscle insulin resistance. Lastly, according to this model antioxidant treatment can target both insulin resistance and mitochondrial dysfunction in skeletal muscle associated with T2D (64, 102, 126).
Increased lipid availability
• ↑FFA; ↑Fatty-acyl CoA; ↑DAG & Ceramide; ↑IMCL

ROS
PKC/JNK/p38MAPK

Increased lipid availability
• ↑FFA; ↑Fatty-acyl CoA; ↑DAG & Ceramide; ↑IMCL

Genetic factors/aging/physical inactivity

Genetic factors

Impaired exercise tolerance

Impaired lipid oxidation

Figure 1.7. Vicious cycle linking lipid excess, insulin resistance and mitochondrial dysfunction in T2D

A proposed pivotal role of ROS in mediating these deleterious changes is highlighted, along with potential protection by antioxidants. Broken lines indicate uncertainty. Under conditions of high fat intake, obesity and in T2D, increased levels of IMCL and lipid intermediates in skeletal muscle promote impairments in insulin signalling and insulin-stimulated glucose disposal via activation of PKCs. Increased lipid availability such as through high fat intake also promotes increased electron transport flux and elevated mitochondrial ROS production, which impairs normal insulin signalling and promotes insulin resistance in skeletal muscle via JNK and p38 MAPK-signalling. Increased ROS in turn causes oxidative damage to the mitochondria, resulting in reduced mitochondrial oxidative capacity and mitochondrial dysfunction. Such a state can promote ectopic lipid storage due to either an overburden on mitochondrial β-oxidation or impaired mitochondrial lipid oxidation, thus worsening existing insulin resistance. These effects however might be ameliorated by treatment with antioxidants (adapted in part from (79)).
1.3.5. Summary and significance #3

At present only limited data exists on ROS levels, markers of oxidative stress and levels of antioxidants in skeletal muscle of people with T2D. Data in rodent models implicate oxidative stress in the causation of skeletal muscle insulin resistance; with antioxidant therapy reversing this defect. Studies looking at the potential ameliorative effects of antioxidant therapies on skeletal muscle ROS and oxidative stress in people with T2D are required. Moreover, studies investigating the association between changes in oxidative stress and improvements in insulin sensitivity in skeletal muscle following antioxidant therapy might offer some mechanistic insights into the importance of oxidative stress and its targeted therapy in people with T2D.

Finally, although mechanisms underlying insulin resistance in skeletal muscle remain contentious; increased ROS appears to play an important signalling role in mediating insulin resistance as well as linking increased lipid availability with mitochondrial dysfunction in insulin resistance.
1.4. Dietary antioxidants for prevention and treatment of T2D

1.4.1. Observational studies

Dietary intake of antioxidant vitamins has been associated with a reduced risk of developing T2D (146-150). Moreover, a recent meta-analysis of nine cohort studies of antioxidant intake and diabetes risk reported a pooled relative risk of 0.87 (95 %CI 0.79, 0.98) for the highest antioxidant intake versus the lowest intake (151). Despite such findings, these studies are limited by their observational designs, and may have been prone to confounding bias by residual dietary and/or lifestyle factors (152). Furthermore, the majority of these studies related prospective diabetes incidence to baseline dietary intake or baseline antioxidant levels, which was estimated decades before incidence was assessed. Changes in dietary antioxidant intake of individuals over this time might not have been accurately accounted for, thus potentially increasing exposure misclassification (153).

1.4.2. Large randomized controlled trials

In contrast to observational studies, evidence from large, long-term randomized controlled trials evaluating the effectiveness of antioxidant supplementation in primary or secondary disease prevention has not been supportive of benefits (152, 154-159). A recent meta-analysis of randomized controlled trials reported no protective effect of antioxidant supplementation on risk of all-cause mortality in primary and secondary prevention of various diseases (160). Moreover, after exclusion of “high-bias risk trials”, this meta-analysis reported an increased risk of all-cause mortality after supplementation with vitamin E, vitamin A and beta-carotene
Literature Review

(160). However, few of the studies included in the meta-analysis were of clear relevance to the treatment of people with T2D. Large scale antioxidant supplementation randomized controlled trials (RCTs) have been limited by factors including reduction of supplementation compliance over time (152, 159), a high-risk study population bias (156, 157), use of poly-antioxidant supplementation regimens rather than single antioxidant regimens (thus introducing potential interactive effects), a lack of strict dietary antioxidant control, and use of self-reported diabetes status (152, 156, 159). Moreover, assessment of oxidative stress was largely ignored in these studies. Thus it is unclear if dosage regimens used were effective enough to improve oxidative stress. Finally, most large scale studies appear to have lacked clear insight into the mode of action of the specific antioxidants used and their biological targets (161, 162), thus making interpretation of their findings difficult.

1.4.3. Randomized controlled trials for the treatment of insulin resistance in T2D

Studies of antioxidant vitamin supplementation in people with T2D have produced mixed results in terms of improving glycaemic control and insulin sensitivity. A recent meta-analysis of 14 intervention studies reported no effect of vitamin E or vitamin C supplementation in people with T2D on fasting glucose and fasting insulin levels (163). However a significant reduction in HbA1c following supplementation was reported ($p=0.0021$; mean difference, $-0.571\%$; 95\% CI $-0.935$, $-0.207$)(163). Notably, the number of studies selected for this meta-analysis was relatively small, and there was an absence of studies that used “gold-standard” glucose clamp methods to assess insulin sensitivity.
Literature Review

Findings from smaller well controlled studies that investigated effects of antioxidant treatments on insulin resistance in people with T2D and which used glucose clamp methods yielded more promising outcomes in terms of improving insulin resistance. In particular, oral or intravenous supplementation with alpha-lipoic acid (164-166), vitamin E (167), vitamin C (168, 169) and glutathione (170) was found to improve insulin sensitivity in people with T2D. Unfortunately a comparison between these studies is rather difficult based on their heterogeneous designs in relation to type, dose, duration and route of supplementation; as well as varying baseline participant characteristics.

1.4.4. Summary and significance #4

Despite consistent associations between dietary antioxidant intake and protection from T2D in observational studies, data from large randomized controlled trials is not generally supportive of benefits of antioxidant supplementation for disease prevention. However, many of these studies suffer from limitations such as inadequate compliance with supplementation, use of high risk population groups, and use of multi-compound antioxidants. Thus, concerns of internal validity of some of the larger studies are brought into question. Smaller, better controlled trials that have used “gold-standard” glucose clamp methods have produced more promising findings supporting antioxidants in the treatment of insulin resistance in T2D. However, it is difficult to arrive at a clear consensus of benefit given the small number and heterogeneity of trials. Future clinical studies should be designed with clear antioxidant-specific biological targets as outcomes as well as key clinical measurements.
1.5. Vitamin C for the treatment of T2D

1.5.1. Biochemistry and functions of vitamin C

Vitamin C is a six-carbon compound that exists in two active forms: in its reduced ascorbic acid (AA) form or in its oxidized dehydroascorbic acid (DHA) form (Figure 1.8). Vitamin C plays an important role in many biological reactions, and is an essential cofactor of numerous hydroxylation reactions (171). For instance, vitamin C acts as a cofactor for iron-dependent dioxygenases (e.g. prolyl & lysyl hydroxylases) which catalyze the hydroxylation of unfolded procollagen chains, leading to the formation of mature collagen (171-173). Among its other functions, vitamin C is also involved in the biosynthesis of catecholamines (174) and carnitine (175). Moreover, vitamin C is a potent water soluble antioxidant in cells and plasma, quenching ROS such as \( \cdot O_2^- \), \( 'OH \) and \( ROO' \) and reducing oxidative stress (176-182). Acting as an antioxidant, AA undergoes a single electron oxidation yielding the ascorbyl radical (Figure 1.8). At physiological pH the ascorbyl radical disproportionates either back to AA or it can donate another electron, forming DHA (183). The fate of DHA is either that of irreversible hydrolysis to diketogulonic acid or it can be reduced back to AA via enzymatic and non-enzymatic mechanisms (184) (Figure 1.9). Notably, glutathione and glutathione-dependent enzymes including glutaredoxins have been shown to be important in this recycling process (183, 185). NADPH-dependent DHA reductases including thioredoxin reductase are also involved in AA recycling (186).
**Figure 1.8.** Conversion between the active forms of vitamin C

Vitamin C in its reduced AA form can be oxidized to the ascorbyl radical, which in turn can be oxidized to DHA. Both the ascorbyl radical and DHA can be reduced back to AA (from (184)).

**Figure 1.9.** Cyclical redox reactions linked to the reduction of DHA to AA

Glutathione (GSH), ascorbic acid (AA), and vitamin E (Vit. E). The oxidized forms of these compounds, respectively, are glutathione disulfide (GSSG), ascorbyl radical (A-radical), dehydroascorbic acid (DHA), and vitamin E-radical (Vit. E radical). The reduction of GSSG requires NADPH as a cofactor (which is produced by cellular glucose metabolism) and is catalyzed by glutathione reductase (not shown). Also shown are several nodal sites where the oxidized forms of the compounds may be lost from the cycles: GSSG may exit from cells; DHA may be irreversibly degraded to diketo-L-gulonic acid; and Vit. E radical may be converted to the relatively nonreactive Vit. E quinone. (adapted from (185)).
1.5.3. Vitamin C nutrition and supplementation

Unlike most other mammals, humans are unable to produce vitamin C endogenously due to absence of the enzyme L-gulonolactone oxidase (187). Therefore sufficient dietary vitamin C must be consumed to prevent vitamin C deficiency and scurvy. Fruits and vegetables are generally good sources of vitamin C, collectively contributing the vast majority of dietary vitamin C intake in Australians (188). Nutrient reference values include a recommended dietary intake (RDI) of 45 mg/day for adult men and women, and a prudent upper limit of 1000 mg/day (189).

The vitamin C RDI in healthy adults might not be sufficient in people with T2D however, as oxidative stress could promote decreased cellular vitamin C transport and/or increased turnover of vitamin C (190). In fact, oxidative stress associated with T2D might partly explain the decreased plasma and leukocyte levels of vitamin C observed in people with T2D (168, 191-194) despite their apparently similar dietary vitamin C intakes compared with non-diabetics (195, 196).

High, supra-dietary doses of vitamin C (≥ 500 mg/day) are not associated with major adverse effects nor specific toxic endpoints (189). The most commonly reported mild adverse effects resulting from high dose oral vitamin C are gastrointestinal disturbances (189). Of potential concern is the formation of oxalate from AA that could crystallize in the urine, forming kidney stones in susceptible individuals (171). Some studies have reported modestly increased urinary oxalate with doses of 1-2 g/day in both stone-forming individuals and non-stone-forming individuals (197-200), leading to suggested recommendations of <2 g/day (197, 198) in individuals who are known stone formers. However, most studies in humans with normal renal function have not reported substantially elevated levels of urinary oxalate following high dose
vitamin C (189, 201). Finally, while supplementation with fat soluble dietary antioxidants including vitamin E and beta-carotene has been associated with an increased risk of morbidity and mortality in randomized controlled trials (160, 202), such negative effects were not found for high dose vitamin C (160). Given its safety profile and its relative deficiency in people with T2D, vitamin C could potentially be an ideal antioxidant treatment for safely targeting oxidative stress and improving insulin sensitivity in people with T2D.

1.5.4. Vitamin C supplementation trials in T2D: effects on insulin sensitivity, oxidative stress and glycaemic control

Randomized controlled trials that have evaluated the impact of vitamin C supplementation on insulin sensitivity, oxidative stress and/or glycaemic control in people with T2D are summarized in Table 1.2. Studies are limited in number and are heterogeneous in relation to factors such as vitamin C dosage, dosage schedule, duration of supplementation, baseline characteristics of participants, and specific measurements undertaken.

One notable study conducted was a placebo-controlled randomized trial in elderly people with T2D that involved four months of vitamin C supplementation (500mg twice daily) in a cross-over manner (169). The study reported significantly improved insulin-mediated whole body glucose disposal (WBGD) and non-oxidative glucose metabolism (NOGM; a semi quantitative estimation of glycogen synthesis rate) following supplementation. HbA1c, plasma cholesterol, triglycerides, fasting insulin and plasma levels of ·O₂⁻ were also found to be significantly improved following supplementation in this study.
From these summarized studies it appears that a threshold dose of 1 g vitamin C per day for ≥6 weeks might be required to elicit favourable outcomes in terms of improved insulin sensitivity, oxidative stress and/or glycaemic control in people with T2D (169, 181, 182, 203-211). With the exception of one study (211) no studies that employed supplementation regimens consisting of < 1 g vitamin C per day and/or durations of < 6 weeks found improvements in relation to systemic oxidative stress and/or glucose metabolism in people with T2D. It should be noted that some reviewed studies did not include a placebo group to evaluate longitudinal effects (207, 209, 210). Furthermore, while some studies in Table 1.2 evaluated markers of systemic oxidative stress (169, 181, 182, 204, 208, 212), with some studies finding improvements following supplementation (169, 181, 182); none of these studies investigated effects of vitamin C on levels of ROS or oxidative stress in skeletal muscle. Improvements in insulin sensitivity were reported in one study (169), but only two studies (169, 205) used “gold-standard” glucose clamp methods (213) to assess insulin sensitivity in people with T2D. Finally, there are no data shown in Table 1.2 for β-cell insulin secretory capacity of study participants. It remains possible that varying baseline insulin secretory capacities of participants and alterations in insulin secretory capacity in response to vitamin C supplementation potentially affected glycaemic control outcomes of the different studies.
### Table 1.2. Randomized trials investigating effects of vitamin C supplementation on insulin sensitivity, oxidative stress and glycaemic control in people with T2D

<table>
<thead>
<tr>
<th>Authors/Study design</th>
<th>Subjects</th>
<th>Subject data*</th>
<th>Duration</th>
<th>Main Measures</th>
<th>Main Findings**</th>
<th>Limitations</th>
<th>Δ VC (μmol/l) Baseline, Post</th>
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<tbody>
<tr>
<td>Ardekani &amp; Shojaiddiny-Ardekani (209) Parallel-group</td>
<td>N=84 (T2D) (41 F, 43 M) (aged: 52.3 ± 9.62 y) Groups: 1) 500 mg VC/day: n=41 2) 1 g VC/day: n=43</td>
<td>DD: 7.62 ± 5.74 y HbA1c: 8.82 ± 1.3 % Drugs: No insulin, no other drugs specified BMI: N/A BP: N/A TC: 198.3 ± 38.1 mg/dl TG: 210.0 ± 65.1 mg/dl Glu: 169.3 ± 34.0 mg/dl</td>
<td>6 weeks treatment: 500mg VC/day (2 x 250 mg) or 1 g VC/day (4 x 250 mg)</td>
<td>Fasting blood glucose, TC, TG, LDL, HDL, HbA1c, Insulin</td>
<td>1 g/day VC: ↓fasting glucose, ↓TG, ↓LDL-C, ↓HbA1c &amp; ↓insulin compared with baseline; no effects elicited with 500 mg/day VC</td>
<td>No placebo control group; not double-blinded; did not assess vitamin C levels</td>
<td>Not Assessed</td>
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<tr>
<td>Authors/Study design</td>
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<td>Bishop, Schorah &amp; Wales (203) Double-blind, cross-over, placebo-controlled</td>
<td>N=50 (n=38 T2D; n=12 T1D) [24 M, 26 W]; Groups: 1) VC/placebo n=25 (aged 51 [19-76] y); 2) Placebo/VC n=25 (aged 60 [33-76] y)</td>
<td>DD: N/A HbA1c: 9.6 ± 1.9 % Drugs: Metformin and/or sulfonylureas; anti-hypertensives BMI: N/A BP: N/A TC: 7.0 ± 1.2 mmol/l TG: 2.8 ± 1.5 mmol/l Glu: 7.5 ± 3.0 mmol/l</td>
<td>2-months treatment with 500mg/day Vitamin C &amp; 2-months placebo, no wash-out</td>
<td>Fasting glucose, cholesterol, TG, HbA1c. Plasma &amp; Leukocyte VC levels</td>
<td>No effect of VC on any clinical measures</td>
<td>Dose possibly too low; compliance not assessed; no wash-out period between treatment arms; included patients with complications &amp; both T1D &amp; T2D</td>
<td>In plasma: VC group: 41.6 ± 26.4, 92.5 ± 29.2 Placebo group: 41.6 ± 26.4, 44.6 ± 21.9 In leukocytes: (μg/10⁶ cells) VC group: 27.6 ± 9.1, 36.0 ± 8.7 Placebo group: 27.6 ± 9.1, 31.4 ± 8.6</td>
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<td>Chen et al. (205) Double-blind, placebo-controlled, parallel-group</td>
<td>N=32 (T2D) [13 M, 19 F]; Groups: 1) Placebo n=17 (aged 47 ± 3 y) 2) VC: n=15 (aged 49 ± 2 y)</td>
<td>DD: 4.3 ±1.1 y HbA1c: 7.5 ± 0.3 % Drugs: anti-hypertensives, anti-diabetics (no insulin) BMI: 34.1 ± 1 SBP: 140 ± 4 mmHg DBP: 77 ± 2mmHg TC: 202 ±11 mg/dl TG: 231 ± 31 mg/dl Glu: 156 ± 11 mg/dl</td>
<td>4 weeks treatment with 800 mg vitamin C/day or placebo</td>
<td>Plasma VC, fasting glucose, fasting insulin, forearm blood flow, insulin sensitivity (using isoglycaemic, hyperinsulinaemic clamp, QUICKI and HOMA)</td>
<td>No effect of VC on insulin sensitivity or clinical measures</td>
<td>Some potential compliance issues; short duration; dose possibly too low</td>
<td>In plasma: VC group: 23 ± 2, 48 ± 6 Placebo group: 21 ± 3, 19 ± 3</td>
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<td>Authors/Study design</td>
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<tr>
<td>Dakhale, Chaudhari &amp; Shrivastava (211) Double-blind, placebo-controlled, parallel group</td>
<td>N=66 (28M, 38F); Groups: 1). VC + metformin: n=33 (aged 48.3±1.4 y); 2). placebo + metformin: n=33 (aged 45.9±1.4 y)</td>
<td>DD: N/A HbA1c: 8.26 ±0.09 % Drugs: Metformin, no psychotropics BMI: N/A BP: N/A TC: N/A TG: N/A Glu: 8.7±0.2 mmol/l</td>
<td>12 weeks treatment with: 500mg VC + 500mg Metformin (both 2 x /day) or placebo + Metformin (both 2 x /day)</td>
<td>Fasting glucose, post-prandial glucose, HbA1c</td>
<td>VC: ↓ fasting &amp; post-prandial glucose &amp; ↓ HbA1c; Placebo: ↓ fasting &amp; post-prandial glucose (but not HbA1c); all changes greater in VC group vs. placebo</td>
<td>Metformin likely confounded findings; no measures of oxidative stress</td>
<td>In plasma: VC group: 14.8 ± 0.5, 25.6 ± 0.6 Placebo group: 13.6 ± 0.3, 15.3 ± 0.6</td>
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<td>Darko et al. (204) Double-blind, placebo-controlled, parallel-group</td>
<td>N=35 (T2D) (12 F, 23 M) Groups: 1). placebo: n=17 (aged 56.6 ± 1.2y) 2). VC: n=18 (aged 55.5 ± 1.8y)</td>
<td>DD: 7.8 ±0.6 y HbA1c: 7.5 ± 0.3 % Drugs: Metformin, Sulfonylureas, anti-hypertensives BMI: 27.9 ± 1.5 SBP: 141 ± 5 mmHg DBP: 80 ± 2 mmHg TC: 5.8 ± 0.3 mmol/l TG: 2.1 mmol/l Glu: 10.9 ± 0.9 mmol/l</td>
<td>3 weeks treatment with vitamin C (3 x 500 mg/day) or placebo</td>
<td>Blood pressure, 8-epi-PGF2α (oxidative stress marker), endothelial function (digital volume pulse, forearm blood flow: FBF), TC, HDL-C, fasting glucose, plasma VC</td>
<td>No effect of VC on any clinical measures</td>
<td>Duration of treatment possibly too short; only assessed one marker of oxidative stress; insulin sensitivity not assessed</td>
<td>In plasma: VC group: 58 ± 6, 122 ± 10 Placebo group: 51 ± 5, 53 ± 5</td>
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<td>Eriksson &amp; Kohvakka (210) Double blind, cross-over</td>
<td>N=56 [n=29 (T1D) n=27 (T2D)] (aged 61 ± 2y)</td>
<td>T2D subjects: DD: 10 ±1 y HbA1c: 9.3 ± 0.3 % Drugs: anti-hyperglycaemics, insulin, others not specified BMI: 28.9 ± 0.8 SBP: 149 ± 3 mmHg DBP: 87 ± 2mmHg TC: 6.2 ±0.2 mmol/l TG: 2.5± 0.2 mmol/l Glu: 10.1 ± 0.6 mmol/l</td>
<td>90 days treatment with magnesium (600 mg/day) &amp; 90 days with Vitamin C (1 g 2x/day), with a 4-week wash-out period and a 90-day run-in period</td>
<td>Systolic/Diastolic BP, fasting glucose,HbA1c, TC, TG</td>
<td>T2D subjects: VC: ↓fasting glucose, ↓HbA1c, ↓TC &amp; ↓TG; Magnesium supplementation had no effect</td>
<td>No placebo control group; did not assess vitamin C levels</td>
<td>Not assessed</td>
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<tr>
<td>Evans et al. (181) Double blind, placebo-controlled, parallel design</td>
<td>N=20 (T2D) (17 M, 3 W) Groups: 1). VC: n=10 (aged 52.7 ± 6.9 y) 2). placebo: n=10 (aged 53.6 ± 7.9 y)</td>
<td>DD: N/A HbA1c: 8.4 % Drugs: Metformin and/or Sulfonylureas BMI: 29.2 ± 4.8 SBP: 151 ±11 mmHg DBP: 85 ± 10 mmHg TC: 5.4 ± 0.7 mmol/l TG: 2.1 ± 0.3 mmol/l Glu: 8.4 ± 0.7 mmol/l</td>
<td>6 weeks with lispro (insulin) + 1 g VC/day or 6-weeks with lispro + placebo</td>
<td>HbA1c; Fasting and postprandial flow-mediated dilation (FMD), TG, cholesterol, insulin, glucose; plasma free radicals using electron paramagnetic resonance (EPR) spectroscopy &amp; lipid peroxidation (TBARS)</td>
<td>VC: ↓fasting &amp; post prandial plasma free radicals &amp; TBARS; No effect of VC on fasting glucose, cholesterol, insulin or HbA1c</td>
<td>No control group without insulin; insulin treatment might have interacted with vitamin C; study possibly underpowered for biochemical indices</td>
<td>Not assessed</td>
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<td>Authors/Study design</td>
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<td>Mazloom et al. (182)</td>
<td>Single blind, placebo-controlled, parallel group</td>
<td>N=30 (T2D) (22 W, 8 M)</td>
<td>DD: 4.57 ± 4.2 y</td>
<td>Fasting and postprandial TG, cholesterol &amp; plasma malondealdehyde (MDA; determined using TBARS)</td>
<td>VC: ↓ fasting &amp; postprandial plasma MDA; no effect of VC on other lipid measures</td>
<td>Single blind only; baseline MDA higher in VC vs. placebo; study possibly underpowered for lipid measures</td>
<td>Not Assessed</td>
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<td>Groups: 1). VC n=14 (aged 47±8.93 y) 2). placebo n=13 (aged 46.61 ± 7.58 y)</td>
<td>HbA1c: N/A</td>
<td>Drugs: Metformin and/or Sulfonylureas BMI: 26.94 ± 4.94 SBP: N/A DBP: N/A TC: 189.4 ± 38.1 mg/dl TG: 174.8±110.4 mg/dl Glu: 131.1±32.8 mg/dl</td>
<td>6 weeks of 1 g VC/day or placebo</td>
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<td>McAuliffe et al. (214)</td>
<td>Double-blind, placebo-controlled, parallel group</td>
<td>N=20 (n=18 T2D; n=2 T1D) (15 M, 5 F);</td>
<td>DD: 12 ± 11 y</td>
<td>No effect of VC on glycaemic control or lipid measures</td>
<td>Study possibly underpowered; half of subjects were taking insulin; included subjects with complications; baseline body-weight higher in VC group</td>
<td>In plasma: VC group: 40.0 (approx.), 84.3 ± 8.0 Placebo group: N/A (no significant change from baseline)</td>
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<td>Groups: 1). VC: n=10 (aged 58±12 y) 2). placebo n=10 (aged 58 ± 12 y)</td>
<td>HbA1c: 7.9 ±1.4 %</td>
<td>Drugs: anti-diabetics &amp; insulin BMI: 31.98 SBP: 147 ± 17mm Hg DBP: 84 ± 9mm Hg TC: N/A TG: N/A Glu: N/A</td>
<td>12 months of 500 mg VC 2x/day or placebo</td>
<td>Albumin excretion rate (AER), HbA1c, TG, TC, HDL-C, SBP &amp; DBP; plasma VC</td>
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<td>Authors/ Study design</td>
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<td>Mullan et al. (206)</td>
<td>N=30 (T2D) (22 M, 8 W) Groups: 1). placebo: n= 15 (aged 57.9 ± 6.6 y); 2). VC: n=15 (aged 61.0 ± 6.5 y)</td>
<td>DD: &lt;10 y HbA1c: 8.1 ± 1.3 % Drugs: Anti-hypertensives &amp; Anti-diabetics (no insulin) BMI: 28.6 ± 4.4 SBP: 142 ±12.6 mmHg DBP: 83.9 ± 4.8 mmHg TC: 5.2 ± 0.8 mmol/l TG: 2.2 ±1.1 mmol/l Glu: 9.3 ± 2.2 mmol/l</td>
<td>4-weeks treatment with 500 mg AA or placebo</td>
<td>Hemodynamic measures: blood pressure, pulse wave analysis; biochemical measures: HbA1c, plasma glucose, plasma insulin, TC, HDL-C, LDL-C, TG; plasma VC</td>
<td>No effect of VC on any biochemical measures</td>
<td>Participation by hypertensive diabetics; dose possibly too low; duration possibly too short</td>
<td>In plasma: VC group: 43.3 ± 19.3, 78.1 ± 19.5 Placebo group: N/A (no significant change from baseline)</td>
</tr>
<tr>
<td>Paolisso et al. (169)</td>
<td>N=40 (T2D) (19 M/21 F) (aged 72 ± 0.5 y)</td>
<td>DD: 8.1 ± 0.3 y Hba1c: 8.1 ± 0.4 % Drugs: Sulfonylureas BMI: 27.7 ± 0.3 SBP: 155 ± 0.6 mmHg DBP: 84 ± 0.4 mmHg TC: 7.2 ± 0.3 mmol/l TG: 2.61±10.09 mmol/l Glu: 8.8 ± 0.4 mmol/l</td>
<td>4 months treatment with 500 mg Vitamin C 2x/day or placebo, with a 30-day wash-out</td>
<td>Plasma VC, Fasting glucose, fasting insulin, cholesterol, TG, GSH, HbA1c, free radicals (O₂), glucose metabolism</td>
<td>↑GSH, ↓TC, ↓LDL-C, ↓TG, ↓HbA1c, ↓O₂, ↓insulin, ↑whole body glucose disposal, ↑non-oxidative glucose metabolism</td>
<td>Clamp data only from half of subjects; did not assess hepatic glucose output; findings might only be relevant to elderly adults</td>
<td>In plasma: VC group: 41.2 ± 5.4, 76.6 ± 3.7 Placebo group: 41.2 ± 5.4, 43.4 ± 2.8</td>
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<td>Authors/ Study design</td>
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<td>Main Findings**</td>
<td>Limitations</td>
<td>Δ VC (μmol/l) Baseline, Post</td>
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<tr>
<td>Tessier et al. (212) Double-blind, placebo-controlled, parallel group</td>
<td>( N = 36 ) (T2D) (28 W, 8 M) Groups: 1) 500 mg VC/day: ( n = 12 ) (aged 72 ± 5 y); 2) 1 g VC/day: ( n = 12 ) (aged 71 ± 4 y); 3) placebo: ( n = 12 ) (aged 72 ± 4 y) DD: 10.2 ± 9.4 y ( HbA1c: 7.4 ± 0.8 % ) Drugs: Metformin, Sulfonylureas, Insulin BMI: 29.5 ± 5.3 SBP: N/A DBP: N/A TC: 4.63 ± 0.76 mmol/l TG: 2.43 ± 0.83 mmol/l Glu: 8.3 ± 2.9 mmol/l</td>
<td>12 weeks of 500 mg VC/day or 1 g VC/day or placebo</td>
<td>Granulocyte levels of VC, vitamin E, GSH, GSSG, lipoprotein lipid peroxidation (TBARS) &amp; lipoprotein particle oxidation; fasting glycaemic &amp; lipid parameters</td>
<td>1 g VC/day: ↑ cellular VC (vs. baseline) &amp; ↑ vitamin E content of LDL; both 1g/day &amp; 500 mg/day: ↑ cellular GSH vs. placebo; no effect of VC on any other measures</td>
<td>Might only be relevant to elderly people with T2D; study might have lacked power for some measures; GSH/GSSG ratio not assessed</td>
<td>In granulocytes: (nmol/mg protein) 1g VC group: 1.45 ± 1.20, 5.66 ± 2.00 500 mg VC group: 3.36 ± 1.99, 5.39 ± 1.90 Placebo group: 2.42 ± 2.01, 2.72 ± 1.88</td>
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<tr>
<td>Toussouls et al. (207) Single blind, controlled, parallel group</td>
<td>( N = 41 ) (T2D) (22 M, 19 W) Groups: 1). Atorvastatin-treated: ( n = 15 ) (aged 58.1 ± 2.6 y) 2). VC-treated: ( n = 13 ) (59.1 ± 2.4 y) 3). Control: ( n = 13 ) (aged 60.9 ± 3.1 y) DD: 8.6 (0.9) y ( HbA1c: 6.29 (0.22) % ) Drugs: No insulin or statins BMI: 29.1 ± 1.1 SBP: N/A DBP: N/A TC: 4.9 ± 0.2 mmol/l TG: 1.7 (1.1-2.4) mmol/l Glu: 7.0 (6.2-8.7) mg/ml</td>
<td>4 weeks of daily 10 mg Atorvastatin (a statin drug) or 2 g vitamin C or control (no supplementati on)</td>
<td>Inflammation markers, hyperaemic forearm blood flow, vasodilatory response to post-ischaemic hyperaemia, cholesterol, TG, fasting glucose</td>
<td>No effect of VC on fasting glucose, TG &amp; cholesterol</td>
<td>No placebo control group; only single blind; possibly too short in duration; included smokers &amp; hypertensives; no measures of oxidative stress</td>
<td>Not assessed</td>
<td></td>
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<tr>
<td>Authors/Study design</td>
<td>Subjects</td>
<td>Subject data*</td>
<td>Duration</td>
<td>Main Measures</td>
<td>Main Findings**</td>
<td>Limitations</td>
<td>Δ VC (μmol/l) Baseline, Post</td>
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</table>
| Upritchard, Sutherland & Mann (208) Placebo-controlled, parallel-group | N=52 (T2D) (32M, 20 F); Groups: 1). VC: n=12 (aged 56±9 y); 2). Vitamin E: n=12 (aged 56 ± 14 y); 3). Tomato Juice: n=15 (aged 63±8 y); 4). Placebo: n=13 (aged 60 ± 6 y) | DD: 1.9 ± 1.3 y  
HbA1c: 6.7 ± 1.0  
Drugs: oral anti-diabetics; anti-hypertensives; aspirin  
BMI: 30.7 ± 6.3  
SBP: 78 ± 27mm Hg  
DBP: 123 ± 40mm Hg  
TC: 5.96 ± 1.16 mmol/l  
TG: 1.75 ± 1.64 mmol/l  
Glu: 8.7 ± 1.5 mmol/l | 4-weeks treatment with: 500mg VC/day or 800IU vitamin E/day or 500ml tomato juice/day or placebo (all groups: a 4-week placebo run-in period) | LDL-oxidation (lag time), C-RP, TC, HDL-C, LDL-C, HbA1c, plasma glucose, sVCAM-1, ICAM-1, α-tocopherol; plasma VC | No effect of VC on any measures | VC dose possibly too low; baseline TG lower in VC vs. other groups; ACE inhibitor drug biased in tomato juice group; study possibly underpowered; study not fully-blinded | In plasma: VC group: 37.7 ± 12.4, 64.7 ± 14.3  
Placebo group: 27.3 ± 15.4, 29.7 ± 21.1 |

(*vitamin C treated subject data; ** relevant to insulin sensitivity, glycaemic control, fasting glucose, fasting insulin & oxidative stress; DD - Diabetes duration; SBP – systolic blood pressure; DBP – diastolic blood pressure; TC – total cholesterol; LDL-C – low density lipoprotein cholesterol; HDL-C – high density lipoprotein cholesterol; TG – triglycerides; BMI – body mass index (in kg/m²); HbA1c – glycosylated haemoglobin; GSH – glutathione; GSSG – oxidized glutathione; HOMA - homeostasis model assessment; QUICKI - quantitative insulin sensitivity check index; VC – vitamin C; sVCAM-1 – soluble vascular cellular adhesion molecule-1; Glu – Fasting glucose; C-RP – C-reactive Protein; ICAM-1 – intercellular adhesion molecule 1; ACE – angiotensin-converting enzyme; TBARS – thiobarbituric acid reactive substances)
1.5.5. Glucose clamp and glucose tracer methods for measurement of insulin sensitivity in people with T2D

Glucose clamp procedures have been used comprehensively in the past to evaluate in vivo insulin sensitivity of people with T2D (215). The basic concept of the glucose clamp is to assess glucose metabolism at an integrated whole-body level when both insulin and glucose levels are kept constant (215). In relation to the commonly used hyperinsulinaemic euglycaemic clamp, plasma glucose concentrations are clamped at euglycaemic levels (normal non-diabetic fasting levels) through the infusion of insulin and variable amounts of glucose. This procedure can be further coupled with the infusion of glucose tracers to estimate the relative contributions of endogenous glucose production (EGP) and peripheral glucose disposal to whole-body glucose metabolism. Glucose tracers share identical kinetic characteristics to glucose, but are distinguishable from glucose for purposes of measurement (216). Several different types of tracer-based methodologies have been used in studies of people with T2D to evaluate peripheral glucose disposal and EGP (readers are referred to (216) for a comprehensive review).

The hot glucose infusion (Hot-Ginf) tracer technique contrasts to the cold glucose infusion (Cold-Ginf) tracer technique previously used in some vitamin C trials involving people with T2D (168, 169) by the additional enrichment of the variably infused glucose used to “clamp” plasma glucose with a small calculated amount of glucose tracer. The addition of tracer to the glucose infusate enables the maintenance of a constant level of plasma tracer enrichment during the clamp procedure. Moreover, the Hot-Ginf method minimizes errors associated with tracer compartmental gradients that can occur during the glucose clamp (217).
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hyperinsulinaemic, euglycaemic clamp, the infusion of non-labelled glucose (Cold-Ginf method) rapidly dilutes plasma tracer levels. This dilution of plasma glucose tracer is not reflective of tracer kinetics in the slower mixing interstitial glucose pool, in which concentrations can remain elevated for some time (217). Furthermore, plasma glucose tracer level measured might not reflect true glucose movements during Cold-Ginf. Since tracer moves down a concentration gradient, some tracer could move from the interstitial glucose pool back into the plasma pool during Cold-Ginf (217). This is unlike the true movement of glucose, which is from the plasma pool into the interstitial pool and finally into cells (217).

Studies utilizing the Cold-Ginf method to calculate WBGD and EGP often report unrealistic negative values for EGP which can be overcome by using the Hot-Ginf method (217). Moreover, the Cold-Ginf method overestimates EGP suppression and underestimates WBGD compared to the Hot-Ginf method. Given that EGP is defective in insulin-stimulated states in T2D (218), previous studies utilizing the Cold-Ginf tracer in people with T2D might have overestimated hepatic insulin sensitivity. Studies are therefore required that couple Hot-Ginf glucose tracer methods with the hyperinsulinaemic, euglycaemic clamp to more accurately assess effects of vitamin C therapy on WBGD and EGP in people with T2D.
1.5.6. Summary and significance #5

Vitamin C supplementation could potentially be a safe antioxidant treatment strategy for reducing ROS levels and improving insulin sensitivity in people with T2D. Studies investigating effects of vitamin C supplementation on levels of insulin sensitivity, markers of oxidative stress and glycaemic control have produced mixed results in people with T2D. Negative findings might be a result of an inadequate dosage and/or duration of vitamin C supplementation employed in studies. To better investigate the efficacy of vitamin C supplementation in people with T2D, randomized controlled trials involving high dose (≥1 g/day) and long-term (≥ six weeks) vitamin C that also incorporate hyperinsulinaemic, euglycaemic clamps and Hot-Ginf glucose tracer methods are required.
1.6. Effects of vitamin C supplementation on vitamin C status in T2D

1.6.1. Assessing vitamin C levels and vitamin C status

At body saturation point it has been estimated that the total body pool of vitamin C is approximately 1500 mg (219, 220), with the majority found in liver and muscle by virtue of their relative mass (221). The approximate concentrations of vitamin C in various tissues in humans are shown in Table 1.3. Tissue concentrations range from relatively high levels (>25 mg/100 g tissue) in adrenal glands, pituitary glands and eye lens to relatively low levels (<5 mg/100 g tissue) in skeletal muscle, testes and thyroid (221).

Table 1.3. Approximate tissue distribution of ascorbic acid in adult humans

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Approximate concentration (mg/100 g tissue)</th>
</tr>
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<tbody>
<tr>
<td>Adrenal glands</td>
<td>30-40</td>
</tr>
<tr>
<td>Pituitary gland</td>
<td>40-50</td>
</tr>
<tr>
<td>Liver</td>
<td>10-16</td>
</tr>
<tr>
<td>Spleen</td>
<td>10-15</td>
</tr>
<tr>
<td>Lungs</td>
<td>7</td>
</tr>
<tr>
<td>Kidneys</td>
<td>5-15</td>
</tr>
<tr>
<td>Testes</td>
<td>3</td>
</tr>
<tr>
<td>Thyroid</td>
<td>2</td>
</tr>
<tr>
<td>Heart Muscle</td>
<td>5-15</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>3-4</td>
</tr>
<tr>
<td>Brain</td>
<td>13-15</td>
</tr>
<tr>
<td>Pancreas</td>
<td>10-15</td>
</tr>
<tr>
<td>Eye Lens</td>
<td>25-31</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.4-1.0</td>
</tr>
<tr>
<td>Saliva</td>
<td>0.07-0.09</td>
</tr>
</tbody>
</table>

(from (221))
There are no reliable functional indices of vitamin C status (222). Thus, static measurements including serum, leukocyte and plasma vitamin C levels are commonly used to assess vitamin C adequacy in humans (222). Plasma concentrations of AA in healthy individuals typically range from 5-90 μmol/l (223). In the National Health and Nutrition Examination Survey (NHANES) II study, deficient vitamin C levels were defined as serum levels <11.3 μmol/l, “normal” levels of vitamin C were defined as serum levels >22.7 μmol/l, while “saturated” levels were defined as serum levels >56.8 μmol/l (224).

In contrast to plasma and serum vitamin C levels, leukocyte vitamin C levels are considered more representative of tissue vitamin C levels (222). In healthy individuals, AA levels in circulating blood cells (leukocytes, monocytes and platelets) were found to be “saturated” after prolonged intakes of 100 mg-400 mg vitamin C/day (225, 226). Yamada et al. (227) reported similar levels of plasma AA in healthy controls, uncomplicated patients with T2D and in patients with complicated T2D. However in the same study leukocyte levels of AA were found to be lower in the people with uncomplicated T2D when compared with healthy controls; and lower still in the people with complicated T2D when compared to individuals with uncomplicated T2D (227).

It is unclear what the best measure of vitamin C adequacy might be in people with T2D. Compared to plasma levels, leukocyte levels are less sensitive to short-term fluctuations in dietary vitamin C intake (228) and drop less rapidly with advancing deficiency (229). On the other hand, establishing levels of vitamin C adequacy on leukocyte concentrations could be limited due to their low relative ascorbic acid holding capacity and lower intake saturation threshold compared to plasma (225,
Moreover, leukocyte vitamin C level has an unclear relationship with ascorbic acid deficiency and predicted saturation of some tissues (231-233).

Based on modelling, skeletal muscle accounts for approximately 65% of the body’s vitamin C stores (219, 220, 234). Moreover, a recent study in healthy individuals with low baseline vitamin C levels demonstrated a stronger correlation of skeletal muscle vitamin C content with both dietary vitamin C intake and plasma vitamin C levels when compared with leukocyte vitamin C content during repletion (233). Thus, skeletal muscle vitamin C concentration could potentially serve as a better marker of total body vitamin C content than either leukocyte or plasma vitamin C alone.

Skeletal muscle concentrations of vitamin C have not yet been investigated in people with T2D. Skeletal muscle vitamin C concentrations in humans are often reported to be between 3-4 mg vitamin C/100 g wet weight tissue (221, 235, 236) as reported in human cadavers (234). In contrast, a more recent investigation in healthy humans found ascorbate levels to be ≈ 1.2 mg/100 g tissue after vitamin C repletion in previously vitamin-C deficient males (233). Differences in measurement and assay techniques for determination of vitamin C concentrations might complicate comparisons between these studies. Schaus (234) measured levels of total vitamin C (AA + DHA) using a 2,4-dinitrophenylhydrazine-based colorimetric assay while Carr et al. (233) measured levels of ascorbic acid using high performance liquid chromatography (HPLC) with electrochemical detection. Future research incorporating more consistent methodology for assessing vitamin C levels in skeletal muscle is required in order to establish an absolute concentration that might constitute an “adequate” level in skeletal muscle.
1.6.2. Effects of vitamin C supplementation on vitamin C status in T2D

Increasing vitamin C to very high levels in the blood via oral dosing is limited by the saturable processes of intestinal absorption (237, 238) and renal reabsorption of ascorbic acid (223, 239, 240). Separate studies in otherwise healthy men and women deplete in vitamin C collectively demonstrated that bioavailability of orally consumed vitamin C was reduced at intakes above 100-200 mg/day (225, 226). Moreover, plasma levels of vitamin C were found to peak at 400 mg/day in women (226) and 1000 mg/day in men (225), with excretion of vitamin C in urine increasing markedly at doses of 500 mg or more.

Quantification of body vitamin C levels before and after supplementation is important in randomized controlled trials that investigate effects of vitamin C so as to confirm that supplementation was effective at increasing vitamin C concentrations. From the studies presented in Table 1.2 (on page 37), it is clear that not all studies quantified body levels of vitamin C. Moreover, there does not appear to be any clear relationship between the relative or absolute reported increases in plasma or leukocyte levels of vitamin C from baseline and improvements in markers of oxidative stress, insulin sensitivity or glycaemic control in people with T2D (169, 203-206, 208, 211). It should be noted however that comparisons between the studies outlined in Table 1.2 might be complicated by differences in baseline participant metabolic characteristics, baseline participant vitamin C levels and study designs employed.

Skeletal muscle vitamin C concentrations of people with T2D have not yet been quantified following vitamin C supplementation. In healthy volunteers with apparently normal baseline plasma vitamin C levels, skeletal muscle vitamin C
concentration increased significantly by 41 % after seven days of supplementation with an antioxidant combination of 1500 mg vitamin C + 120 mg coenzyme Q + 345 mg α-tocopherol (241). On the other hand, a recent bioavailability study in otherwise healthy males with low baseline ascorbate levels reported an increase in skeletal muscle ascorbate levels by ≈ 350 % over baseline following 42-days supplementation with only 53 or 212 mg of vitamin C (233). Baseline vitamin C levels might confound comparisons in these studies however. Participants in the study by Carr et al. (233) were marginally deficient in plasma vitamin C prior to supplementation while participants in the study of Hellston et al. (241) had an adequate mean plasma vitamin C concentration (242). The dose-response tissue vitamin C saturation curve is likely characterized by a steep portion (at low tissue vitamin C concentrations) and a flatter portion (at high tissue vitamin C concentrations) (225, 226). Therefore, it could be speculated that participants in the study by Carr et al. (233) were positioned on the steeper portion of the skeletal muscle dose-response curve while participants in the study by Hellston et al. (241) were on the flatter portion of this curve. Thus, despite a lower vitamin C supplementation dose in the study by Carr et al. (233) compared with Hellston et al. (241), the relative increase in skeletal muscle vitamin C concentration was greater following supplementation.
1.6.3. Summary and significance #6

Plasma and/or leukocyte levels of vitamin C provide useful, but limited measures of body vitamin C content. Skeletal muscle vitamin C concentration might represent a potentially better indicator of body levels given that skeletal muscle contains the majority of the body’s vitamin C. However it is unclear from studies what concentration might constitute an “adequate” concentration of vitamin C in skeletal muscle. Measurement of vitamin C concentrations in skeletal muscle before and after vitamin C supplementation is important in antioxidant studies focussing on effects in skeletal muscle. Measurement of changes in vitamin C concentration following supplementation can provide confirmation that vitamin C supplementation is able to increase intramuscular vitamin C content so as to exert its antioxidant effects in muscle.
1.7. Skeletal muscle AA versus DHA levels in T2D

1.7.1. Skeletal muscle levels of AA and DHA

It is widely believed that DHA is present only at very low levels in plasma of healthy individuals (i.e. <2 % of total vitamin C) (223, 243). However, DHA levels as high as 10-60 % of total plasma vitamin C has been reported in the literature in various population groups (244). In contrast to plasma, there is a paucity of data reporting on AA and DHA levels in human tissues. Like plasma it is widely believed that the majority of vitamin C in tissues is in the reduced AA form compared with the oxidized DHA form (221, 223, 243). However mixed data exists in animals, suggesting that the relative AA and DHA content of tissues might differ both within and between different species (245-248). In guinea pigs, who like humans cannot synthesize ascorbic acid endogenously, the majority of vitamin C in the adrenal glands, brain, testis and liver after being fed a diet adequate in vitamin C was reported to be in the reduced, AA form; whereas the majority of vitamin C in the heart, spleen, thymus and skeletal muscle was in the oxidized DHA form (246, 247).

Improper handling or treatment of samples prior to analysis has been suggested to result in increased oxidation of AA to DHA and diketogulonic acid, thus leading to artifactually increased DHA levels and a potential irreversible loss of vitamin C (243). Poor sample handling after collection includes a failure to minimize sample contact with oxygen, and a failure to freeze, acidify, and combine the sample with a metal-chelating agent immediately after collection (243). Thus, a failure to minimize sample handling effects and artifactual AA oxidation might confound some of the findings reporting elevated DHA concentrations.
To our knowledge, levels of AA and DHA have not yet been investigated in skeletal muscle of people with T2D. Given that skeletal muscle is a key site of oxidative stress and insulin resistance in T2D, determining levels of both the reduced AA and oxidized DHA forms in muscle might be important in characterizing the oxidative stress milieu in people with T2D.

1.7.2. Skeletal muscle DHA/AA: a marker of oxidative stress in T2D?

Lower levels of AA and/or higher levels of DHA have been reported in plasma of people with T2D when compared with non-diabetic individuals (168, 191, 192, 194, 249-251). One possible explanation for this discrepancy between healthy individuals and people with T2D is an increased oxidation of AA to DHA as a result of oxidative stress in people with T2D (223).

Plasma DHA/AA has previously been regarded as a marker of oxidative stress in T2D (249, 252). An increased DHA/AA ratio in plasma was reported in people with T2D compared with non-diabetics (249, 252). Three weeks of vitamin C supplementation (500mg twice daily) increased plasma AA and reduced DHA/AA in people with T2D and in non-diabetics (249). However when supplementation continued for six weeks, the improved levels of AA and DHA/AA observed at three weeks had reverted back towards baseline levels in the people with T2D but not in the healthy participants (249). In relation to these findings, the authors suggested a disturbance in AA metabolism in the people with T2D (249). Compliance was not reported in the latter study however. Thus it remains possible that compliance with supplementation deteriorated over time in the people with T2D. Furthermore, other markers of oxidative stress and tissue DHA/AA levels were not assessed during this study.
Literature Review

Considering that plasma levels of vitamin C are sensitive to short-term dietary fluctuations, tissue levels of DHA/AA might represent a better marker of oxidative stress than plasma DHA/AA. In an experimental animal model of diabetes (253), significantly reduced levels of AA and increased levels of DHA were found in diabetic liver tissue compared with normal liver tissue. Increased tissue levels of DHA but reduced tissue levels of AA in diabetes are believed to be a result of increased cellular oxidation of AA to DHA (194) and/or due to impaired recycling of cellular DHA to AA (253). There is a current lack of data reporting on tissue DHA/AA levels in humans. Given that skeletal muscle constitutes the majority of body vitamin C, evaluation of the DHA/AA redox ratio in skeletal muscle might offer further insight into the oxidative stress milieu of human tissues. Moreover, skeletal muscle DHA/AA could potentially serve as a useful marker of tissue oxidative stress in people with T2D.

1.7.3. Summary and significance #7

Oxidative stress might increase the DHA/AA ratio in plasma and tissues of people with T2D through increased oxidation of AA to DHA. Assessment of intramuscular levels of AA, DHA and DHA/AA in people with T2D is currently unexplored. It is of interest to quantify these levels in skeletal muscle of people with T2D given the importance of skeletal muscle as a site of oxidative stress and dysfunctional insulin action in T2D. Studies investigating changes in intramuscular DHA/AA following vitamin C supplementation in association with other markers of oxidative stress could help to better establish intramuscular DHA/AA as an important redox marker of oxidative stress in T2D.
1.8. Vitamin C transporters in skeletal muscle

1.8.1. AA transport in skeletal muscle: effects of T2D and vitamin C supplementation

Vitamin C levels in the body are regulated by vitamin C transporters. Vitamin C in its reduced AA form can be taken up by most organs via sodium-dependent vitamin C transporters (SVCTs) including SVCT1 and SVCT2 (254). Uptake occurs with a stoichiometry of two sodium cations transported for every ascorbate anion co-transported (255, 256). The high-capacity, low-affinity carrier SVCT1, expressed predominantly in epithelial cells and in the liver and kidney (254) (Figure 1.10), appears to be mainly involved in absorption of dietary ascorbic acid and in kidney-based reabsorption (257). SVCT1 thus appears to play a role in maintaining circulating levels of AA and in regulating whole-body vitamin C homeostasis (257). On the other hand, expression of the low-capacity, high-affinity carrier SVCT2 is more widespread, being expressed in diverse tissues and cells such as the brain, choroid plexus, Schwann cells, skeletal muscle, spleen and endothelial cells (184, 254, 258) (Figure 1.10). SVCT2 appears to be primarily involved in maintaining intracellular AA levels in metabolically active tissues (257).
Gene and protein expression of SVCT2 reflects ascorbic acid transport in skeletal muscle cells (236, 260). Previous studies investigating transport kinetics of AA in C2C12 mouse cells calculated AA uptake in time-dependent and concentration-dependent experiments using Michaelis-Menten kinetics (236, 260). These studies
matched AA transport activity with protein and gene expression levels of SVCT2 in muscle (236, 260).

Levels of SVCT2 have been little explored in human skeletal muscle. Early studies using northern blot techniques failed to detect SVCT2 mRNA in human skeletal muscle (261). However, Low et al. (258) recently detected SVCT2 protein in human lumbar skeletal muscles using immunohistochemistry. Intracellular levels of AA might exert a negative feedback-type regulation of SVCT abundance, such that expression of SVCTs are increased when intracellular levels of AA are low but are reduced when levels of AA are high (262, 263). Expression of SVCT2 in skeletal muscle might also be regulated by intracellular redox state (236). *In vitro* studies utilizing murine skeletal muscle cell lines have shown that when intracellular levels of oxidants increase, expression of SVCT2 is also increased (236, 260). Conversely, SVCT2 transporter expression was shown to be down-regulated when muscle cells were treated with the antioxidant lipoic acid (236).

Therefore, given the above regulatory characteristics of SVCT2, it could be hypothesized that SVCT2 transporter expression will be increased in skeletal muscle in people with T2D as a consequence of intracellular oxidative stress. Moreover, it could also be hypothesised that expression levels of SVCT2 will be decreased in diabetic skeletal muscle following AA supplementation due to increased intracellular antioxidant levels and/or reduced oxidant levels. Studies of vitamin C supplementation in people with T2D are now required to test out these hypotheses.
1.8.2. DHA transport in skeletal muscle: Effects of T2D and vitamin C supplementation

Unlike AA, transport of DHA occurs via facilitative transport by hexose transporters. Specifically, GLUT1, GLUT3 and GLUT4 have been implicated in the transport of DHA \textit{in vitro} (264-266). DHA is believed to be rapidly reduced to AA following cellular uptake (184). In conditions characterized by chronic oxidative stress such as T2D, cellular uptake of DHA might be of increased importance to facilitate a cellular supply of AA since the pro-oxidative environment might lead to increased oxidation of extracellular AA to DHA (184).

Given its predominance as a glucose transporter in skeletal muscle, GLUT4 might play an important role in mediating skeletal muscle DHA transport. It has been demonstrated \textit{in vitro} that DHA uptake by GLUT4 is increased markedly following insulin stimulation (266). However an impaired insulin-mediated translocation of GLUT4 to the plasma membrane in people with T2D could impair DHA transport as well as glucose transport in skeletal muscle.

GLUT1 and GLUT3 are expressed at low levels in human skeletal muscle (267, 268). Studies have reported reduced (44) or unaltered (42, 45, 267, 269) protein levels of GLUT1 in membrane fractions of muscle in people with T2D. Decreased expression of GLUT3 in skeletal muscle of severely insulin-resistant individuals has also been found (267). However, it has been argued that the presence of erythrocytes and perineurial cells in muscle samples could result in artifactualy elevated levels of GLUT1 and GLUT3 respectively, thus complicating findings of these studies (268).

DHA uptake by skeletal muscle cells is impaired by high glucose levels \textit{in vitro} (270). Specifically, reduced DHA uptake in rat L6 skeletal muscle cells incubated with
glucose was found to be the result of a combination of competition between glucose and DHA for available GLUT transporters and a hyperglycaemia-mediated down-regulation of GLUT1 expression (270). Hyperglycaemia associated with T2D might therefore impair cellular uptake of DHA by muscle cells via a direct down-regulation of GLUT1 transporter levels and/or through an increased competition between glucose and DHA for available GLUT transporters. This could in turn promote decreased intracellular AA levels. Effects of vitamin C supplementation on GLUT expression levels in skeletal muscle of people with T2D has not yet been explored. Given the non-specificity of GLUT transporters for DHA, it would be difficult to ascertain if effects of vitamin C supplementation on GLUT transporter expression were a result of direct (DHA-related) or indirect (glucose metabolism-related) modulation. Additional investigations of DHA transport to protein expression analyses would be required to further elucidate direct versus indirect effects of supplementation on GLUT expression in skeletal muscle.

1.8.3. Summary and significance #8

Skeletal muscle vitamin C transporters regulate intramuscular vitamin C concentrations. Assessment of vitamin C transporter levels in T2D could therefore be important in understanding altered tissue levels of vitamin C in T2D. The effects of vitamin C supplementation on gene and protein expression of SVCT2 and GLUT transporters in skeletal muscle in people with T2D are presently unknown. Expression of SVCT2 might be increased in skeletal muscle of people with T2D, consistent with decreased levels of AA and/or increased levels of ROS. Moreover, vitamin C supplementation could potentially normalise elevated SVCT2 expression in people with T2D.
1.9. Effects of vitamin C supplementation on nitric oxide metabolism

1.9.1. Skeletal muscle nitric oxide synthase levels in T2D

Nitric oxides (NO) are bioactive chemicals synthesized endogenously from L-arginine via various nitric oxide synthases (NOS) (271) (Figure 1.11A). NO acts as a signalling molecule in the regulation of central biological processes in almost all tissues (272). Both endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) are expressed in skeletal muscle (273). Inducible nitric oxide synthase (iNOS) is not normally expressed abundantly in skeletal muscle but can be induced in response to inflammatory stress (142), obesity (274) and endotoxin-induced stress (273). Impaired NO production in skeletal muscle has been implicated in reduced recruitment of microvasculature and glucose uptake in response to insulin stimulation (275-277). NO appears to potentiate glucose transport in skeletal muscle via a signalling pathway involving the activation of guanylate cyclase and increased cyclic GMP levels (278). Impaired skeletal muscle basal and insulin-stimulated total NOS activities were found in people with T2D (279, 280). Altered NOS activity found in T2D appears to be isoform-specific however, with endothelial NOS (eNOS) activity being reduced but inducible NOS (iNOS) activity being increased (142).
NO is also capable of reacting with \cdot O_2^- in tissues to form ONOO^−, a highly reactive nitrogen species (RNS) (111). Thus, although NO production appears to be beneficial in normal glucose metabolism, it can produce detrimental effects at high concentrations. In particular, NO levels can increase dramatically in response to iNOS induction (142). iNOS-induced NO can react with \cdot O_2^−, in turn increasing ONOO^− levels rapidly and causing impaired skeletal muscle insulin sensitivity through nitration of key insulin signalling proteins (282-284). Therefore, measurement of levels of specific NOS isoforms could prove valuable in the assessment of NO metabolism in diabetic skeletal muscle when compared with levels of total NO and total NOS activity alone.

**Figure 1.11. Nitric oxide production by human eNOS**

Modelling of human eNOS activity. A: Nitric oxide (NO) and L-citrulline (L-Cit) are formed via eNOS from L-arginine (L-Arg) and oxygen (O_2). Tetrahydrobiopterin (BH_4) serves as a cofactor for NO production by NOS. Ascorbic acid (AsCH) can act to maintain BH_4 in its reduced state by reducing the BH_4 radical trihydrobiopterin (BH_3) back to BH_4. B: Increased oxidative stress, in particular peroxynitrite (ONOO^−) can cause oxidation of BH_4 and promote uncoupling of eNOS activity. This enhances the formation of superoxide (\cdot O_2^-) rather than NO via eNOS (From (281)).
1.9.2 Effect of vitamin C supplementation on nitric oxide synthase levels in T2D

Through its antioxidant scavenging of ·O₂⁻, ascorbic acid is theoretically able to minimize the interaction between ·O₂⁻ and NO and reduce ONOO⁻ formation. Another outcome of this antioxidant action is that less NO is inactivated by ·O₂⁻ and more is available for biological activity in skeletal muscle (285). ROS/RNS including ·O₂⁻ and ONOO⁻ were shown to inactivate nNOS via oxidation of the NOS cofactor tetrahydrobiopterin (BH₄), causing impaired NO production and increased ·O₂⁻ production in human kidney cells transfected with rat nNOS (286). On the other hand, repletion of decreased BH₄ levels resulting from excess ·O₂⁻ and ONOO⁻ formation can restore nNOS activity and NO production in these cells (286). ONOO⁻-induced uncoupling of eNOS via BH₄ oxidation has also been demonstrated in cultured BAEC cells (287) (Figure 1.11B).

A proposed specific role of ascorbic acid in vivo additional to its general antioxidant effects is to maintain BH₄ in its reduced state (288, 289). Ascorbic acid is able to reduce the BH₄ radical (trihydrobiopterin, BH₃) back to BH₄ and prevent eNOS uncoupling (287) (Figure 1.11A). Enhanced NOS activity and BH₄ levels were found in aortic walls of C57BL/6J mice following prolonged oral vitamin C supplementation (290). Furthermore, supplementation with ascorbic acid, L-arginine and BH₄ was found to increase calcium-dependent NOS (nNOS & eNOS) activity and eNOS protein expression; and reduce nitrotyrosine levels (a marker of ONOO⁻ stress) in skeletal muscle of ischaemic mice (291). In this latter study, the addition of vitamin C to combined L-arginine and BH₄ supplementation potentiated total eNOS protein expression but did not further increase NOS activity in ischaemic skeletal muscle. Interestingly, ascorbic acid has also been shown to stimulate eNOS activity in human
endothelial cells through a mechanism involving activation of AMP-kinase (AMPK) that is independent of BH$_4$ (292).

In light of findings of impaired NOS activity in T2D and the potential mechanistic role of ascorbic acid in NO metabolism through BH$_4$-related and/or antioxidant-related effects, an investigation of effects of vitamin C supplementation on NOS activity and NOS isoform expression levels in skeletal muscle of people with T2D is warranted.

**1.9.3. Summary and significance #9**

In addition to its general antioxidant actions, vitamin C also appears to play an important role in normal nitric oxide metabolism by maintaining the NOS cofactor BH$_4$ in its reduced state. Vitamin C supplementation could potentially enhance impaired skeletal muscle NOS activity in people with T2D, thus facilitating enhanced glucose uptake through improved nitric oxide production and decreased ROS/RNS-induced inactivation of key components of insulin signalling transduction. Studies investigating effects of vitamin C supplementation on markers of oxidative stress, antioxidant enzymes, NOS activity and activation levels of key insulin-signalling subunits in skeletal muscle could prove useful in linking redox-related mechanisms of action of vitamin C in skeletal muscle with improvements in insulin signalling and insulin sensitivity.
1.10 Overall summary & significance

Antioxidant vitamin C therapy offers the potential of a relatively safe and effective treatment for skeletal muscle insulin resistance in T2D through its scavenging of excess ROS, a key underlying factor in the pathophysiology of T2D.

Clinical studies investigating effects of antioxidant vitamin C supplementation on insulin resistance, glycaemic control and oxidative stress in people with T2D have reported mixed findings. Studies finding no benefits were arguably too short in duration (<6 weeks) and/or used vitamin C dosages that were too low (<1 g/day) to elicit improvements in oxidative stress and clinical outcomes. Furthermore, the effect of vitamin C supplementation on insulin resistance in skeletal muscle versus liver using the hyperinsulinaemic, euglycaemic clamp coupled to “Hot-Ginf” glucose tracer techniques in people with T2D has not been studied to date.

Since skeletal muscle is a major site of ROS production and insulin resistance in people with T2D, investigations looking at the antioxidant effects of vitamin C at this pivotal site are warranted. Currently, effects of vitamin C supplementation on levels of vitamin C, oxidative stress, insulin signalling and activation of NOS in skeletal muscle have not been explored in people with T2D. Furthermore, the effects of vitamin C supplementation on levels of oxidized versus reduced forms of vitamin C and expression of vitamin C transporters SVCT2 and GLUT1 in skeletal muscle, which are important in the context of oxidative stress and regulation of vitamin C levels respectively, have not yet been investigated in people with T2D. Future studies targeting these research shortcomings are now required.
1.11 Research aims, hypotheses and conceptual overview

1.11.1 Research Aims

To investigate the effects of chronic, high dose oral vitamin C supplementation on:

1) Gluoregulatory control in people with T2D, including:
   - Skeletal muscle insulin sensitivity and endogenous glucose production
     *(primary measures)*
   - Clinical diabetes measures including HbA1c, fasting glucose, fasting insulin and lipids *(secondary measures)*

2) Vitamin C concentrations in people with T2D, including:
   - Concentrations of total vitamin C in skeletal muscle and plasma

3) Oxidative stress in people with T2D, including:
   - Skeletal muscle levels of F2-Isoprostanes, GSH/GSSG and DCFH oxidation

4) Levels of oxidized versus reduced forms of vitamin C in people with T2D, including:
   - Skeletal muscle levels of ascorbic acid and dehydroascorbic acid

5) Vitamin C transporter expression levels in people with T2D, including:
   - SVCT2 protein abundance in skeletal muscle

6) Nitric oxide synthase levels in people with T2D, including:
   - Activation and expression levels of nitric oxide synthase in skeletal muscle
1.11.2. Research Hypotheses

1) High-dose and long-term vitamin C supplementation will improve skeletal muscle insulin sensitivity and insulin-stimulated non-oxidative glucose metabolism in people with T2D

2) Vitamin C supplementation will increase vitamin C concentrations in skeletal muscle and plasma of people with T2D

3) Vitamin C supplementation will reduce ROS/RNS levels and improve markers of oxidative stress including F2-Isoprostanes, GSH/GSSG and DCFH oxidation in skeletal muscle of people with T2D

4) Vitamin C supplementation will increase AA levels and improve the DHA/AA ratio in skeletal muscle and plasma of people with T2D

5) Vitamin C supplementation will promote a decrease in protein expression of sodium-dependent vitamin C transporter SVCT2 in skeletal muscle of people with T2D

6) Vitamin C supplementation will increase total NOS activity in skeletal muscle of people with T2D

1.11.3. Conceptual overview of research

These research aims and hypotheses were addressed through the undertaking of a clinical trial that investigated effects of vitamin C supplementation in people with T2D (Figure 1.12).
**Figure 1.12.** Conceptual overview of thesis research project

Key foci of aims and hypotheses of the thesis relate to data obtained from a clinical trial of vitamin C supplementation in people with T2D
2.0. VITAMIN C SUPPLEMENTATION IN HEALTHY MALES

2.1. Background

Prior to the major project of this thesis, a study was undertaken in healthy individuals in whom several key skeletal muscle measurements were made. The study was undertaken as part of an honours project by student researcher, Raquel Baptista. The study investigated the bioavailability of high dose oral vitamin C supplementation in skeletal muscle of healthy young males. Key targets of investigation were muscle and plasma vitamin C concentrations, skeletal muscle antioxidant and oxidative stress levels, and skeletal muscle SVCT2 transporter expression. The study provides insights into effects of vitamin C supplementation in basal human skeletal muscle, which is of relevance to the major project of the thesis. Study participant recruitment and data collection was undertaken by Raquel Baptista; and the project was conceived and designed by Dr Glenn Wadley. Sample analyses of vitamin C concentration and antioxidant enzyme levels as detailed within a manuscript recently accepted in Free Radical Biology and Medicine Journal (293) were conducted by both Raquel Baptista and the current author. SVCT2 western blotting was undertaken solely by the current author. The development and optimisation of all analyses including the measurement of plasma and skeletal muscle vitamin C concentrations using HPLC, the antioxidant and oxidative stress measurements, and SVCT2 western blotting were undertaken by the current author. Manuscript preparation was also predominantly completed by the current author. Thus, this chapter of the thesis has been devoted to aspects of this research study in which the current author had a major contribution.
2.2. Introduction

Vitamin C is a potent water-soluble antioxidant that scavenges ROS and reduces oxidative stress \textit{in vitro} and \textit{in vivo} (176, 178, 179). Skeletal muscle contains the largest proportion (~65\%) of total body vitamin C (219, 234) with approximately 3-4 mg ascorbic acid per 100 g of tissue (234). Skeletal muscle is also a key site of ROS production (117) and intramuscular ascorbic acid likely acts to reduce elevated muscle ROS levels (260), which can occur acutely during bouts of physical exercise (116) and chronically in individuals with insulin resistance (64). High dose antioxidant vitamin C supplementation could potentially benefit individuals with skeletal muscle and systemic oxidative stress including people with T2D (141, 169). Oral vitamin C supplementation increases skeletal muscle vitamin C concentrations in humans (233, 241). However, the time course of vitamin C accumulation in skeletal muscle during high dose vitamin C supplementation is unclear and remains to be determined. Establishing a time course accumulation of vitamin C in skeletal muscle during supplementation could help to optimize vitamin C supplementation regimens that target skeletal muscle oxidative stress in disease.

Transport of ascorbic acid into cells occurs via specific sodium-dependent vitamin C transporters (SVCT1 & SVCT2) at a stoichiometry of two sodium ions to one ascorbic acid molecule (255, 256). Of these transporters, only SVCT2 has been identified in mature skeletal muscle (258, 260). Studies in various human cell types suggest that SVCT2 expression is inversely regulated by intracellular ascorbic acid concentration (259, 262). Thus, interventions aimed at increasing tissue vitamin C concentrations could potentially result in decreased tissue SVCT2 expression and attenuated vitamin
C accumulation in accordance with intracellular vitamin C content. In mice who are capable of producing vitamin C endogenously, oral vitamin C supplementation is largely ineffective at increasing tissue vitamin C concentrations due to strict control exerted by SVCT transporters (294). The situation could be different in humans, however, who are incapable of producing vitamin C endogenously. Moreover, the effect of vitamin C supplementation on SVCT2 protein expression in human skeletal muscle has not yet been investigated \textit{in vivo}.

In contrast to its potential clinical benefits, high dose vitamin C supplementation may be detrimental to exercise-related metabolic adaptations including mitochondrial biogenesis, antioxidant enzyme induction and insulin sensitization in healthy individuals (118, 295, 296). Compared with exercise training studies, there is a relative lack of investigation into basal effects of chronic antioxidant supplementation in healthy human skeletal muscle. Strobel et al. (297) reported decreased markers of mitochondrial biogenesis in skeletal muscle of healthy rats following high dose antioxidant vitamin E supplementation that was independent of exercise training. It is possible therefore that redox changes induced by antioxidant supplementation could also impair skeletal muscle mitochondrial biogenesis and antioxidant concentrations basally in humans. Among the findings of Strobel et al. (297) was a decrease in skeletal muscle citrate synthase activity following antioxidant supplementation. Citrate synthase activity exhibits a strong association with mitochondrial content in healthy human skeletal muscle (298). Therefore, the measurement of citrate synthase activity before and after vitamin C supplementation can provide insight into effects of regular high dose vitamin C intake on mitochondrial content in skeletal muscle.
Given the potential for high dose vitamin C supplementation to modulate SVCT2 expression, antioxidant concentrations, oxidative stress and mitochondrial content in skeletal muscle, studies in humans are required to clarify these issues. Thus, the aims of the present study were to firstly, investigate the time course effects of high dose vitamin C supplementation on vitamin C concentrations in skeletal muscle and plasma of healthy individuals; secondly, to investigate effects of vitamin C supplementation on SVCT2 transporter expression levels; and thirdly, to investigate effects of vitamin C supplementation on markers of oxidative stress, antioxidant concentrations and mitochondrial content in healthy individuals.
Materials and methods

Subjects

Healthy male participants (n=8) aged 18-40 years were recruited for the study through local community- and university-wide advertising. Potential participants completed a prior medical screening session and questionnaire to rule out any cardiovascular, pulmonary, renal or metabolic disease. Individuals were excluded if they were smokers, if they suffered from a bleeding disorder or heart murmur, if they were highly trained athletes or if they were consuming any vitamin supplements. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Deakin University Human Research Ethics Committee (Ethics approval # 2011-021). Written informed consent was obtained from all subjects.

Research design

The study was a double-blinded, placebo-controlled trial that involved consumption of a capsule containing 500 mg ascorbic acid (capsule ingredients: 500 mg L-ascorbic acid, microcrystalline cellulose, vegetable cellulose, vegetable magnesium stearate; Solgar Inc., Leonia NJ) or a gelatine-based placebo capsule (ingredients: 560 mg gelatine, 8 mg calcium carbonate, vegetable magnesium stearate, vegetable cellulose; Solgar Inc., Leonia NJ) twice daily with morning and evening meals for six weeks. The study had a cross-over design and a wash-out period of at least four weeks separated the two six-week treatment arms (mean ± SEM wash-out: 44.1 ± 3.8 days). Treatment order was implemented in a randomized counterbalanced manner to participants. Concealment and randomization of treatments was undertaken by a
third party individual not directly involved in the trial. Vitamin C and placebo capsules were almost identical in appearance and were provided to participants in identical sealed opaque bottles. Supplements were commercially available products purchased through online retail stores. Capsules randomly selected from vitamin C and placebo bottles were tested for vitamin C content using HPLC. Vitamin C capsules tested in this manner were found to contain >99.9% of the expected ascorbic acid level while placebo capsules contained 0% ascorbic acid. The dose of vitamin C used in the present study (500 mg twice daily) was chosen because it represents a prudent upper limit for vitamin C intake based on estimations of a no observed adverse effect level (189). Moreover, this daily supplemental dose also represents a commonly recommended intake of vitamin C using commercially available vitamin C tablets and capsules.

Participants visited the clinical research facility at Deakin University in Melbourne for morning testing (07:00) in an overnight (10-12h) fasted state (from 21:30) on days 0 (baseline), 1, 7 and 42 during each six-week treatment period. To minimize effects of acute vitamin C ingestion on vitamin C measurements, vitamin C and placebo capsules were not consumed by participants on the morning of trials. Participants were asked to maintain their usual physical activity and dietary intake during the trial. Participants were requested to avoid any strenuous exercise during the 24 hours prior to each testing day. Participants were also asked to consume the same originally self-reported 24-h dietary intake on the day preceding each testing day. There were no differences reported for energy intake, macronutrient composition or vitamin C intake during the pre-trial 24-h period between any trial days when calculated using Foodworks® 2009 software (v.6.0.2562, Xyris Software, Kenmore Hills, Australia) (data not shown).
Muscle biopsies

Muscle biopsy samples were taken on days 0, 1, 7 and 42 during supplementation periods for assessment of skeletal muscle vitamin C concentration, protein expression (SVCT2 and antioxidant enzymes), antioxidant enzyme activities and citrate synthase activity. Muscle sampling was performed according to the percutaneous needle biopsy sampling method of Bergstrom (299) modified to include suction. Samples of approximately 100-200 mg were obtained. After the brief removal of visible blood and connective tissue from samples, approximately 50 mg of the muscle was collected for vitamin C analysis and the remaining sample was rapidly snap-frozen and stored in liquid nitrogen until sample analysis. For vitamin C analysis, fresh muscle was homogenized on ice 1:20 (wt:vol) in phosphate-buffered saline using a glass-glass Tenbroeck homogenizer (Wheaton, Millville, NJ). The homogenate was then mixed with an equal volume of 0.1 g/ml metaphosphoric acid and stored airtight at -80 °C until analysis. The remainder of the homogenate was stored separately at -80 °C for antioxidant enzyme assays. Biopsy samples were taken from the same thigh during each arm of the trial, approximately 1-2 cm proximal to the previous sampling site.

Blood sampling

Fasting blood samples were collected in lithium heparin-containing tubes on days 0, 1, 7 and 42 to assess effects of vitamin C intake on plasma vitamin C concentration and antioxidant enzyme activities. Samples taken were immediately centrifuged at 3000 g for 15 minutes at 4 °C to separate out the plasma portion. Plasma was mixed
with an equal volume of 0.1 g/ml metaphosphoric acid for vitamin C analysis. Plasma samples were stored at -80 °C until analysis.

_Vitamin C analysis_

Total vitamin C (ascorbic acid + dehydroascorbic acid) concentrations were measured in muscle and plasma samples using HPLC coupled with UV detection (Agilent technologies Inc. 1100 series, Santa Clara, CA). Methods of analysis were adapted from Lykkesfeldt (243) and Wechtersbach & Cigic (300). Samples were eluted on a Luna C18 column (particle size 3 μm, 150 x 4.6 mm i.d., Phenomenex Inc., Torrance, CA) connected to a Security Guard C18 column (Phenomenex Inc.). The mobile phase was adapted from Lykkesfeldt (243) to contain 0.5 % (vol:vol) methanol, and was pH adjusted to 4.8 using orthophosphoric acid. Samples were combined with an equal volume of a 0.05 g/ml metaphosphoric acid solution containing 5mmol/l EDTA and 10mmol/l tris (2-carboxyethyl) phosphine. Samples were then flushed with a light stream of nitrogen gas for 20 seconds and incubated at 20°C for 120 minutes to facilitate reduction of sample DHA to ascorbic acid. 45 μl was injected onto the column for analysis. Column flow rate was 0.6 ml/min and ascorbic acid was detected at a wavelength of 265 nm. The ascorbic acid peak was verified by spiking muscle and plasma samples with known amounts of ascorbic acid (obtaining recoveries of 94-104 % of expected values) and through treatment of samples with 20 units ascorbate oxidase to selectively eliminate the ascorbic acid peak. Quantification of peak area for ascorbic acid was made using Agilent ChemStation software (v. B.03; Agilent technologies, Santa Clara, CA). Sample concentrations were determined from linear
(r²>0.99) standard curves generated from ascorbic acid standards prepared daily in mobile phase.

Western blot analyses

Muscle samples (~20-30 mg) were homogenized on ice in a freshly prepared ice-cold buffer consisting of 50 mmol/l Tris (pH 7.5), 1 mmol/l EDTA, 10 % (vol:vol) glycerol, 1 % (vol:vol) Triton-X, 50 mmol/l NaF, 5 mmol/l NaPP, 1 mmol/l DTT, 1 mmol/l PMSF, and 5 μl/ml protease inhibitor cocktail (Sigma P8340). Samples were then centrifuged for 15 minutes at 13,000 g and supernatants frozen at -80 °C until analysis. Tissue lysates were solubilised in Laemmli sample buffer and equal amounts of protein (75 μg protein) were boiled for 5 minutes at 90 °C and then loaded onto 10 % polyacrylamide gels. All samples for an individual were run on the same gel in the trial order undertaken. Gels were subjected to electrophoresis and electrotransferred onto PVDF membranes. Membranes were blocked in 0.05 g/ml bovine serum albumin, followed by an overnight incubation at 4 °C in a primary SVCT2 goat polyclonal antibody (sc-9926; Santa Cruz). Membranes were washed and incubated in an anti-goat secondary antibody (LI-COR IRDye® 680LT or 800LT, LiCor Biosciences, Lincoln, USA) at room temperature for 1h. Detection and quantification of target proteins were made using the Odyssey® Infra-red Imaging system (LiCor Biosciences). Results are presented as measured integrative intensity as done previously (301).

A mouse polyclonal GAPDH antibody (Sigma-Aldrich) was used as a protein loading control for SVCT2. There were no effects of treatment found for GAPDH expression (data not shown). Additional control samples containing protein amounts below and
above sample amounts were loaded onto each gel to confirm the linear range of quantification for both target protein and loading control (302).

Enriched human blood platelet fractions were used as positive controls for SVCT2 western blotting. For platelet isolation, whole blood was collected from a single participant in EDTA tubes containing citrate buffer and centrifuged at 200 g for 15 mins. The platelet rich fraction was then mixed 10:1 (vol:vol) with acid-citrate-dextrose buffer and centrifuged at 900 g for 15 mins. The washed pellet was re-suspended in a buffer containing 25 mmol/l tris (pH 8.0), 1 mmol/l EDTA, 0.05 g/ml SDS and 5 μl/ml protease inhibitor cocktail (Sigma P8340) and subjected to ultrasonication (262).

Antioxidant assays

Muscle and plasma samples were prepared and undertaken in accordance with recommended procedures by assay kit manufacturers and did not include any known interfering compounds. Catalase activity was determined using a commercially available assay kit (#707002, Cayman Chemical Company). In short, the assay involved the reaction of catalase with methanol in the presence of hydrogen peroxide and the colorimetric detection of formaldehyde formed at 540 nm with 4-amino-3-hydrazino-5-mercapto-1,2,4-trizole. Total SOD activity (including all SOD isoforms) was determined using a commercial assay kit (#706002, Cayman Chemical Company) that utilized a tetrazolium salt to detect superoxide radicals produced by xanthine oxidase and hypoxanthine. Total antioxidant capacity was assessed using a commercial assay kit (#709001, Cayman Chemical Company). The assay measured the capacity of all antioxidants within a sample to inhibit oxidation of 2,2’-Azino-di-
[3-ethylbenzthiazoline 6-sulphonic acid] to its radical cation by metmyoglobin. A lower absorbance for a sample at 750 nm relative to a Trolox standard curve indicated a higher antioxidant concentration in that sample. Total glutathione concentration of samples was assessed using a commercially available kit (Bioxytech ® GSH/GSSG-412™, OxisResearch, Portland, OR). The assay involved the reaction of dithiobis-2-nitrobenzoic acid with glutathione in the presence of glutathione reductase. The rate of change of absorbance at 412 nm was measured following sample treatment with NADPH, with the observed rate reflecting total glutathione concentration. Finally, glutathione peroxidase (GPx) activity was measured using a commercially available assay kit (#703102, Cayman Chemical). In this assay GPx activity was measured indirectly via glutathione reductase and NADPH-dependent recycling of glutathione disulphide formed from the reduction of cumene hydroperoxide by GPx. The rate of decrease in absorbance at 340 nm was reflective of GPx activity in a sample.

**DCFH Assay**

The probe 2',7' – dichlorodihydrofluorescein (DCFH) has previously been used to measure levels of oxidant species in rodent skeletal muscle (303, 304). While use of this probe is limited by non-specificity of reaction with oxidant species (305), it allows for a convenient general measurement of oxidative stress in samples (305). Muscle samples (0.01 mg protein) prepared in the same lysis buffer as for western blot samples were added in duplicate to 96-well fluorescent plates to which 290 μl assay buffer (130 mmol/l KCl, 5 mmol/l MgCl₂, 20 mmol/l Na₂HPO₄, 20 mmol/l tris-HCl and 30 mmol/l D-glucose, pH 7.4 (304)) was added. The reaction was initiated with addition of 1.2μl DCFH-DA (2,7-Dichlorodihydrofluorescein diacetate; Cayman
Vitamin C study in healthy males

Chemical) dissolved in methanol (5 μmol/l final concentration). To facilitate deacetylation of DCFH-DA to DCFH by sample esterases, plates were incubated at 37°C for 15 minutes in the dark. DCFH was then rapidly oxidized to fluorescent DCF in the presence of sample ROS and reactive nitrogen species(306). The rate of change in fluorescence was measured using a Biotek® Synergy 2 spectrophotometer (Biotek Instruments, Inc., Winooski, VT) with an excitation wavelength of 485 nm and an emission wavelength of 528 nm over 30 minutes. The rate of change of sample fluorescence was linear for at least 60 minutes over a range of different protein concentrations. After 60 minutes, 1667 μmol/l H₂O₂ was added to all samples, causing a rapid increase in fluorescence in all samples that increased linearly for at least 30 minutes. This demonstrated that added DCFH was not saturated by sample oxidant levels. An H₂O₂ standard curve was created in pooled muscle homogenates, and the rate of fluorescence change was linear for added H₂O₂ concentrations (0-3330 μmol/l) over 60 minutes (r²=0.97). Sample concentration was expressed as H₂O₂ equivalents (in μmol) per mg protein. Treatment of samples with 1mmol/l glutathione (an H₂O₂ scavenger) attenuated the increase in sample-induced fluorescence by approximately 60 %, while treatment of samples with 1mmol/l ascorbic acid (a general ROS/RNS scavenger) attenuated the increase in fluorescence by approximately 90 % (data not shown).

Citrate synthase assay

Muscle samples were prepared and homogenized in the same lysis buffer as for western blotting. In brief, the assay involved the reaction of acetyl coenzyme A with oxaloacetate and the subsequent reaction of formed thiol-bound coenzyme A with
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DTNB. This produced a detectable spectrophotometric product at 412 nm. An extinction coefficient of 13,600 M⁻¹.cm⁻¹ for DTNB (307) was used in calculations and citrate synthase activity was expressed per gram total protein.

**Total protein analysis**

Total protein content of samples used for western blotting, antioxidant assays and citrate synthase activity was determined using the bicinchoninic acid assay (308) with bovine serum albumin as the reference standard.

**Statistical analyses**

Normality of data was tested via the Shapiro-Wilk test. Data found to violate Gaussian normality were log-transformed prior to parametric testing. The two-way repeated measures ANOVA was used to compare vitamin C and placebo treatments over time points for all measurements. For significant treatment versus time interactions found, Bonferroni-corrected multiple comparisons were used to determine specific time points at which the treatments differed. Correlation coefficients were used to evaluate relationships between SVCT2 protein expression and plasma and skeletal muscle vitamin C concentration. A p value of p<0.05 was used for all statistical tests. All statistical analyses were performed using GraphPad Prism (v. 6.0; La Jolla, CA) and SPSS software (v.21.0, IBM Corp).
2.4. Results

Participants

All eight enrolled participants completed the trial as outlined. Participants were aged 25.9 ± 2.3 years and had a mean height and weight of 178.9 ± 3.8 cm and 81.8 ± 4.2 kg respectively. Baseline plasma vitamin C concentrations of participants were 58.1 ± 6.7 μmol/l while skeletal muscle concentrations were 2.9 ± 0.2 mg/100 g wet weight. All participants had adequate baseline plasma vitamin C concentrations according to plasma cut-off levels reported by Jacob & Sotoudeh (242). Baseline skeletal muscle concentrations were also consistent with previously reported levels in age-matched humans (234). No adverse side effects such as gastrointestinal complications (189) were reported by participants during either treatment of the study.

Effects of supplementation on plasma and skeletal muscle vitamin C levels

Analysis using two-way repeated measures ANOVA found increased plasma vitamin C concentrations after 1 day compared with placebo ($p<0.001$). Plasma vitamin C did not significantly increase during vitamin C treatment after this time but remained significantly different to placebo at 7 days ($p<0.001$) and 42 days ($p<0.001$) (Figure 2.1A). Vitamin C supplementation significantly increased skeletal muscle vitamin C concentrations after seven days of supplementation compared with placebo ($p=0.027$) and remained significantly higher after 42 days ($p=0.001$) (Figure 2.1B).
Vitamin C study in healthy males

![Figure 2.1](image)

**Figure 2.1.** Vitamin C supplementation increases vitamin C concentration in healthy males.

A. plasma vitamin C during 42 days of supplementation. B. skeletal muscle vitamin C during 42 days of supplementation. The last vitamin C or placebo capsule was consumed 10-12 hours prior to sample collection on days 1, 7 and 42. Data points are mean ± SEM. Data are for n=8 participants. * Denotes significantly different to placebo p<0.05. Effect of vitamin C supplementation on vitamin C concentrations in healthy males

**SVCT2 protein expression**

Skeletal muscle SVCT2 protein data required log transformation in order to approximate Gaussian normality prior to ANOVA analysis. There was no significant treatment x time interaction effect, although vitamin C treatment increased SVCT2 expression during supplementation compared with placebo (p=0.006 main effect of treatment, Figure 2.2). An increase in skeletal muscle SVCT2 expression occurred in all subjects after one day of vitamin C supplementation, albeit variable, with increases between 19% and 613 % compared with baseline levels (Figure 2.2). The increase in SVCT2 expression after one day of supplementation correlated significantly with plasma vitamin C measured at day 1 (r=0.559, p=0.032; Figure 2.3A).
In addition, the measured skeletal muscle SVCT2 expression at day 1 predicted the skeletal muscle vitamin C concentration observed at day 7 ($r=0.692$, $p=0.005$; Figure 2.3B); and also the change in skeletal muscle vitamin C concentration from day 1 to day 7 ($r=0.551$, $p=0.036$). These latter correlations demonstrate a temporal relationship between the increase in SVCT2 expression and subsequent increase in skeletal muscle vitamin C concentration.

**Figure 2.2.** Vitamin C supplementation increases skeletal muscle SVCT2 protein expression in healthy individuals.

Top: an example western blot for a participant on days 0, 1, 7 and 42; bottom: SVCT2 protein expression in skeletal muscle during 42 days of supplementation. Data are presented as individual data and as mean $\pm$ SEM. Statistical analyses were conducted on data from $n=7$ participants due to an incomplete data set for one participant. *Denotes significantly different to placebo, $p<0.05$. 

Vitamin C study in healthy males

Figure 2.3. Plasma and skeletal muscle vitamin C concentrations correlate with skeletal muscle SVCT2 protein expression.

A. Spearman’s correlation of plasma vitamin C concentration at t=1 day and the change in SVCT2 protein expression from t=0 to t=1 day. B. Spearman’s correlation of SVCT2 protein expression at t=1 day and muscle vitamin C concentration at t=7 day. A single data point is missing due to insufficient sample for SVCT2 measurement in one participant.

Antioxidants

Data for muscle catalase activity, plasma catalase activity and muscle antioxidant capacity required log-transformation in order to approximate Gaussian normality prior to parametric ANOVA analyses. There were no main effects of treatment or interaction effects observed for the antioxidant measurements in muscle (Figure 2.4) or plasma (data not shown).
Vitamin C study in healthy males

**Figure 2.4.** Vitamin C supplementation does not alter basal skeletal muscle antioxidant enzyme activities and concentrations over 42 days in healthy individuals.

A. Total SOD activity. B. Catalase activity. C. Total antioxidant capacity. D. Total glutathione concentration. Data are mean ± SEM. All analyses were conducted with n=8 participants.

**DCFH oxidation**

Vitamin C treatment had no significant effect on DCFH oxidation in skeletal muscle using two way repeated measures ANOVA (Figure 2.5).
**Figure 2.5.** Vitamin C supplementation does not affect basal skeletal muscle DCFH oxidation over 42 days in healthy individuals.

*Data points are mean ± SEM. Data are for n=8 participants.*

**Citrate synthase activity**

Vitamin C supplementation had no significant effect on skeletal muscle citrate synthase activity (Figure 2.6) when comparing treatments pre- and post-supplementation using two way repeated measures ANOVA (p=0.13 for treatment x time interaction).

**Figure 2.6.** Vitamin C supplementation does not affect skeletal muscle citrate synthase activity after 42 days in healthy individuals.

*Data points are mean ± SEM. Data are for n=8 participants.*
2.5. Discussion

A major finding of the present study was that seven days of vitamin C supplementation (500 mg twice daily) was required to cause a significant increase in vitamin C concentration in skeletal muscle to significantly increase in healthy males with adequate baseline vitamin C concentrations. Vitamin C concentration did not increase further in skeletal muscle after seven days of vitamin C supplementation, although the increase compared to placebo was greatest (~1.3 times placebo) at 42 days. In contrast, plasma vitamin C was more sensitive to vitamin C supplementation, increasing as early as one day post supplementation and reaching a concentration of approximately 1.8 times the placebo level at 42 days. Previously the time course accumulation of vitamin C in skeletal muscle during vitamin C supplementation had not been well characterized in healthy individuals. Indeed, a placebo-controlled study by Hellsten et al. (241) reported similar changes in muscle (~1.4 times) and plasma (~1.8 times) vitamin C to our study after seven days of supplementation with a high dose antioxidant combination including 1500 mg of ascorbic acid in healthy males. However it was uncertain from the Hellsten et al. (241) study how quickly vitamin C concentrations had increased in muscle during supplementation and/or if vitamin C levels would have continued to increase beyond seven days if supplementation had persisted for longer. Another study (233) found supplementation with vitamin C in the form of golden kiwi fruit (53 or 212 mg vitamin C/day) to effectively increase skeletal muscle ascorbate concentrations in marginally vitamin C-deficient males after 42 days. Again it was uncertain from this latter study how quickly muscle vitamin C concentrations had increased in response to vitamin C supplementation. Based on the current findings it is proposed that in future clinical studies investigating effects of exogenous vitamin C in skeletal muscle, at least seven days of high dose
supplementation should be undertaken to ensure skeletal muscle vitamin C concentration is significantly elevated.

A novel finding in the present study was that high dose vitamin C supplementation significantly increased SVCT2 protein expression in skeletal muscle of healthy males. A significant correlation found between the change in skeletal muscle SVCT2 expression and plasma vitamin C concentration would appear to confirm an augmentative effect of supplemental vitamin C on skeletal muscle SVCT2 expression. Findings from previous *in vitro* and *in vivo* experimental studies investigating non-muscle cells and tissue suggest that intracellular ascorbic acid concentration could exert a negative feedback regulation on cellular SVCT2 expression (262, 309). This could potentially limit the effectiveness of high dose vitamin C supplementation in elevating tissue vitamin C levels. However, recent evidence suggests that substrate regulation of SVCT2 expression varies in different types of cells and tissues (310-312). Moreover, findings of this study show that SVCT2 expression increases in human muscle in response to high dose supplementation, therefore likely facilitating intramuscular vitamin C accumulation. This is supported by a significant correlation between SVCT2 expression at t=1 day and the increased skeletal muscle vitamin C concentration measured at t=7 days. Although no significant treatment x time interaction was found, it was clear that the largest difference in SVCT2 expression between treatments occurred at t=1 day (average 2.5-fold greater expression in the vitamin C group). After this time there was only a 1.5-fold average elevation in SVCT2 expression during vitamin C supplementation compared with placebo. Importantly, no decrease in SVCT2 expression below the baseline level was observed after t=1 day during vitamin C supplementation despite an increased skeletal muscle vitamin C concentration. Therefore it appears that skeletal muscle vitamin C concentrations
achievable with this high dose regimen do not negate the intramuscular transport of ascorbic acid in healthy individuals. It should be noted that SVCT2 transport activity was not assessed in this study. A previous study in C2C12 myotubes observed an increase in ascorbic acid transport in association with increased levels of SVCT2 protein expression (236). Thus, SVCT2 protein expression appears to be a good indicator of SVCT2 transporter activity in skeletal muscle.

Vitamin C supplementation did not alter skeletal muscle oxidative stress, as assessed by oxidation of DCFH. This finding is perhaps not surprising given the healthy, vitamin C-sufficient status of our participants. Levine et al (226) found no effect of high dose vitamin C supplementation on systemic lipid peroxidation measures in healthy individuals. On the other hand, high dose vitamin C has been found to improve systemic oxidative stress markers in individuals with chronic oxidative stress (169, 179, 181). The effect of high dose vitamin C on markers of oxidative stress in skeletal muscle of clinical populations with chronic oxidative stress has been little explored however. This will be the focus of the main study in this thesis.

High dose vitamin C supplementation had no effect on total antioxidant capacity or on antioxidant enzyme activities in skeletal muscle. Khassaf et al. (313) similarly found no effect of eight weeks of supplementation with 500mg vitamin C per day on skeletal muscle catalase or SOD activities in healthy males. In another study, lower skin glutathione concentrations were observed in healthy individuals after eight weeks of supplementation with 500mg vitamin C per day during which time skin vitamin C concentrations increased significantly (314). In the latter study the authors speculated that vitamin C supplementation could have promoted a reduction in endogenous antioxidant levels due to their displacement by increased exogenous
Vitamin C study in healthy males

ascorbic acid. Tissue-specific effects of vitamin C supplementation might underlie these different findings in skin and skeletal muscle.

No change in muscle citrate synthase activity was found following vitamin C supplementation. Previous studies in humans and animals have reported attenuating effects of antioxidant supplementation on skeletal muscle mitochondrial biogenesis with or without exercise training (118, 295-297). In contrast, findings of the current study suggest that high dose vitamin C supplementation is unlikely to significantly alter basal antioxidant enzyme activities or mitochondrial content in healthy males. These results lend support to recent studies in healthy humans and animals (315-317) that found no impairment in skeletal muscle mitochondrial biogenesis or antioxidant enzyme induction following high dose antioxidant supplementation. For clinical populations who have oxidative stress and impaired mitochondrial biogenesis in skeletal muscle (92, 141), high dose vitamin C could potentially enhance skeletal muscle antioxidant capacity without further impairing mitochondrial content.

This study demonstrated that high dose oral vitamin C supplementation significantly increases skeletal muscle vitamin C concentrations in healthy males after seven days. Therapeutic interventions aimed at increasing vitamin C concentrations in skeletal muscle might therefore require at least seven days to significantly enhance muscle vitamin C. Supplementation with Vitamin C also increased SVCT2 protein expression in skeletal muscle. Given the potential for regular high dose vitamin C to safely increase skeletal muscle vitamin C without impairing muscle antioxidant activities or mitochondrial content, research of its effects in clinical populations with skeletal muscle oxidative stress are warranted.
3.0. CLINICAL TRIAL METHODS

3.1. Clinical trial characteristics

3.1.1. Study design

The clinical trial was a placebo-controlled trial in people with T2D that involved oral consumption of a capsule containing 500 mg ascorbic acid or a placebo twice daily in the morning and evening for four months (mean ± SEM: 121 ± 1.8 days; Figure 3.1). The study had a cross-over design, and a wash-out period of one month (34.6 ± 2.7 days) separated the two treatments. The trial was undertaken in a double-blinded manner, with a third party individual not directly involved in the study responsible for the assignment of participants to treatments and the concealment of treatment order. Treatment order was randomized via coin toss.

![Figure 3.1: Basic structure of clinical trial](image)

*Figure 3.1: Basic structure of clinical trial*
3.1.2. Study population

It was planned a priori that 12-24 females and males with T2D aged 35-70 years would be recruited to participate in the clinical trial. Sample size was chosen based on: (a) calculations using an online power analysis (318) of data from relevant published research (169, 181) and our own data from the study in Chapter 2 (on page 85); and (b) allowance for participant drop-outs during the trial as a result of the long study duration and invasiveness of study procedures used (Table 3.1). Based on this power analysis, 12-24 participants would provide high power (>90 %) to detect ≈ 50 % changes in whole-body insulin sensitivity, whole-body non-oxidative glucose metabolism and skeletal muscle vitamin C concentration (Table 3.1). On the other hand, 12-24 participants would provide a somewhat underpowered (<50 % power) sample size to confidently detect changes in fasting plasma insulin and HbA1c (169). The proposed sample size also has a high power (>90 %) to detect changes in markers of oxidative stress. However, oxidative stress data used in the power analysis was from plasma only, so calculated power might not be directly applicable to skeletal muscle outcomes. Both females and males were chosen for the clinical trial since T2D affects genders at a similar rate (5). Also, the age range of 35-70 years was chosen since diabetes prevalence is elevated within this age range in Australia (5). Moreover, this age group has not previously been investigated using this study design.
**Table 3.1. Power determination of key variables in major study**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Measure</th>
<th>Δ Mean*</th>
<th>SD†</th>
<th>N (80 %)‡</th>
<th>Power (n=12)§</th>
<th>Power (n=24)ǁ</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insulin Sensitivity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paolisso et al. (169)</td>
<td>WBGD</td>
<td>11.9 (μmol/kg FFM/min)</td>
<td>2.68</td>
<td>4</td>
<td>99%</td>
<td>100%</td>
<td>very relevant high power</td>
<td>WBGD not Δ Rd</td>
</tr>
<tr>
<td>Paolisso et al. (169)</td>
<td>NOGM</td>
<td>11.6 (μmol/kg FFM/min)</td>
<td>2.68</td>
<td>4</td>
<td>99%</td>
<td>100%</td>
<td>very relevant high power</td>
<td>calculated from WBGD not Δ Rd</td>
</tr>
<tr>
<td><strong>Oxidative stress</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Evans et al. (181)</td>
<td>ROS</td>
<td>1.7 (arbitrary)</td>
<td>0.8</td>
<td>10</td>
<td>92%</td>
<td>99%</td>
<td>Relevant high power</td>
<td>in plasma</td>
</tr>
<tr>
<td>Evans et al. (181)</td>
<td>TBARS</td>
<td>9.17 (arbitrary)</td>
<td>1.17</td>
<td>4</td>
<td>100%</td>
<td>100%</td>
<td>Relevant high power</td>
<td>in plasma</td>
</tr>
<tr>
<td>Paolisso et al. (169)</td>
<td>GSH</td>
<td>0.22 (μmol/l)</td>
<td>0.19</td>
<td>14</td>
<td>73%</td>
<td>96%</td>
<td>relevant, good power</td>
<td>in plasma; only an antioxidant measure</td>
</tr>
<tr>
<td><strong>Vitamin C levels</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mason et al. (293)</td>
<td>Total vitamin C in muscle</td>
<td>0.96 (mg/100g wet weight)</td>
<td>0.64</td>
<td>6</td>
<td>99%</td>
<td>99%</td>
<td>very relevant high power</td>
<td>in healthy muscle</td>
</tr>
<tr>
<td>Mason et al. (293)</td>
<td>Total vitamin C in plasma</td>
<td>55.89 (μmol/l)</td>
<td>22.7</td>
<td>4</td>
<td>99%</td>
<td>100%</td>
<td>very relevant high power</td>
<td>in healthy plasma</td>
</tr>
</tbody>
</table>

(*minimal detectable mean difference; †standard deviation; ‡n required for at least 80% power; §power with minimum study sample number; ¶power with maximum study sample number; WBGD-whole body glucose disposal; FFM – fat-free mass; NOGM-non-oxidative glucose metabolism; Rd-skeletal muscle insulin sensitivity; TBARS – thiobarbituric acid reactive substances; GSH – reduced glutathione)

### 3.1.3. Inclusion/exclusion criteria

Inclusion/exclusion criteria for participation included: HbA1c >6.5 % and <10.0 %; total cholesterol <6.5 mmol/l; HDL cholesterol >0.9 mmol/l; triglycerides <4.0 mmol/l; body mass index <35 kg/m²; systolic blood pressure <160 mmHg; diastolic blood pressure <90 mmHg; a non-smoker; no major medical illness apart from T2D; no history of cardiovascular, renal, respiratory or neurological disease; no heart murmur condition or bleeding disorder; no incidence of haemochromatosis or iron
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overload disorder; not taking any diabetic medications other than metformin (≤ 2000 mg total per day), a sulfonylurea and/or a dipeptidyl-peptidase-4 inhibitor (≤ 2 diabetes medications in total); not taking any vitamin or herbal supplements during the month prior to participation; not taking any medications known to influence insulin sensitivity; not engaging in regular intensive exercise training; and not pregnant or planning a pregnancy during the trial. It was furthermore expected that participants demonstrated stable glucose control (Δ HbA1c ± 0.5 % for at least 6 months) prior to the trial. Final approval for participation was granted by our medical doctor (Dr Andrew Garnham) following a medical screening.

3.1.4. Supplementation

Vitamin C capsules contained 500 mg L-ascorbic acid, microcrystalline cellulose, vegetable cellulose and vegetable magnesium stearate (Solgar, Inc, Leonia, NJ) and placebo capsules contained 560 mg gelatine, 8 mg calcium carbonate, vegetable magnesium stearate and vegetable cellulose (Solgar, Inc, Leonia, NJ). Vitamin C and placebo capsules were almost identical in appearance and were provided to participants in identical sealed opaque bottles. Supplements were commercially available products purchased through online retail stores. Capsules randomly selected from vitamin C and placebo bottles were tested for vitamin C content using HPLC. Vitamin C capsules tested in this manner were found to contain >99.9 % of the expected ascorbic acid level while placebo capsules contained 0 % ascorbic acid (data not shown). The dose of vitamin C used (500 mg twice daily) was chosen because it represents a prudent upper limit for vitamin C intake based on estimations of a no observed adverse effect level (189). Moreover, this daily supplemental dose also
Clinical trial methods

represents a commonly recommended intake of vitamin C using commercially available vitamin C tablets and capsules.

3.1.5. Ethics statement

This study was approved by the Deakin University Human Research Ethics Committee (Ethics approval # 210-256). The study was also retrospectively registered in the Australian and New Zealand Clinical Trials registry (registration # ACTRN12614000239662). Participants were required to provide written consent in order to participate and to provide biological samples for analyses.
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3.2. Participant recruitment and screening

A number of different strategies were employed to recruit participants for the T2D clinical trial. These included:

- Melbourne-wide general practitioner (GP) Network (Medicare local) newsletter articles
- Flyer postings at local GP clinics
- Flyer postings at community health centres
- Flyer postings around Deakin University
- Online advertising on the Diabetes Australia Victoria website
- Regular advertising in the Herald Sun newspaper
- Newspaper advertising in the local Whitehorse Leader newspaper
- Oral presentations at Diabetes Support Groups in Melbourne
- A radio interview on 3WBC community radio
- Online community and university noticeboard advertising
- Online clinical research trial website advertising

Participants tentatively approved via telephone and email communications attended a pre-test screening session at Deakin University (Figure 3.2). During this session the following tasks were undertaken: (a) a medical questionnaire was completed; (b) a fasting blood sample was taken; (c) height, weight and blood pressure were measured; (d) a plain language statement of the study was provided and explained in detail; and (e) informed consent for both trial participation and sample provision for research purposes was provided by potential participants. Informed consent from the individual’s GP was further expected in order to participate in the study. This
latter requirement was based on the necessity to withdraw from anti-diabetic medications for two consecutive days on four occasions throughout the trial (see Section 3.3.2 on page 103). Based on clinical screening outcomes and pathology blood test results, eligible participants commenced the clinical trial. A complete summary of individual characteristics of all screened individuals can be found in Appendix 10.1 on page 266.

**Figure 3.2.** Flowchart of participant recruitment and screening in the clinical trial

**Telephone/email screening (n=83)**
- not included due to:
  - medication incompatibility (n=18)
  - trial-related issues (n=16)
    - clinical disease (n=4)
    - recent diagnosis (n=4)
    - smoking (n=2)
  - other/unable to follow-up (n=14)

**Clinical screening (n=25)**
- not included due to:
  - HbA1c low/high (n=7)
  - lifestyle/medication (n=2)
    - withdrew (n=3)

**Participants (n=13)**
- completed (n=7)
- withdrew (n=6) due to:
  - medication-related (n=3)
  - personal-related (n=3)
3.3. Pre-testing preparation

3.3.1. Diet and physical activity checklist

During the seven days prior to the first and third testing sessions (i.e. at pre-supplementation timepoints), participants completed a diet checklist to evaluate usual dietary vitamin C intake. Guided by portion size guides and photographic examples, participants were asked to tick the appropriate portion size box that approximated their daily intake of a specified vitamin C-rich food item. The checklist was derived from the AUSNUT database and included foods and beverages that constitute the largest proportion of dietary vitamin C in the adult Australian diet (188) (Appendix 10.2 on page 267). It also included less commonly consumed foods that are considered rich sources vitamin C and foods and beverages fortified with vitamin C. For quantifying vitamin C intake, foods and beverages consumed were able to be directly and objectively entered into the Foodworks® 2009 software (Xyris Software, Highgate Hill, QLD) for determination of daily intakes. A 24-h diet recall was undertaken for the day prior to the first testing session and this diet was replicated prior to all subsequent testing sessions for each participant.

On day seven, participants completed a brief physical activity survey to assess physical activity performed over the previous week. The survey was derived from the Active Australia Questionnaire (319) which has previously been used in several state and national Australian surveys (319). Estimation of physical activity energy expenditure (in kcals) was undertaken using this data as done previously (320). An example completion of one day of the diet checklist along with the physical activity recall survey is included in Appendix 10.2 on page 267.
Clinical trial methods

3.3.2. Diabetes medication withdrawal

Forty-eight hours prior to each testing session, participants were requested to stop taking their anti-diabetic medications so as not to interfere with measurements associated with insulin and glucose metabolism. Participants were asked to monitor their blood glucose levels during this period of medicine withdrawal; and if glucose levels were observed to increase by more than 3 mmol/l above normal during this time, they were advised to recommence their medications and be withdrawn from the study. During the study no participant deviated by more than 3 mmol/l above their usual blood glucose level during this medication withdrawal period.

Participants fasted from 21:30 on the day prior to testing and visited the laboratory at 07:00 for testing. Participants were asked not to take any medications on the morning of testing days, including vitamin C or placebo supplements.
3.4. Clinical trial testing

3.4.1. Fasting blood samples

At the commencement of each testing day, fasting blood samples were collected for assessment of glycated haemoglobin (HbA1c), fasting glucose, fasting insulin, serum lipids (total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides), renal and hepatic function, plasma vitamin C, plasma F₂-Isoprostanes, plasma lactate, whole-blood glutathione (GSH) and glutathione disulphide (GSSG), and plasma inflammation markers (adiponectin, resistin, interleukin-6, interleukin-8, interleukin-10 and TNF-α; Figure 3.3). Plasma samples taken for vitamin C analysis were combined with an equal volume of 10 % (w/v) metaphosphoric acid (MPA) containing 5mmol/l ethylenediaminetetraacetic acid disodium salt dihydrate (disodium EDTA). For F₂-Isoprostane measurements, 0.005 % (w/v) 3,5-Di-tert-4-butylhydroxytoluene (BHT) was added to plasma. For GSSG measures, 3mmol/l 1-methyl-2-vinylpyridiniumtrifluoromethanesulfonate (M2VP) was added to whole blood samples. Samples collected were either immediately relocated for standard pathology testing (Healthscope Pathology, Melbourne, VIC) or were frozen at -80°C for later analyses.
Following collection of a fasting blood sample, participants underwent infusion of 6,6-D$_2$ glucose tracer. After 150 minutes of basal tracer equilibration, a hyperinsulinaemic, euglycaemic clamp was commenced with infusion of insulin and a 10% glucose solution containing 1% 6,6-D$_2$ glucose. Muscle biopsies and indirect calorimetry were undertaken during basal and insulin-stimulated conditions at the indicated time points. Regular blood samples were taken for measurement of blood glucose and plasma tracer enrichment. Testing was concluded with a measurement of body composition using dual-energy X-ray absorptiometry (DXA).
3.4.2. 6, 6-D2 glucose tracer infusion

A primed intravenous infusion of the stable 6, 6-D2 glucose tracer (99.1 % isotopic purity; Cambridge Isotope Laboratories, Inc., Andover, MA) began at 07:30 on each testing day (Figure 3.3). An infusion bolus of 3 mg tracer/kg bodyweight infused over five minutes was followed by a constant infusion rate of 2.4 mg tracer/kg/h for the remainder of the infusion periods (321). During the infusion periods, plasma samples were taken from a catheterized heated hand vein (≈ 55 °C). Arterialization of the venous blood was regularly verified by measuring oxygen saturation of the blood to be between 90-98 % (322) using an ABL 800 glucose analyser (Radiometer Medical ApS, Brønshøj, Denmark). A basal equilibration period of 150 minutes was allowed for the tracer to reach a steady enrichment level in the body (323). Basal plasma samples for tracer measurements were taken at: -150, -60, -30, -20, -10 and 0 mins prior to clamp commencement. Basal tracer calculations (see Section 4.1.3 on page 112) were determined during the final 30 minutes of the basal period. Figure 3.4 illustrates the pooled change in plasma 6, 6-D2 glucose enrichment as measured during the clinical trial. Levels of tracer MPE in plasma were relatively stable when measured from 90 minutes after commencement of the basal tracer infusion until the end of the hyperinsulinaemic, euglycaemic clamp.
Clinical trial methods

Figure 3.4. 6, 6-D$_2$ glucose tracer enrichment in plasma during the T2D clinical trial

Moles percent excess (MPE) of 6, 6-D$_2$ glucose in plasma samples of trial participants during the glucose tracer infusion procedures after correction for fasting glucose levels. Time = 0 corresponds to commencement of the hyperinsulinaemic, euglycaemic clamp. Data are mean ± SEM (n=32 clamps).

3.4.3. Hyperinsulinaemic, euglycaemic clamp

At time (t) = 0 minutes, a primed intravenous infusion of short-acting insulin (Actrapid, Novo Nordisk, Novo allé, Bagsvaerd) was commenced through a catheter inserted into the arm contralateral to the glucose tracer infusion. A bolus of 9 mU/kg delivered over one minute was followed by a constant infusion rate of 40 mU/m$^2$/min for 180 minutes. Blood glucose measurements were taken at 5-minute intervals during the clamp (Figure 3.3) and glucose was measured using an ABL 800 glucose analyser (Radiometer Medical ApS). Blood glucose was clamped at 5.5 mmol/l by means of a variable infusion of 10 % glucose (Baxter Laboratories, Boronia, VIC) based
Clinical trial methods

on methods of DeFronzo et al. (324). Glucose concentrations of glucose infusates
were measured using the ABL800 analyzer rather than assumed. Glucose infused
contained 1% 6,6-D2 tracer (mean tracer enrichment: 1.14 ± 0.04 MPE, n=32 clamps)
in order to avoid errors associated with inter-compartmental tracer gradients (217).
In the event that participants hadn’t achieved a stable glucose infusion rate by t=180
minutes, the clamp was extended until glucose infusion rates were stable (+/- 10%)
for at least 30 mins. Thereafter, clamp durations were held constant for each
participant for all trials undertaken (mean clamp duration: 196.7 ± 3.7 min, n=32
clamps). Glucose infusion was continued after completion of the insulin infusion
procedure in order to prevent potential residual hypoglycaemic effects of insulin.
Participants were also provided with a small meal at this time in order to restore basal
glucose levels. Once glucose levels of participants had returned to basal levels, the
glucose infusion was stopped.

Plasma samples were taken for tracer measurements during the clamp at t=30, 60,
90, 120, 130, 140, 150, 160, 170, 180 and if necessary every 10 minutes thereafter
until completion of the clamp. Insulin-stimulated tracer calculations (see Section
4.1.3 on page 112) were determined during the final 60 mins of the clamp. Samples
were taken for analysis of insulin levels at t=0, 30, 60, 120, 150 and 180 minutes. A
plasma sample was also taken at t=180 mins for assessment of plasma lactate.
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3.4.4. Indirect calorimetry

Participants breathed expired air into Douglas bags through a mouthpiece-nose-clip indirect calorimetry system (Hans Rudolph, Inc., Shawnee, KS) to calculate resting rates of carbohydrate oxidation, fat oxidation, energy expenditure and respiratory exchange ratio (RER). It was previously reported that mouthpiece-nose-clip indirect calorimetry systems produce similar values for VO\(_2\), VCO\(_2\), RER and resting energy expenditure when compared with ventilated hood systems (325). On each testing occasion, participants underwent a 10-15 minute period of familiarization with the indirect calorimetry system. This was followed by testing for 15-minute periods during both the basal and insulin-stimulated periods (Figure 3.3), from which the final 10-minutes of expired gas was collected for subsequent calculations. A small sample of the expired gas was assessed for % O\(_2\) and % CO\(_2\) using a Moxus modular metabolic system (AEI technologies, Pittsburgh, PA). The metabolic system was calibrated prior to use on each testing day. Ventilation rate was calculated after determining the total volume of expired air using a dry gas metre (Harvard Apparatus, Holliston, MA). VO\(_2\) and VCO\(_2\) were then calculated using standard equations after conversion to STPD values. Rates of carbohydrate and fat oxidation were determined using the equations of Frayn et al. (326) assuming urinary nitrogen excretion as a constant value of 0.009 g/min (327) (see Appendix 10.3 on page 281 for equations). RER was calculated as the ratio of carbon dioxide production to oxygen uptake (VCO\(_2\)/VO\(_2\)).
3.4.5. Muscle biopsies

Muscle biopsy samples were taken firstly during the final 30 minutes of the basal period (basal sample); and secondly, after 60 minutes of insulin infusion during the hyperinsulinaemic, euglycaemic clamp (insulin-stimulated sample; Figure 3.3). Muscle sampling was performed according to the percutaneous needle biopsy sampling method of Bergstrom (299). Briefly, a local anaesthetic (Lidocaine) was injected into the fascia of the vastus lateralis muscle and a small scalpel incision was made. The biopsy needle, with an inside cutting cylinder, was inserted through the incision site into the underlying muscle belly. Samples of ~100-200 mg were obtained via syringe suction. After the brief removal of visible blood and connective tissue, samples were rapidly frozen in liquid nitrogen and stored until analysis. Subsequent biopsy samples on the same day were taken approximately 1-2 cm proximal to the previous sampling site. The sampling leg was alternated on different trial days.

3.4.6. Dual-energy X-ray Absorptiometry

Body composition including fat-free mass, fat mass and percent body fat, were determined using a Lunar Prodigy whole-body scanner (GE Medical Systems, Madison, WI) in Total Body scan mode after completion of the clamp procedure (Figure 3.3). Briefly, the participant lay still in the supine position and was subjected to low-dose X-rays from a mobile scanner. The different body components (fat, lean body mass & bone) produce distinct X-ray absorption profiles whereby fat and lean composition can be calculated from the linear decomposition of the localised tissue X-ray attenuation according to calibrated samples (328).
4.0. ANALYTICAL RESEARCH METHODS

4.1. Glucoregulatory Measurements

4.1.1. Glucose, lipid, organ function & inflammation measures

Fasting blood samples collected for HbA1c, serum cholesterol (total, LDL-C & HDL-C), serum triglycerides and serum renal and hepatic function were measured according to standardized laboratory procedures by a commercial pathology laboratory (Healthscope Pathology, Melbourne, VIC). Whole blood fasting glucose was measured using an ABL 800 glucose analyser (Radiometer Medical ApS). General indices of basal glucose control including Homeostasis Model Assessment (HOMA)-IR (insulin resistance), HOMA-S (insulin sensitivity) and HOMA-B (beta cell function) were calculated using fasting values for glucose and insulin with an online HOMA-2 calculator (http://www.dtu.ox.ac.uk/HOMACalculator). Basal plasma inflammation-related measures, including adiponectin, resistin, interleukin-6, interleukin-8, interleukin-10 and TNF-α were measured using a human adipokine magnetic bead multiplex assay (Cat # HADK1MAG-61K; EMD Millipore, Billerica, MA) and a human cytokine magnetic bead multiplex assay (Cat # HCYTOMAG-60K; EMD Millipore) according to the manufacturer’s instructions on a Bio-Plex Multi-plex system (Bio-Rad Laboratories, Hercules, CA).

4.1.2. Plasma insulin

Plasma insulin was calculated during all trials at time points: t=-150 (fasting), t=0, t=30, t=60, t=120, t=150 and t=180 mins using a sandwich ELISA assay (Cat # EZHI-14K; EMD Millipore) according to the manufacturer’s instructions. Insulin levels
obtained using this specific assay kit have previously been shown to produce insulin concentrations approximately 50% of the absolute values obtained using radioimmununassay methods (329). Thus, for purposes of comparability with previous studies, insulin concentrations of fasting \((t=-150)\) and insulin-stimulated \((t=180)\) plasma samples were also measured using a human-specific radioimmunassay kit (Cat # HI-14K; EMD Millipore) on an automatic gamma counter (Wallac Wizard 1470, Waltham, MA).

4.1.3. 6, 6 D₂-glucose tracer measurements

Enrichment of 6, 6-D₂ tracer in plasma samples was assessed using electron-ionizing gas chromatography-mass spectrometry (GC-MS). Methods were adapted from McConell et al. (330). In brief, 50 μl of plasma was deproteinized with 50 μl 0.3 mol/l BaOH₂ and 50μl 0.3 mol/l ZnSO₄. 80 μl of supernatant was then dehydrated and subsequently derivatized with 100 μl of a 2:3 pyridine/acetic anhydride solution into glucopyranose pentaacetate derivatives. Samples were injected using a 1:50 split ratio onto a HP-5MS 5 % Phenyl Methyl Siloxane column (30.0 m x 250 μm x 0.25 μm; Agilent technologies, Santa Clara, CA) connected to an Agilent 6890 Gas Chromatograph. Target compounds were detected via an Agilent 5975 Mass Spectrometer. The GC program consisted of a 20 °C/min ramp starting at 100 °C. A final temperature of 260 °C was then held for five minutes. Hydrogen was used as the carrier gas with a flow rate of 1.1 ml/min. The mass ratio of derivatized tracer (100 m/z) to derivatized glucose (98 m/z) was used in the determination of tracer MPE:
**Analytical methods**

\[
MPE = \frac{100 \text{ m/z} - (100/98 \ [0 \text{ MPE}] \times 98 \text{ m/z})}{100 \text{ m/z} + 98 \text{ m/z} - (100/98 \ [0 \text{ MPE}] \times 98 \text{ m/z}) - (98/100 \ [100 \text{ MPE}] \times 100 \text{ m/z})}
\]

(where 100/98 [0 MPE] = 100/98 m/z ratio determined from 0 MPE standard; and 98/100 [100 MPE] = 98/100 m/z ratio determined from 100 MPE standard)

Sample MPE was determined from standard curves created from 6, 6-D and standards ranging from 0 to 12.5 MPE. MPE of glucose and tracer infusates used in trials was measured via GC-MS and not assumed. Calculations of rate of glucose disappearance (Rd) and rate of glucose appearance (Ra) were calculated according to formulas of Steele for single-pool, non-steady-state conditions (331) modified for use with stable tracers (323). The pool fraction was assumed to be 0.65 and the glucose space as 25% in calculations (323).

Clamp Rd was calculated as the average of each 10-minute Rd value during the final 60 minutes of the euglycaemic clamp. Basal Rd was calculated as the average of each 10-minute Rd value during the final 30-minutes of the basal period. Insulin-stimulated glucose disposal (ΔRd) was calculated as the difference between clamp Rd and basal Rd. A failure to account for effects of basal glucose disposal on glucose disposal during the clamp can lead to an underestimation of the defect in insulin-stimulated glucose disposal in T2D (332, 333). Clamp Ra, basal Ra and ΔRa were calculated in the same manner as for Rd. Glucose metabolic clearance rate (MCR) was calculated as Rd divided by blood glucose level. Clamp MCR, basal MCR and ΔMCR were calculated in the same manner as for Rd. Endogenous glucose production (EGP) was calculated as the difference between Ra and rate of exogenous glucose infusion. Clamp EGP, basal EGP and ΔEGP were calculated in the same manner as for Rd. Non-oxidative glucose metabolism (NOGM) was calculated as the difference between Rd
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and carbohydrate oxidation rate determined using indirect calorimetry. Clamp NOGM, basal NOGM and Δ NOGM were calculated in the same manner as for Rd. Equations used to determine tracer-derived variables are provided in Appendix 10.3 on page 281. The optimization of GC-MS methods is discussed in Section 5.1 on page 127.
4.2. HPLC measurements

4.2.1. Vitamin C & uric acid concentrations

Ascorbic acid (AA) and total ascorbic acid (AA + dehydroascorbic acid [DHA]; TAA) concentrations were measured in muscle and plasma samples using high performance liquid chromatography (HPLC) coupled with UV detection (Agilent technologies Inc. 1100 series, Santa Clara, CA). Methods of analysis were adapted from Lykkesfeldt (243) and Wechtersbach & Cigic (300). Samples were eluted on a Luna C18 column (particle size 3 μm, 150 x 4.6 mm i.d., Phenomenex Inc., Torrance, CA) connected to a Security Guard C18 column (Phenomenex Inc., Torrance, CA). 1.0 L mobile phase consisted of: (A) 129 mmol/l sodium dihydrogen phosphate monohydrate, 215 mmol/l sodium acetate anhydrous, 1 mmol/l disodium EDTA and 189 μmol/l n-docyltrimethylammonium chloride dissolved in 990 ml Milli-Q®, combined with (B) 36.6 μmol/l tetraoctylammonium bromide dissolved in 10ml methanol. The combined solution was pH-adjusted to 4.8 using orthophosphoric acid. The mobile phase was filtered under vacuum through a 0.45 μm filter.

Frozen muscle samples (25-35 mg) were homogenized in a glass-glass Duall tissue grinder (Kimble Chase, Rockwood, TN) on ice in 5% (w/v) MPA containing 5 mmol/l disodium EDTA. Muscle sample homogenates and defrosted plasma samples were centrifuged for 3 minutes at 16,000 g. Sample supernatants were then filtered through 0.2 μm syringe filters and 200 μl was transferred to two amber autosampler vials, to which an either an equal volume of a 5 % (w/v) MPA solution containing 5mM disodium EDTA (for AA analysis) or an equal volume of a 5% (w/v) MPA solution containing 5mM disodium EDTA and 20 mmol/L tris (2-carboxyethyl) phosphine (TCEP; for TAA analysis) was added. Vials were flushed with a light stream of nitrogen.
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gas for 20-30 seconds and capped. To facilitate reduction of sample DHA to AA, TAA samples were incubated at 20 °C for 120 minutes. 45 μL of sample was injected onto the column for analysis. Column flow rate was 0.6 ml/min. AA was detected at a wavelength of 265 nm and had a retention time of approximately 3.2 minutes. DHA was calculated as the difference between TAA and AA. The percentage of DHA as a fraction of TAA (DHA/TAA) was considered as a measure of the vitamin C redox status.

Measurement of uric acid (UA) in samples served as an endogenous internal standard for the calculation of DHA as previously recommended (243). UA was measured at a UV wavelength of 300 nm and had a retention time of approximately 6 minutes. AA standards were prepared fresh daily in mobile phase and both AA and UA standards, ranging from 0.63 μmol/l to 100 μmol/l, were run daily to generate standard curves for calculation of sample concentrations. All linear regression standard curves were \( r^2 > 0.99 \). HPLC optimization experiments are outlined in Section 5.2 on page 134.
4.3. Oxidative stress measurements

4.3.1. F₂ Isoprostanes

Muscle samples (25-35 mg) were homogenized 1:20 (w/v) in a 0.1 mol/l phosphate buffer containing 1 mmol/l EDTA and 0.005 % (w/v) BHT using polypropylene pellet grinders on ice. This was followed by 2 x 20 s bursts of ultrasonication on ice (Vibracell™ Sonics & Materials inc., Newtown, CT). Muscle homogenates and plasma samples (stored in 0.005% BHT) were mixed 1:1 with 15 % KOH, flushed with a light stream of nitrogen gas for 20-30 s, and incubated for 60 minutes at 40 °C to facilitate hydrolysis of esterified F₂-Isoprostanes. This allows for the measurement of total F₂-Isoprostanes (i.e. bound + free) in samples. Following this step, 8 x sample volumes of ice-cold ethanol containing 0.01 % (w/v) BHT were added to samples. Samples were incubated on ice for 10 minutes and then centrifuged for 10 minutes at 3300 g to precipitate out proteins. Sample supernatants were then dried using vacuum centrifugation (HetoVac; Heto, Scandinavia). Dried samples were re-suspended in 30 % acetic acid (final pH 4.0), centrifuged to remove any remaining undissolved debris, and then applied to C18 reverse-phase solid phase extraction columns (Item #400020 Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions. F₂-Isoprostanes in both skeletal muscle and plasma samples were eluted in a 1 % methanol: ethyl acetate solution, dried under vacuum centrifugation, and then diluted 1:3 in 8-Isoprostanes EIA kit assay buffer (Cayman Chemical). Total F₂-Isoprostane concentrations were determined in samples using a commercial EIA assay kit (8-Isoprostane EIA kit; Cayman Chemical) according to the manufacturer’s instructions. Plasma samples prepared in the manner above reportedly correlate highly ($r^2=0.99$) with F₂-Isoprostane measurements by GC/MS methods. Data from
optimization experiments in muscle and plasma are provided in Section 5.3.1 on page 141.

4.3.2. DCFH assay

2',7'-dichlorofluorescein (DCF) was used as a fluorescent probe to measure levels of reactive species in skeletal muscle homogenates (304, 305). This assay was undertaken twice, firstly as a stand-alone oxidative stress assay; and secondly as a post-hoc investigation of oxidative stress in both basal and insulin-stimulated muscle. In the first experiment, insulin-stimulated muscle samples (≈10-15 mg) were homogenized 1:50 (w/v) in assay buffer consisting of 130 mmol/l KCl, 5 mmol/l MgCl₂, 20 mmol/l NaH₂PO₄, 20 mmol/l Tris-HCl and 30 mmol/l glucose, pH 7.4 (304). In the second experiment, basal and insulin-stimulated muscle samples were homogenized 1:10 (w/v) in a buffer consisting of 25 mmol/l Tris-HCl (pH 7.4), 1mM EDTA and 1mM EGTA. Samples in the second experiment were then diluted 1:5 in the above assay buffer. In both experiments, 10 μl of sample was added in duplicate to 96-well fluorescent plates, to which 290 μl of assay buffer was further added. The reaction was initiated with addition of 1.2μL 2',7-Dichlorodihydrofluorescein diacetate (DCFH-DA; Cayman Chemical) dissolved in methanol (5 μmol/l final concentration). To facilitate deacetylation of DCFH-DA to DCFH by sample esterases, plates were incubated at 37 °C for 15 minutes in the dark. DCFH was then rapidly oxidized to fluorescent DCF in the presence of sample reactive species, including ROS such as H₂O₂, HO· and ROO'; and RNS such as ·NO and ONOO⁻ (306).

The rate of change in fluorescence was calculated using a Biotek® Synergy 2 spectrophotometer (Biotek Instruments, Inc., Winooski, VT) with an excitation...
wavelength of 485 nm and an emission wavelength of 528 nm every 3-minutes over 60 minutes. After 60 minutes, samples were spiked with 1667 μmol/l H₂O₂. The addition of H₂O₂ caused a rapid increase in fluorescence in all samples over a linear range for at least 30 minutes. This demonstrated that added DCFH was not saturated by oxidant levels of samples. Sample concentration was expressed as H₂O₂ equivalents (μmol) per mg protein. Δ DCF fluorescence was calculated as the difference between clamp DCF fluorescence and basal DCF fluorescence. The measurement of Δ DCFH oxidation could reflect an insulin-stimulated antioxidant measurement in people with type 2 diabetes (334, 335). Optimization experiments for the DCFH assay in skeletal muscle are provided in Section 5.3.2 on page 144.

**4.3.3. GSH and GSSG**

Muscle samples (~45 mg) were freeze-dried and then powdered. Visible portions of connective tissue and fat were removed and samples were portioned out and weighed as two samples: one for total glutathione (TGSH) and one for GSSG. For TGSH analysis, muscle samples were homogenized 1:80 (w/v) in 5% MPA. For GSSG analysis, muscle samples were homogenized 1:80 (w/v) in a buffer containing 5% MPA and 3 mmol/l M2VP. M2VP acts as a scavenger of GSH in samples, thus allowing for the determination of GSSG levels only (336). Homogenized samples were centrifuged for 15 minutes at 16,000 g to pellet out protein. Sample supernatants were diluted for assay: 1:40 for GSH and 1:8.5 for GSSG based on prior assay optimization for linearity based on protein concentration and time (see Appendix 10.4 on page 282). Glutathione concentration of samples was measured based on methods of Tietze (337) using a commercially available assay kit (Bioxytech ®...
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GSH/GSSG-412™, OxisResearch, Portland, OR). The assay involved the reaction of dithiobis-2-nitrobenzoic acid (DTNB) with glutathione in the presence of glutathione reductase. In this reaction, all GSSG present in the sample is reduced to GSH, allowing for the measurement of TGSH. The rate of change of absorbance at 412 nm reflects TGSH concentration (337); and was measured over three minutes following sample treatment with nicotinamide adenine dinucleotide phosphate (NADPH). Reduced GSH was calculated according to the formula: GSH = TGSH – 2 x GSSG. GSH and GSSG levels of blood samples were determined according to the assay kit recommended methods. The ratio of reduced GSH to GSSG (GSH/GSSG) was considered as a measure of oxidative stress. All assay plates included protein sample controls that demonstrated the linearity of the assay in relation to the protein concentration of samples (see Appendix 10.4 on page 282).
4.4. Muscle enzyme activity and metabolite measures

4.4.1. Nitric oxide synthase activity

Basal and insulin-stimulated samples (~10 mg) were homogenized 1:10 (w/v) in a buffer containing 250 mmol/l Tris-HCL (pH 7.4), 10 mmol/l EDTA and 10 mmol/l EGTA (item #10007063, Cayman Chemical) using polypropylene pellet grinders on ice. Samples were then centrifuged for 10,000 \( g \) for 5 minutes at 4 °C. Supernatants were assayed for total NOS activity using a radioactive-based assay kit (item #781001; Cayman Chemical) according to the manufacturer’s instructions. The assay is based on the biological conversion of L-arginine to nitric oxide and L-citrulline by NOS. In brief 5 μl of sample supernatant was added to 45 μl of reaction mixture containing 25 mmol/l Tris-HCl (pH 7.4), 3 μmol/l tetrahydrobiopterin (BH4), 1 μmol/l flavin adenine dinucleotide, 1 μmol/l flavin adenine mononucleotide, 1 mmol/l NADPH, 1 μl \( [^{3}H] \) arginine (1 μCi/μl), 75 μmol/l CaCl\(_2\) and 0.1 μmol/l calmodulin (all final concentrations indicated). Samples were then incubated at room temperature for 30 minutes followed by the addition of 400 μl of Stop buffer containing 50 mmol/l HEPES (pH 5.5) and 5 mmol/l EDTA. Equilibrated resin was added to samples (1:5 v/v), which were transferred to spin cups and centrifuged at 22,000 \( g \) for 5 minutes. Sample eluates were then transferred to scintillation vials and radioactive citrulline was quantitated using a liquid scintillation counter (Tricarb\(^{®}\), Perkin Elmer, Waltham, MA). A positive control (murine recombinant iNOS) was used in experiments. To determine sample NOS activity, citrulline counts from a “blank” sample treated with the NOS inhibitor nitro-L-arginine (L-NNA) was subtracted from each sample count. Optimization experiments are presented in Section 5.4 on page 147. NOS activity was expressed as pmol citrulline formed per gram protein per minute.
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4.4.2. Superoxide dismutase activity

Muscle samples (10-15 mg) were homogenized (1:50) in a buffer consisting of 100 mmol/l NaH₂PO₄ (pH 7.0) with 1 mmol/l EDTA and 0.5 % Triton-X100 using polypropylene pellet grinders on ice. A prior optimization of lysis buffer was undertaken for this assay (see Appendix 10.5 on page 284). Samples were centrifuged at 1500 g for 10 minutes to remove cellular debris. Supernatants were then diluted 1:10 in assay buffer consisting of: 50 mmol/l Tris-HCl (pH 8.0), 0.1 mmol/l diethylenetriaminepentaacetic acid (DTPA) and 0.1 mmol/l hypoxanthine for assay. Total superoxide dismutase activity (including Cu/Zn SOD, MnSOD and FeSOD) was determined using a commercial spectrophotometric assay kit (item #706002; Cayman Chemical) that utilized a tetrazolium salt to detect ·O₂⁻ produced by xanthine oxidase and hypoxanthine. All assay plates included protein sample controls that demonstrated the linearity of the assay in relation to the protein concentration of samples (see Appendix 10.5 on page 284).

4.4.3. Citrate synthase activity

Citrate synthase activity was assessed as a marker of mitochondrial content in skeletal muscle (338). Muscle samples were prepared as for the SOD assay above with an additional freeze-thaw cycle. Assay buffer consisting of 66 mmol/l Tris (pH 8.3), 0.6 mmol/l Acetyl CoA, 0.1 mmol/l DTNB and 0.6 mmol/l oxaloacetate (all final concentrations) was added to 5μl sample supernatant in 96-well plates and the change in absorbance was read over 5 minutes at 412 nm on a spectrophotometer. The assay was based on the reaction of acetyl-CoA with oxaloacetate and the
subsequent reaction of the formed thiol-bound CoA-SH with DTNB (307). Citrate synthase activity was expressed as \( \mu \text{mol per gram protein per minute} \) assuming an extinction coefficient of 13,600 M\(^{-1}\)cm\(^{-1}\) for DTNB (339).

**4.4.4. Muscle glycogen assay**

Freeze-dried powdered muscle samples (\( \approx 1 \text{ mg} \)) were assessed for glycogen concentration as previously described (340). In brief, samples were incubated in 2M HCl for two hours at 95-100 °C followed by neutralization with 0.667 mol/l NaOH. Samples were then assayed in a buffer cocktail consisting of 50 mmol/l Tris, 1 mmol/l MgCl\(_2\), 0.5 mmol/l DTT, 0.3 mmol/l ATP (pH 7.0), 50 \( \mu \text{mol/l} \) NADP\(^+\) and G-6-P-DH. 2.5 \( \mu \text{l} \) of sample was combined with 250 \( \mu \text{l} \) of buffer and read on a fluorometer at 365 nm. Hexokinase was then added to samples which were incubated in the dark for 1h. Sample absorbances were then read spectrophotometrically (341) and glycogen concentration determined from NADH and glucose standards. Glycogen concentration was assessed in both basal and insulin-stimulated muscle samples.

**4.4.5. Total protein analysis**

Total protein content of muscle samples used in assays was determined using the bicinchoninic acid assay (308) with bovine serum albumin as the reference standard.
4.5. Western blot analyses

4.5.1. SVCT2 and NOS isoform expression

Muscle samples (20-25 mg) were homogenized on ice in a freshly prepared ice-cold buffer consisting of 50 mmol/l Tris (pH 7.5), 1 mmol/l EDTA, 10 % (v/v) Glycerol, 1 % (v/v) Triton X, 50 mmol/l NaF, 5 mmol/l NaPP, 1 mmol/l DTT, 1 mmol/l PMSF, and 5 μl/ml protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO #P8340, consisting of 104 mmol/l AEBSF, 80 μmol/l Aprotinin, 2 mmol/l Leupeptin, 4 mmol/l Bestatin, 1.5 mmol/l Pepstatin A and 1.4 mmol/l E-64)(340). Samples were then centrifuged for 15 minutes at 13,000 g and supernatants frozen at -80 °C until analysis.

4 x Loading buffer (40 % v/v glycerol, 285 mmol/l SDS, 250 mmol/l Tris [pH 6.8], 400 mmol/l DTT and 1 % w/v bromophenol blue) was added to muscle samples (total protein: 20 μg for nNOS, 30 μg for eNOS and 60 μg for SVCT2), boiled for 5 minutes at 95 °C, and then loaded into 8-10 % polyacrylamide gels. The protein loading amount for each target protein was determined from previous optimization experiments that established the optimal range of linearity for protein quantification (refer to Section 5.5 on page 149 for SVCT2 optimization). Gels were subjected to electrophoresis and transferred onto PVDF membranes via electroblotting. Membranes were blocked in 5 % bovine serum albumin, followed by an overnight incubation at 4 °C with a mouse monoclonal anti-nNOS antibody (BD Biosciences; Franklin Lakes, NJ, diluted 1:1000), a mouse monoclonal anti-eNOS antibody (BD Biosciences, diluted 1:1000) or a goat polyclonal anti-SVCT2 antibody (S-19; Santa Cruz Biotechnology, Dallas, TX, diluted 1:400). Membranes were then incubated with a relevant secondary antibody (LI-COR IRDye® 680LT, LiCor Biosciences, Lincoln, USA). Detection and quantification of target proteins were made using the Odyssey® Infra-
red Imaging system (LiCor Biosciences). A primary mouse monoclonal antibody for iNOS (Santa Cruz) was also tested on muscle samples ranging from 20-80 μg protein, but no detectable reactive band was observed despite a strong band found for recombinant iNOS (data not shown).

A mouse monoclonal α-tubulin antibody (Cell Signaling, Beverly, MA) was used as a protein loading control for nNOS and eNOS. A mouse polyclonal GAPDH antibody (Sigma-Aldrich) was used as a protein loading control for SVCT2 due to the co-localization of α-tubulin with SVCT2 on western blots. In addition, on each gel, control samples containing protein amounts both below and above the target protein amount were loaded. These additional control samples demonstrated a linearity of quantification in the range of sample loading for both the target protein and the loading control. For example, loading 20ug and 40ug of total protein for the control sample on each gel/membrane resulted in a doubling of signal intensity (302). Target proteins were normalized to loading controls for statistical analyses.

Rat gastrocnemius muscle samples were used as positive controls for both nNOS and eNOS expression in human muscle homogenates. Enriched human blood platelet fractions were used as positive controls for SVCT2 western blotting in muscle homogenates. For platelet isolation, whole blood was collected from a single participant in EDTA tubes containing citrate buffer and centrifuged at 200 g for 15 minutes. The platelet rich fraction was then mixed 10:1 with acid-citrate-dextrose buffer and centrifuged at 900 g for 15 minutes. The washed pellet was re-suspended in a buffer containing 25 mmol/l Tris (pH 8.0), 1 mmol/l EDTA, 0.5 % SDS and 5 μl/ml protease inhibitor cocktail (Sigma P8340) and subjected to ultra-sonication (262). Optimization of western blotting is outlined in Section 5.5 on page 149.
4.6. Statistics

Prior to parametric analyses, data normality was evaluated using a Shapiro Wilk test. When datasets violated the assumption of Gaussian normality, they were log-transformed. Vitamin C (VC) and placebo (PLA) trials were compared using the two-way repeated measures ANOVA with SPSS software (v. 21, IBM Corporation, Armonk, NY). Statistically significant treatment versus time interactions found were further assessed using Bonferroni-corrected multiple comparisons with Prism software (v.6.0, GraphPad Software Inc., La Jolla, CA). For select measures of glucose metabolism and oxidative stress that involved both basal and insulin-stimulated measurements, a general effect of hyperinsulinaemia (basal vs. insulin-stimulated) was determined using paired t-tests with pooled data for treatment (VC, PLA) and time (Pre treatment, Post treatment). Paired t-tests were also used to compare self-reported vitamin C intake and physical activity energy expenditure between VC and PLA trials. Pearson’s correlations were used to assess associations between vitamin C concentration and oxidative stress and glucoregulatory measures; and for testing the repeatability of vitamin C intake between trial #1 and trial #2 using the 7-day diet checklist. Data are presented as mean ± SEM and as individual values. Log-transformed data are presented as geometric mean ± 95% CI. p<0.05 was considered significant for all analyses. No significant differences were found for any measured variable at baseline when comparing vitamin C and placebo trials using two-tailed paired t-tests (data not shown). Power analyses from previous published data (169) and our own data (Chapter 2, on page 85) determined that n=6 participants were required to complete the trial to ensure >80 % power for both a 50 % increase in levels of insulin-stimulated Rd (+11.9 ± 2.7 μmol/kg FFM/min; mean increase ± SD) and muscle vitamin C (+9.6 ± 0.6 μg/g tissue) (318) (see Table 3.1 on page 97).
5.0. OPTIMIZATION OF ANALYTICAL METHODS

5.1. Optimization of GC-MS methods

Determination of 6, 6-D_2 glucose tracer enrichment using GC-MS required prior optimization experiments to validate specificity and sensitivity of measurements. The protocol was based on quantification of the ratio of 100 m/z to 98 m/z mass products detected after sample derivatization with acetic anhydride and pyridine and electron ionization as done previously (330). The protocol required adaptation from helium to hydrogen as a carrier gas. A wide spectrum mass scan of derivatized 6, 6-D_2 glucose tracer standards and plasma samples was firstly undertaken to confirm the specificity of the 100/98 m/z ratio for discriminating 6, 6-D_2 glucose in samples. Figure 5.1A illustrates two glucose pentaacetate stereoisomers identified in a scan of a plasma sample spiked with a small amount (≈ 5 % MPE) of 6, 6-D_2 glucose tracer.

Figure 5.1B shows the total ion mass scan within the first stereoisomer peak. It can be seen that 98 m/z is of much higher abundance when compared with 100 m/z in human plasma. Similarly, Figures 5.1C and 5.1D illustrate peaks of a derivatized sample of 99.1 % isotopically pure 6, 6-D_2 glucose and its mass scan. In contrast to plasma, 100 m/z has proportionally higher abundance than 98 m/z in 6, 6-D_2 glucose (Figure 5.1D). Figure 5.1E shows selected peaks of a 50 % MPE 6, 6-D_2 glucose standard and its mass scans when favouring either the 100 m/z peak (Figure 5.1F) or the 98 m/z peak (Figure 5.1G). It can be seen that the 98 m/z and 100 m/z masses are similar in terms of their relative abundance in this mixed sample. Collectively these preliminary scans demonstrate that 98 m/z and 100 m/z mass products are sensitive to changes in 6, 6-D_2 glucose concentrations in samples.
Figure 5.1. Verification of 100/98 m/z ratio for quantification of 6, 6-D₂ glucose in samples

Total ion chromatograms and mass scans for: A & B: a plasma sample with ≈ 5 % MPE 6, 6-D₂ glucose; C & D: neat 6,6-D₂ glucose; and E-G: 50 % MPE 6, 6-D₂ glucose (refer to text for their descriptions).
A more sensitive GC-MS method was subsequently developed to selectively monitor only the 98 m/z and 100 m/z products in samples. The GC program consisted of a 20 °C/min ramp starting at 100 °C. A final temperature of 260 °C was then held for five minutes. Hydrogen flow rate was set at 1.1 ml/min. A split ratio injection of 1:50 was chosen based on a trade-off between high sample throughput and sample sensitivity. A lower split ratio allowed improved sensitivity, however it proved to shorten the life of columns and produce a more frequent need to clean the mass spectrometer ion source. The two glucose pentaacetate stereoisomers eluted sequentially, but the separation was adequate for quantification purposes. Only the first of the two stereoisomers was used in calculations of MPE. Typical chromatographic outputs are shown in Figure 5.2 for neat plasma, plasma after 90 minutes of tracer infusion, a 25 % MPE standard, and a 99 % MPE standard with the selective ion mode selected.

Due to the potential of sample matrix effects of added 6, 6-D₂ glucose, standards created in aqueous glucose solution versus plasma were compared. Both aqueous glucose and plasma standards were similar and linear in the range of 0 - 10 MPE as required for the current study (Figure 5.3). Thus, aqueous glucose 6, 6-D₂ glucose standards were used in the current study for determination of plasma sample MPE (Figure 5.4).
Analytical method optimization

A

98 m/z abundance

B

98 m/z abundance

C

98 m/z abundance

100 m/z abundance

100 m/z abundance

100 m/z abundance
**Figure 5.2. Typical chromatographic output for the GC-MS calculations**

98 & 100 m/z peaks and shown for a plasma sample (A), a sample of the same plasma after glucose tracer was infused for 90 minutes (B), a 25 % MPE 6, 6-D\textsubscript{2} glucose standard (C), and a 99 % MPE 6, 6-D\textsubscript{2} glucose standard (D).

**Figure 5.3. Comparison of aqueous and plasma 6, 6-D\textsubscript{2} glucose standards**

Each point on the curve represents a mean of two different derivatized standard samples for a given concentration. 6, 6-D\textsubscript{2} glucose standards were diluted in either equimolar aqueous glucose or equimolar human plasma.
Figure 5.4. A typical standard curve of 6, 6-D$_2$ glucose produced in aqueous glucose

Each point on the curve represents the mean 100/98 m/z ratio of duplicate standards.

Derivatized plasma and aqueous 6, 6-D$_2$ glucose standard samples were stable for at least 24 h at room temperature as evidenced by no change in the 100/98 ratio over a range of different MPE concentrations (data not shown). Finally, a retrospective investigation of the stability of samples when stored at -80 °C was undertaken by re-testing samples for one participant twelve months after the first testing occasion. There was only marginal change in the calculated MPE for all samples after this time (Figure 5.5). Furthermore, the insulin-stimulated calculations for Rd and EGP were very similar after this time (Figure 5.5).
Figure 5.5. Evaluation of stability of plasma tracer samples stored at -80°C

Data points are $n=1$ (mean of duplicate injections). Samples indicated with open boxes were derivatized and measured using GC-MS two weeks after collection and storage at -80 °C. Samples indicated with closed boxes were derivatized and measured 12 months after collection and storage at -80 °C. All samples underwent a single freeze-thaw process. Moles percent excess of 6, 6-D$_2$ glucose is normalized to fasting glucose concentration. $R_d$ and $EGP$ were calculated during the final 60 minutes of the clamp.
Analytical method optimization

5.2. Optimization of HPLC methods

Determination of concentrations of AA in skeletal muscle homogenates and plasma using HPLC methodology required prior optimization experiments to validate specificity and sensitivity of measurements. The protocol was based in part on methodology of Lykkesfeldt (243). Adaptations to the method were required based on the different UV-based detection system compared with electrochemical detection (243) and also to optimize identification of AA in skeletal muscle. Preliminary experiments using neat AA and UA standards enabled the generation of linear standard curves using the HPLC system (Figure 5.6). AA proved highly unstable in aqueous solution, and required the addition of 5 mmol/l EDTA or preparation in mobile phase to ensure stability of standards for measurements. Given the capacity of the HPLC system to measure AA in a linear range of relevance to the biological samples, it was then necessary to determine sensitivity and specificity in skeletal muscle and plasma samples.

![Linear standard curves generated for ascorbic acid (A) and uric acid (B) using HPLC](image.png)

*Figure 5.6. Linear standard curves generated for ascorbic acid (A) and uric acid (B) using HPLC*

*Data points are the mean of duplicate measurements*
Analytical method optimization

Through a large number of experimental manipulations that included altering mobile phase constituent concentrations and pH, modifying sample preparation techniques, and changing run method and UV detection parameters; the most optimal conditions were established for detection of AA and TAA in samples. Illustrative examples of differences in chromatographic output before and after some of these manipulations are outlined in Figure 5.7. Typical chromatograms for muscle and plasma AA and TAA after all of these changes are shown in Figure 5.8. To validate the established conditions for sensitivity and specificity of AA in samples, recovery studies were undertaken for AA and TAA in skeletal muscle (Figure 5.9) and plasma. These studies involved spiking both skeletal muscle and plasma samples with known amounts of AA and comparing the measured concentrations with expected concentrations (Table 5.1). A similar process was also undertaken for UA in both skeletal muscle (Figure 5.10 & Table 5.2 on page 139) and plasma. Recoveries of 93 – 108 % of expected concentrations were found for AA and UA in recovery studies undertaken in both muscle and plasma. Finally, to additionally verify the specificity of the AA peak, samples were treated with the selective AA oxidizer, ascorbate oxidase, in order to selectively attenuate the ascorbic acid peak. An example of this experiment in plasma is illustrated in Figure 5.11). In this example, it can be seen that the AA peak is almost undetectable following 30 minutes of treatment with ascorbate oxidase.

To measure system stability and inter-day variability of AA and UA over the course of all sample testing days, a control plasma sample was run on each day. The control sample demonstrated excellent stability over time, with overall intra-day and inter-day coefficients of variation for peak area of < 5 % for both AA and UA (data not shown).
**Figure 5.7.** Some key methodological alterations made to improve ascorbic acid detection in samples.

A (L-R): improved separation of AA by decreasing the mobile phase methanol content from 25% to 1% (plasma sample); B (L-R): reduced interference of TAA in samples by dissolving 10 mmol/l TCEP in 5% MPA solution and incubating at room temperature for 120 mins instead of using a McIlvaine-TCEP buffer (plasma sample); C (L-R): improved separation of AA in skeletal muscle TAA samples by increasing the mobile phase pH from 4.70 to 4.80. AA – ascorbic acid, UA - uric acid.
Analytical method optimization

Figure 5.8. Typical chromatograms for ascorbic acid in samples

A: Reduced ascorbic acid in skeletal muscle; B: total ascorbic acid in skeletal muscle; C: reduced ascorbic acid in plasma; D: total ascorbic acid in plasma. AA – ascorbic acid
**Figure 5.9.** An example of an ascorbic acid recovery study in skeletal muscle

Chromatograms illustrate the addition of varying amounts of ascorbic acid to a skeletal muscle sample. AA: Ascorbic acid.

**Table 5.1.** Results of AA recovery study in skeletal muscle

<table>
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<tr>
<th>Added (in μmol/l)</th>
<th>Added (in μg/g)</th>
<th>Concentration (μg/g)</th>
<th>Expected (μg/g)</th>
<th>Recovery (%)</th>
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<td>100.66</td>
<td>101.91</td>
<td>108.82</td>
<td>93.65</td>
</tr>
</tbody>
</table>
Figure 5.10. An example of a uric acid recovery study in skeletal muscle

Chromatograms illustrate addition of varying amounts of uric acid to a skeletal muscle sample. UA: uric acid.

Table 5.2. Results of UA recovery study in skeletal muscle

<table>
<thead>
<tr>
<th>Added (μmol/l)</th>
<th>Added (μg/g)</th>
<th>Concentration (μg/g)</th>
<th>Expected (μg/g)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.00</td>
<td>5.82</td>
<td>5.82</td>
<td>-</td>
</tr>
<tr>
<td>5.00</td>
<td>38.43</td>
<td>44.09</td>
<td>44.25</td>
<td>99.63</td>
</tr>
<tr>
<td>25.00</td>
<td>192.15</td>
<td>186.29</td>
<td>197.97</td>
<td>94.10</td>
</tr>
<tr>
<td>50.00</td>
<td>384.30</td>
<td>384.29</td>
<td>390.12</td>
<td>98.50</td>
</tr>
</tbody>
</table>
Figure 5.11. Treatment of a plasma sample with ascorbate oxidase

A: chromatogram of a sample pre-treatment; B: the same sample after 30 minutes incubation with 20 units ascorbate oxidase at 25 °C, pH 5.5-adjusted (note: sample in B. is diluted 1:2 compared to A.)
5.3. Optimization of oxidative stress measures

5.3.1. Optimization of F₂-Isoprostanes assay

Demonstration of suitability of the F₂-Isoprostanes ELISA (8-Isoprostanes, Cayman Chemical) for human skeletal muscle was required based on a prior lack of data in human skeletal muscle homogenates using the assay. Initial experiments using human muscle homogenates, rat muscle homogenates and human plasma demonstrated a non-linearity of quantification with respect to protein concentration and/or dilution using the assay. It was therefore necessary to remove sample interferences through additional purification steps prior to assay. Furthermore, given the presence of F₂-Isoprostanes in both free and esterified forms in vivo (342), an hydrolysis step was also undertaken to enable measurement of total F₂-Isoprostanes in both muscle and plasma samples. It was found that single use reverse phase C18 solid phase extraction columns (Item #400020 Cayman Chemical, Ann Arbor, MI) were more reliable in allowing a consistent measurement of total F₂-Isoprostanes in both skeletal muscle and plasma samples when compared with multi-use commercially available purification columns (Item # 10367, Cayman Chemical; data not shown). Sample hydrolysis, deproteinization and purification steps were undertaken according to recommended methods of the assay kit manufacturer. Linearity of the 8-Isoprostanes assay was then established in both skeletal muscle (Figure 5.12) and plasma (Figure 5.13) with respect to total protein concentration and sample dilution in assay buffer respectively. All assay plates included sample controls that demonstrated the linearity of the assay in relation to the sample protein concentration and/or dilution (Figure 5.14).
Optimization experiment involving three different human muscle samples and their estimated concentrations using the 8-Isoprostanes assay over a range of different protein concentrations. The most optimal collective linear range for sample concentration in the assay is circled, with 1.67 mg/ml used in the assay.

Optimization experiment involving two different extracted human plasma samples and their estimated concentrations using the 8-Isoprostanes assay over a range of different dilutions. The most optimal collective linear range for sample dilution in the assay is circled, with a dilution of 1:3 (plasma: assay buffer) used in the assay.
Analytical method optimization

Figure 5.14 Control samples in skeletal muscle and plasma F2-Isoprostanes assays

Demonstration of linearity of sample protein concentration and dilution in F2-Isoprostanes assays for (A) skeletal muscle and (B) plasma respectively. Data are mean ± SEM (n=3 for skeletal muscle; n=2 for plasma).
5.3.2. Optimization of DCFH assay

The DCFH assay was adapted for use in 96-well fluorescent plates from previous studies in skeletal muscle homogenates (304, 305). This required a number of optimization experiments to extensively verify the accuracy of the assay. Firstly, the linearity of the assay with respect to both protein concentration and time were investigated using a few different homogenization buffers (Figure 5.15). Samples were homogenized in DCFH assay buffer (130 mmol/l KCl, 5 mmol/l MgCl₂, 20 mmol/l NaH₂PO₄, 20 mmol/l Tris-HCl and 30 mmol/l glucose, pH 7.4 (304)) or in western blot lysis buffer (50 mmol/l Tris (pH 7.5), 1 mmol/l EDTA, 10% (v/v) Glycerol, 1% (v/v) Triton X, 50 mmol/l NaF, 5 mmol/l NaPP, 1 mmol/l DTT, 1 mmol/l PMSF, and 5 μl/ml protease inhibitor cocktail [Sigma Aldrich, # P8340]) or in a buffer consisting of 25 mmol/l Tris-HCl (pH 7.4), 1 mmol/l EDTA and 1 mmol/l EGTA. Samples homogenized in all three buffers produced approximately linear rates of fluorescence change over a range of different protein concentrations when measured every 3 minutes during a 60 minute period (Figure 5.15). However, values were lower in the western blot lysis buffer, and thus this buffer was not chosen for use in subsequent assays. Based on these experiments, a sample protein concentration of 2 mg/ml (0.067 mg/ml final assay concentration) was chosen for use in assays. Both the DCFH assay buffer and the Tris/EDTA/EGTA buffer were used in assays. All assay plates included protein sample controls that demonstrated the linearity of the assay with respect to protein concentration of samples (data not shown).
Figure 5.15. Optimization experiments for DCFH assay in muscle homogenates

Optimization included use of different protein concentrations and homogenization buffers. Change in DCF fluorescence was linear over 60 mins and concentrations were calculated over 30 minutes. The most optimal buffers and linear range tested are circled.

Skeletal muscle homogenates exhibited a profound matrix effect with respect to the measured DCF fluorescence after addition of H₂O₂ to samples based on predicted recoveries using an aqueous DCFH-H₂O₂ standard curve (data not shown). Thus, for quantification of DCF fluorescence in samples, an H₂O₂ standard curve was created in pooled muscle sample homogenates. The rate of fluorescence change was shown to be approximately linear for added H₂O₂ concentrations (0-3330 μmol/l) over 60 minutes in all experiments (Figure 5.16). To further demonstrate the redox sensitivity of the assay, samples were treated with either 1 mmol/l GSH (an H₂O₂ scavenger) or 1 mmol/l ascorbic acid (a general ROS/RNS scavenger) in a series of experiments (Figure 5.17). Measured change in fluorescence was attenuated by approximately 60
% in samples after treatment with GSH, while change in fluorescence in samples was decreased by approximately 90% after treatment with ascorbic acid.

![Graph](image)

**Figure 5.16.** Typical standard curve for hydrogen peroxide generated in muscle homogenates

Change in DCF fluorescence in pooled muscle homogenates was linear over 60 mins and concentrations were calculated over 30 minutes.

![Graph](image)

**Figure 5.17.** Effect of redox modulation on DCF fluorescence in skeletal muscle samples

Pooled results of n=10 experiments for demonstration of sensitivity of the DCFH assay to treatment with hydrogen peroxide and antioxidants glutathione (GSH) and ascorbic acid (AA). SAMPLE refers to a human muscle sample. The amount of H₂O₂ added varied in experiments; although 1 mmol/l of GSH and AA were added in each experiment. Data are mean ± SEM.
5.4. Optimization of enzyme activity assays

5.4.1. Optimization of NOS activity assay

Optimization experiments were required to establish the optimal conditions for undertaking the NOS activity assay in skeletal muscle samples. Firstly, linearity of the assay was determined with respect to muscle protein concentration and time. An experiment was conducted where samples ranging from 2.5-10 mg/ml protein were assayed for 15 minutes or 30 minutes at 37 °C according to assay kit instructions. It was demonstrated that the assay was approximately linear for a sample protein concentration between 5mg/ml and 10mg/ml after 15 minutes (Figure 5.18A), but only for 5 mg/ml samples between 15 and 30 minutes. A second experiment was then undertaken to evaluate the temperature conditions of assay for a 5 mg/ml sample over 15-45 minutes. The sample was incubated at either room temperature or 37 °C (Figure 5.18B), for 15 minutes, 30 minutes or 45 minutes. Similar to findings of the first optimization experiment, the assay was linear with respect to time using a 5 mg/ml sample. However, in this experiment, the room temperature incubation produced a slightly more linear trajectory of change in measured radioactive counts between 15-45 minutes when compared with the 37 °C incubation; although they were almost identical after 45 minutes (Figure 5.18B). Therefore, 30-minute room temperature assay incubations using samples containing 5mg/ml protein were used in the final assays.
Figure 5.18. Optimization experiments for the NOS activity assay in skeletal muscle

A: The experiment demonstrated linearity between 5 mg/ml and 10 mg/ml protein samples after 15 mins and also for the 5 mg/ml protein sample between 15 and 30 minutes at 37 °C. B: The experiment demonstrated approximate linearity for the assay performed with 5 mg/ml protein samples over 45 minutes at either room temperature or 37 °C.
5.5. Optimization of western blotting methods

It was necessary to evaluate the effectiveness of western blotting to discriminate SVCT2 protein in human skeletal muscle homogenates. Previous data in non-muscle human cell types has shown that human SVCT2 migrates to approximately 50 kDa using SDS western blotting as compared with 65-75 kDa for murine SVCT2 (236, 256, 259, 260, 262). A goat polyclonal antibody, S-19 (Santa Cruz biotechnology, Inc), was chosen for western blotting as it has been widely used for SVCT2 detection in published research (e.g. (236, 262, 343, 344)). Furthermore, according to the antibody manufacturer’s product data sheet (http://datasheets.scbt.com/sc-9926.pdf), human SVCT2 has a molecular weight of 50 kDa.

Given the previous detection of SVCT2 in human platelets in vivo using this particular antibody, whole blood was collected from a participant for isolation of a platelet-rich portion to serve as a positive control. Both the platelet-rich positive control and human skeletal muscle homogenates were run on a 10 % SDS gel using western blotting. As expected, platelet-rich samples produced a strong reactive band at ≈50 kDa (Figure 5.19). Skeletal muscle also produced a distinct reactive band at ≈50 kDa (Figure 5.19). However, additional bands were also detected at ≈80 kDa and ≈45 kDa (a doublet band) in skeletal muscle, but not in platelets. Since these bands were not detected in the platelet samples, it could be argued that they are not SVCT2-specific. However, one cannot discount the possibility that the higher molecular weight band detected is a glycosylated form; and that the lower molecular weight doublet band is a shorter isoform of SVCT2 in skeletal muscle. In fact, a shorter inactive but inhibitory isoform of SVCT2 has been detected in various cells (345). It should be noted, however, that the 45 kDa doublet was not detectable in all participants’ skeletal...
muscle. In consideration of this information, only the 50 kDa band was consequently chosen for SVCT2 quantification in skeletal muscle.

**Figure 5.19. Detection of SVCT2 in skeletal muscle and platelets using western blotting**

The left lane is a human skeletal muscle homogenate (=100 μg protein), the middle lane is a human platelet-rich sample, and the third lane is a molecular weight ladder.

In order to determine the optimal linear range for detection of SVCT2 in skeletal muscle using western blotting, an optimization experiment based on protein amount loaded was undertaken. Protein amounts ranging from 20 – 100 μg per lane were loaded in duplicate to determine the most optimal amount to load (Figure 5.20). The reactive band quantification was approximately linear across this entire loading range, with proportional increases relative to the amount loaded. A consistent salient band was detectable from approximately 40 μg upwards. Thus, 60 μg was chosen as the loading amount for SVCT2 western blotting. A similar process for determining
Analytical method optimization

protein loading amount used for SVCT2 was also undertaken for both nNOS and eNOS in skeletal muscle, establishing optimal loading amounts of 20 μg and 30 μg total protein respectively (data not shown).

Figure 5.20. Optimization of SVCT2 protein loading for western blotting in skeletal muscle

Top: A representative western blot involving a range of different total protein amounts for optimization of the linear protein quantification range for SVCT2 in skeletal muscle homogenates. Bottom: Measured integrative intensity output from this same experiment in duplicate. Data is mean ± SEM.

On each gel run during the study for SVCT2, nNOS and eNOS, pooled muscle was loaded at amounts less than and more than the amount of total protein loaded for samples in order to demonstrate linearity of quantification for target proteins. An example western blot and summary data for SVCT2, eNOS and nNOS during the study are shown in Figure 5.21.
**Figure 5.21. Control samples run on western blots for SVCT2, nNOS and eNOS**

Doubled protein control samples run on each western blot for (A) SVCT2 and the loading control GAPDH, (B) nNOS and the loading control α-Tubulin, and (C) eNOS and the loading control α-Tubulin demonstrate a doubling of signal intensity. In each image, a representative blot is provided in which each protein amount is loaded in duplicate. At the bottom of each image is the summary data from all western blots during the study. Data are mean ± SEM.
6.0. RESULTS

6.1. General Results

6.1.1. Participant characteristics

Of the thirteen participants that commenced the trial, seven participants were compliant with trial requirements. Three participants were withdrawn during the trial due to alterations in their anti-diabetic medication regimens, and three participants withdrew for personal reasons relating to family, work and health concerns respectively. Capsule compliance, as determined by the equation: 
\[
\text{number of capsules consumed} / \text{number of capsules expected for full compliance} \times 100 \%
\]
were: vitamin C (VC) trial: 97.5 ± 0.9 % and placebo (PLA) trial: 96.6 ± 2.0 %. General characteristics of participants that commenced the trial compared with compliant participants are presented in Table 6.1. Unpaired two-tailed t-test comparisons revealed no significant differences (\(p>0.05\)) between compliant and non-compliant participants for any baseline characteristics (data not shown). Overall, participants had stable moderate glucose control as shown by the HbA1c measures (range 6.5 - 9.4 %) prior to the trial; and twelve out of the thirteen participants were on single or dual oral anti-diabetic medications (see Appendix 10.1 on page 266). Participants were middle-aged (range 40.3 - 68.7 y) and had excess total body adiposity (range 29.5 - 44.0 %) (346). Additional medications for lipid control, blood pressure control and health-specific indications used by participants were maintained during the course of the study (see Appendix 10.1 on page 266).
**Results**

*Table 6.1. Baseline participant characteristics*

<table>
<thead>
<tr>
<th></th>
<th>All Participants</th>
<th>Compliant Participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (Gender)</td>
<td>13 (12 male, 1 female)</td>
<td>7 (6 male, 1 female)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>57.9 ± 2.5</td>
<td>59.4 ± 3.5</td>
</tr>
<tr>
<td>Years since diabetes diagnosis</td>
<td>5.2 ± 1.4</td>
<td>4.1 ± 1.1</td>
</tr>
<tr>
<td>Diabetes treatment (n treatment)</td>
<td>11 Met*, 5 Sulf†, 5 Dpp-4‡, 1 Diet§</td>
<td>7 Met, 2 Sulf, 4 Dpp-4</td>
</tr>
<tr>
<td>Principal ethnicity</td>
<td>10 Caucasian/3 Asian</td>
<td>6 Caucasian/1 Asian</td>
</tr>
<tr>
<td>Height (m)</td>
<td>172 ± 2</td>
<td>173 ± 3</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>89.9 ± 3.1</td>
<td>91.6 ± 3.8</td>
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<tr>
<td>BMI (kg.m⁻²)</td>
<td>30.5 ± 0.8</td>
<td>30.8 ± 1.3</td>
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<tr>
<td>Fat-free mass (kg)</td>
<td>59.3 ± 2.1</td>
<td>59.3 ± 3.3</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>34.7 ± 1.3</td>
<td>36.1 ± 2.1</td>
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<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>135.7 ± 4.0</td>
<td>141.4 ± 5.4</td>
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<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>82.5 ± 2.0</td>
<td>83.1 ± 3.4</td>
</tr>
<tr>
<td>Plasma vitamin C (μmol.l⁻¹)</td>
<td>45.1 ± 3.1</td>
<td>46.6 ± 5.1</td>
</tr>
<tr>
<td>M-value (mg.kg FFM⁻¹.min⁻¹)ǁ</td>
<td>3.5 ± 0.3</td>
<td>3.6 ± 0.5</td>
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<tr>
<td>HbA1C (%)</td>
<td>7.6 ± 0.2</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td>Fasting glucose (mmol.l⁻¹)</td>
<td>9.6 ± 0.5</td>
<td>9.5 ± 0.7</td>
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<tr>
<td>Total cholesterol (mmol.l⁻¹)</td>
<td>4.6 ± 0.3</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol.l⁻¹)</td>
<td>2.4 ± 0.2</td>
<td>2.2 ± 0.3</td>
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<tr>
<td>HDL-cholesterol (mmol.l⁻¹)</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Triglycerides (mmol.l⁻¹)</td>
<td>2.1 ± 0.3</td>
<td>1.7 ± 0.8</td>
</tr>
<tr>
<td>Serum urea (mmol.l⁻¹)</td>
<td>6.3 ± 0.5</td>
<td>6.2 ± 0.7</td>
</tr>
<tr>
<td>Serum creatinine (μmol.l⁻¹)</td>
<td>81.8 ± 2.8</td>
<td>79.7 ± 4.5</td>
</tr>
<tr>
<td>GGT (U.l⁻¹)#</td>
<td>40.8 ± 7.9</td>
<td>48.3 ± 13.2</td>
</tr>
<tr>
<td>AST (U.l⁻¹)**</td>
<td>22.8 ± 1.8</td>
<td>24.6 ± 2.1</td>
</tr>
<tr>
<td>ALT (U.l⁻¹)††</td>
<td>35.3 ± 4.0</td>
<td>36.6 ± 4.6</td>
</tr>
</tbody>
</table>

(Data are expressed as mean ± SEM; *Metformin; †Sulfonylurea; ‡DPP-4 inhibitor; §Diet-only treated; ǁM-value – rate of whole-body glucose metabolism calculated during the final 60 minutes of the hyperinsulinaemic, euglycaemic clamp in 20-minute intervals according to formula of DeFronzo et al. (324); #GGT - serum gamma glutamyl transpeptidase; **AST - serum aspartate aminotransferase; ††ALT - serum alanine aminotransferase)
6.1.2. Adverse effects

One participant reported minor gastrointestinal discomfort during the early stages of the VC trial, although symptoms disappeared after 1-2 weeks. No other adverse events were reported during either VC or PLA trials.

6.1.3. Basal glucose, lipids, body composition and inflammation

There were no statistically significant effects of vitamin C treatment on HbA1c, fasting glucose, HOMA indices, serum cholesterol, triglycerides, renal function or hepatic function when compared with placebo in compliant participants (Table 6.2). Vitamin C supplementation also had no significant effect on plasma levels of adiponectin, resistin, interleukin-6, interleukin-10 or TNF-α (p>0.05 for all comparisons; Table 6.2). A significant treatment x time interaction was found for plasma interleukin-8 (p=0.050; Table 6.2), reflecting increased pre-post levels with vitamin C supplementation compared with decreased pre-post levels with placebo supplementation. However, in relation to this interaction, post-hoc tests were not statistically significant, suggesting an equivocal effect of supplementation on plasma interleukin-8 levels.
### Results

**Table 6.2. Changes in basal characteristics of compliant participants**

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Vitamin C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>91.2 ± 3.9</td>
<td>90.1 ± 3.7</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>172.4 ± 3.0</td>
<td>172.5 ± 2.9</td>
</tr>
<tr>
<td>BMI (kg.m⁻²)</td>
<td>30.7 ± 1.3</td>
<td>30.3 ± 1.1</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>36.1 ± 2.2</td>
<td>35.0 ± 1.9</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>31.3 ± 2.1</td>
<td>30.2 ± 1.9</td>
</tr>
<tr>
<td>Fat-free mass (kg)</td>
<td>59.1 ± 3.4</td>
<td>59.5 ± 3.2</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.3 ± 0.2</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td>Glucose (mmol.l⁻¹)</td>
<td>10.3 ± 0.7</td>
<td>10.0 ± 0.5</td>
</tr>
<tr>
<td>HOMA-IR*</td>
<td>3.4 ± 0.3</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>HOMA-S (%)</td>
<td>30.3 ± 2.0</td>
<td>31.1 ± 3.0</td>
</tr>
<tr>
<td>HOMA-B (%)</td>
<td>57.8 ± 6.7</td>
<td>59.4 ± 7.1</td>
</tr>
<tr>
<td>Total cholesterol (mmol.l⁻¹)</td>
<td>3.9 ± 0.3</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol.l⁻¹)</td>
<td>2.1 ± 0.1</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol.l⁻¹)</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Triglycerides (mmol.l⁻¹)</td>
<td>1.7 ± 0.3</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Adiponectin (μg.ml⁻¹)</td>
<td>10.1 ± 2.5</td>
<td>9.6 ± 2.0</td>
</tr>
<tr>
<td>Resistin (pg.ml⁻¹)</td>
<td>20.9 ± 2.5</td>
<td>20.0 ± 3.1</td>
</tr>
<tr>
<td>Interleukin-6 (pg.ml⁻¹)</td>
<td>10.3 ± 6.5</td>
<td>11.9 ± 5.6</td>
</tr>
<tr>
<td>Interleukin-8 (pg.ml⁻¹)</td>
<td>4.8 ± 0.5‡</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>Interleukin-10 (pg.ml⁻¹)</td>
<td>11.8 ± 2.8</td>
<td>10.7 ± 2.9</td>
</tr>
<tr>
<td>TNF-α (pg.ml⁻¹)†</td>
<td>9.7 ± 1.4</td>
<td>9.6 ± 1.6</td>
</tr>
<tr>
<td>Serum urea</td>
<td>5.3 ± 0.5</td>
<td>5.6 ± 0.5</td>
</tr>
<tr>
<td>Serum creatinine (μmol.l⁻¹)</td>
<td>78.1 ± 5.4</td>
<td>79.4 ± 5.9</td>
</tr>
<tr>
<td>eGFR (ml.min⁻¹.1.73m⁻²)§</td>
<td>83.7 ± 2.9</td>
<td>84.9 ± 2.4</td>
</tr>
<tr>
<td>GGT (U.l⁻¹)</td>
<td>46.3 ± 13.0</td>
<td>47.4 ± 14.1</td>
</tr>
<tr>
<td>AST (U.l⁻¹)</td>
<td>23.6 ± 2.4</td>
<td>24.1 ± 3.1</td>
</tr>
<tr>
<td>ALT (U.l⁻¹)</td>
<td>39.0 ± 7.2</td>
<td>39.6 ± 8.8</td>
</tr>
</tbody>
</table>

*(Data are expressed as mean ± SEM; * as calculated using an online Homeostasis Model-2 calculator [http://www.dtu.ox.ac.uk/HOMACalculator/]; † tumor-necrosis factor-alpha; ‡ indicates main treatment x time interaction; § eGFR-estimated age-adjusted glomerular filtration rate)*
6.1.4. Diet and physical activity measurements

There were no significant differences between VC and PLA trials for self-reported vitamin C intake (VC: 105.6 ± 14.4 mg/day vs. PLA: 99.0 ± 20.7 mg/day, \( p = 0.651 \)) or physical activity energy expenditure (VC: 440.6 ± 95.5 kJ/day vs. PLA: 748.0 ± 188.7 kJ/day, \( p = 0.054 \)) in compliant participants when compared using two-tailed paired t-tests. To further investigate reliability of the vitamin C dietary checklist to assess self-reported vitamin C intake throughout the trial, Pearson’s correlations were undertaken to compare intake in trial #1 and trial #2. The vitamin C dietary checklist was found to be a highly repeatable measure of dietary vitamin C intake in individuals as evidenced by significant correlations between trial #1 and trial #2 vitamin C intake after normalisation to muscle \((r = 0.86, p = 0.013)\) or plasma \((r = 0.97, p < 0.001)\) vitamin C concentration (Figure 6.1).

![Figure 6.1](image)

**Figure 6.1.** Reliability of the 7-day diet checklist for assessment of vitamin C levels

*Repeatability of self-reported vitamin C intake using the 7-day diet checklist for assessment of vitamin C levels in individuals after normalisation to skeletal muscle (A) and plasma (B) vitamin C concentration using Pearson’s correlations*
Results

6.2. Glucose and substrate metabolism

6.2.1. Plasma insulin, clamp glucose and glucose infusion rate

Plasma insulin was significantly increased at t=30 minutes during the hyperinsulinaemic, euglycaemic clamp compared with t=0 minutes ($p<0.001$) and did not change after this time when assessed using the human insulin ELISA kit (Figure 6.2). There was no effect of vitamin C treatment on plasma insulin at any time point when assessed using the human ELISA kit (Figure 6.2). Despite lower absolute values reported for insulin with the ELISA kit compared with the RIA kit, fasting insulin values correlated highly between assays ($r=0.93$, $p<0.001$) using Pearson’s correlation. Similar to the ELISA results, no significant effect of treatment on either fasting ($p=0.352$ for treatment x time interaction; Figure 6.3A) nor clamp ($p=0.086$ for treatment x time interaction; Figure 6.3C) insulin levels were observed when assessed using the human insulin RIA kit.

Figure 6.2. Effect of supplementation on plasma insulin levels as measured using the human ELISA kit

Values are mean ± SEM; Pre: pre supplementation, Post: post supplementation
Figure 6.3. Effect of supplementation on fasting and clamp plasma insulin levels using the human RIA kit

A & B: fasting insulin; C & D: steady-state clamp insulin; A & C: values are mean ± SEM; B & D: values are individual data.

Neither blood glucose nor the glucose infusion rate differed significantly between vitamin C and placebo trials at pre- and post- supplementation time points during the hyperinsulinaemic, euglycaemic clamp (Figure 6.4). Blood glucose decreased progressively over time until 120 minutes during the clamp, after which time it remained stable at 5.5 mmol/L (Figure 6.4). In contrast, the glucose infusion rate increased progressively during the clamp until 120 minutes, after which time it did not change significantly (Figure 6.4).
Figure 6.4. Effect of supplementation on blood glucose and glucose infusion rate during the hyperinsulinaemic, euglycaemic clamp

Blood glucose (left y axis data) and glucose infusion rate (right y axis data) during the clamp pre- and post-supplementation. Data are mean ± SEM; Placebo pre- and post-trials are indicated with negative error bars; Vitamin C pre- and post-trials are indicated with positive error bars; GIR – glucose infusion rate.
Results

6.2.2. Rates of glucose disappearance and metabolic clearance of glucose

Vitamin C treatment had no effect on basal Rd (Figure 6.5A), clamp Rd (Figure 6.5A) or MCR (Table 6.3). There was a trend for clamp Rd to increase following vitamin C after correcting for plasma insulin (Rd/I) ($p=0.078$ for treatment x time interaction). $\Delta$ Rd (insulin-stimulated glucose disposal) significantly increased following vitamin C treatment when compared with placebo ($p=0.009$ for treatment x time interaction; Figure 6.5C). $\Delta$ Rd remained significantly elevated following VC treatment after correcting for the change in plasma insulin during the clamp ($p=0.013$ for treatment x time interaction). $\Delta$ MCR was also significantly increased following vitamin C treatment ($p=0.025$ for treatment x time interaction; Table 6.3).

6.2.3. Endogenous glucose production

EGP decreased significantly from basal levels during the hyperinsulinaemic, euglycaemic clamp by approximately 74 % independent of treatment and time ($p<0.001$). However, vitamin C treatment had no significant effect on basal EGP, clamp EGP or $\Delta$ EGP (Table 6.3).

6.2.4. Rates of substrate oxidation and non-oxidative glucose metabolism

There were no significant differences between basal and clamp values for carbohydrate oxidation rate or resting energy expenditure rate ($p>0.05$). Vitamin C treatment had no effect on basal or insulin-stimulated rates of carbohydrate or lipid oxidation, resting energy expenditure, RER or plasma lactate (Table 6.3). There was
also no significant effects of vitamin C treatment on basal or clamp NOGM (Figure 6.6A); although there was a tendency for Δ NOGM to improve with vitamin C treatment ($p=0.065$ for treatment x time interaction; Figure 6.6C).

6.2.5. Muscle glycogen

There was no significant difference between basal and clamp measures of muscle glycogen independent of treatment and time ($p=0.077$; Table 6.3). Vitamin C treatment had no significant effect on levels of basal muscle glycogen, clamp muscle glycogen or Δ muscle glycogen when compared with placebo ($p>0.05$; Table 6.3).
Results

**Figure 6.5.** Effect of supplementation on rate of glucose disappearance

A & B: \( \text{Rd} \) measured during the final 30 minutes of basal conditions and the final 60 minutes of the hyperinsulinaemic, euglycaemic clamp; C & D: \( \Delta \text{Rd} \); A & C: values are mean ± SD; B & D: data are individual values; *denotes significantly different to pre, \( p<0.05 \); #denotes significantly different to placebo, \( p<0.05 \); PLA – placebo; VC – vitamin C.
# Results

Table 6.3. Substrate kinetics pre- and post-supplementation

<table>
<thead>
<tr>
<th></th>
<th>PLACEBO</th>
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<th>VITAMIN C</th>
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<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>MCR (ml.kg FFM⁻¹.min⁻¹)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Basal</td>
<td>2.1 ± 0.2</td>
<td>1.9 ± 0.3</td>
<td>2.3 ± 0.2</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Clamp</td>
<td>4.3 ± 0.4</td>
<td>4.7 ± 0.4</td>
<td>4.3 ± 0.5</td>
<td>5.3 ± 0.8</td>
</tr>
<tr>
<td>Delta</td>
<td>2.2 ± 0.3</td>
<td>2.8 ± 0.3</td>
<td>2.0 ± 0.5</td>
<td>3.5 ± 0.6 *†</td>
</tr>
<tr>
<td>EGP (μmol.kg FFM⁻¹.min⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>18.6 ± 1.9</td>
<td>16.8 ± 1.9</td>
<td>20.2 ± 2.2</td>
<td>14.5 ± 2.3</td>
</tr>
<tr>
<td>Clamp</td>
<td>4.3 ± 0.6</td>
<td>5.2 ± 1.4</td>
<td>4.8 ± 1.3</td>
<td>4.2 ± 1.4</td>
</tr>
<tr>
<td>Delta</td>
<td>-14.3 ± 1.6</td>
<td>-11.5 ± 1.5</td>
<td>-15.4 ± 2.4</td>
<td>-10.3 ± 3.3</td>
</tr>
<tr>
<td>‡CHO ox (μmol.kg FFM⁻¹.min⁻¹)§</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>16.6 ± 5.5</td>
<td>11.6 ± 2.3</td>
<td>13.7 ± 2.8</td>
<td>13.2 ± 5.2</td>
</tr>
<tr>
<td>Clamp</td>
<td>17.8 ± 3.9</td>
<td>17.1 ± 4.2</td>
<td>16.6 ± 4.0</td>
<td>14.1 ± 3.2</td>
</tr>
<tr>
<td>Delta</td>
<td>1.2 ± 2.2</td>
<td>5.5 ± 2.3</td>
<td>2.9 ± 3.4</td>
<td>0.8 ± 4.9</td>
</tr>
<tr>
<td>‡Fat ox (μmol.kg FFM⁻¹.min⁻¹)ǁ</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Basal</td>
<td>1.02 ± 0.39</td>
<td>0.98 ± 0.18</td>
<td>1.17 ± 0.23</td>
<td>0.93 ± 0.32</td>
</tr>
<tr>
<td>Clamp</td>
<td>0.70 ± 0.34</td>
<td>0.46 ± 0.21</td>
<td>0.86 ± 0.30</td>
<td>0.83 ± 0.31</td>
</tr>
<tr>
<td>Delta</td>
<td>-0.31 ± 0.21</td>
<td>-0.52 ± 0.24</td>
<td>-0.30 ± 0.24</td>
<td>-0.09 ± 0.41</td>
</tr>
<tr>
<td>‡Respiratory exchange ratio</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.86 ± 0.04</td>
<td>0.84 ± 0.01</td>
<td>0.84 ± 0.02</td>
<td>0.84 ± 0.04</td>
</tr>
<tr>
<td>Clamp</td>
<td>0.89 ± 0.03</td>
<td>0.89 ± 0.03</td>
<td>0.87 ± 0.03</td>
<td>0.86 ± 0.03</td>
</tr>
<tr>
<td>Delta</td>
<td>0.03 ± 0.02</td>
<td>0.05 ± 0.00</td>
<td>0.03 ± 0.03</td>
<td>0.02 ± 0.04</td>
</tr>
<tr>
<td>‡Resting energy expenditure (J.kg FFM⁻¹.min⁻¹)</td>
<td></td>
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</tr>
<tr>
<td>Basal</td>
<td>86.7 ± 9.4</td>
<td>69.9 ± 11.0</td>
<td>83.0 ± 3.4</td>
<td>77.5 ± 9.6</td>
</tr>
<tr>
<td>Clamp</td>
<td>79.9 ± 9.2</td>
<td>69.6 ± 11.4</td>
<td>81.7 ± 8.7</td>
<td>72.7 ± 7.3</td>
</tr>
<tr>
<td>Delta</td>
<td>-6.9 ± 2.9</td>
<td>-0.2 ± 4.0</td>
<td>-1.3 ± 5.6</td>
<td>-4.8 ± 4.0</td>
</tr>
<tr>
<td>Plasma lactate (mmol.l⁻¹)</td>
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</tr>
<tr>
<td>Basal</td>
<td>1.39 ± 0.07</td>
<td>1.10 ± 0.07</td>
<td>1.26 ± 0.06</td>
<td>1.21 ± 0.07</td>
</tr>
<tr>
<td>Clamp</td>
<td>1.06 ± 0.09</td>
<td>1.11 ± 0.12</td>
<td>1.13 ± 0.11</td>
<td>1.07 ± 0.08</td>
</tr>
<tr>
<td>Delta</td>
<td>-0.33 ± 0.12</td>
<td>0.01 ± 0.14</td>
<td>-0.13 ± 0.12</td>
<td>-0.14 ± 0.11</td>
</tr>
<tr>
<td>Muscle glycogen (mmol.kg⁻¹ dry weight)</td>
<td></td>
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</tr>
<tr>
<td>Basal</td>
<td>394.0 ± 38.5</td>
<td>356.7 ± 11.6</td>
<td>362.1 ± 15.9</td>
<td>397.9 ± 57.6</td>
</tr>
<tr>
<td>Clamp</td>
<td>428.1 ± 48.4</td>
<td>398.7 ± 21.6</td>
<td>399.2 ± 29.8</td>
<td>493.5 ± 92.9</td>
</tr>
<tr>
<td>Delta</td>
<td>34.1 ± 34.9</td>
<td>42.0 ± 30.3</td>
<td>37.1 ± 32.0</td>
<td>95.6 ± 104.7</td>
</tr>
</tbody>
</table>

(Data are expressed as mean ± SEM; * indicates significantly different to placebo; † indicates significantly different to pre, p<0.05; ‡n=6 only - one participant was removed from analysis due to systematic hyperventilation during indirect calorimetry; § CHO ox – carbohydrate oxidation rate; ¶ Fat ox – fat oxidation rate)
**Figure 6.** Effect of supplementation on non-oxidative glucose metabolism

A & B: Basal and clamp NOGM; C & D: Δ NOGM; A & C: values are mean ± SEM; B & D: data are individual values; data is only n=6 due to systematic hyperventilation by one participant; PLA – placebo; VC – vitamin C.
6.3. Vitamin C concentrations

6.3.1. Plasma and skeletal muscle total ascorbic acid

Vitamin C supplementation significantly increased concentrations of total ascorbic acid in plasma ($p=0.005$ for treatment x time interaction; Figure 6.7A) and skeletal muscle ($p=0.017$ for treatment x time interaction; Figure 6.7C) compared with placebo.

![Graphs showing vitamin C levels in plasma and skeletal muscle pre- and post-supplementation](image)

**Figure 6.7.** Vitamin C levels in plasma and skeletal muscle pre- and post-supplementation

A & B: Plasma TAA; C & D: skeletal muscle TAA; values are mean ± SEM; *denotes significantly different to pre, $p<0.05$; #indicates significantly different to placebo, $p<0.05$. 
6.4. Reduced versus oxidized vitamin C concentrations

6.4.1. Plasma and skeletal muscle AA and DHA concentrations

Vitamin C supplementation significantly increased reduced AA concentrations in both plasma ($p=0.004$ for treatment x time interaction; Figure 6.8A) and skeletal muscle ($p=0.017$ for treatment x time interaction; Figure 6.9A) compared with placebo. Vitamin C supplementation had no effect on DHA levels in either plasma ($p=0.807$ for treatment x time interaction; Figure 6.8C) or skeletal muscle ($p=0.230$ for treatment x time interaction; Figure 6.9C). There was a trend ($p=0.051$) for the DHA/TAA redox ratio to decrease in plasma following vitamin C compared with placebo (Figure 6.8E). This was a result of a higher pre-supplementation DHA/TAA level in the vitamin C group when compared with placebo and post vitamin C levels. Vitamin C had no significant effect on skeletal muscle DHA/TAA ($p=0.176$ for treatment x time interaction; Figure 6.9E).
Results

Figure 6.8. Effect of supplementation on levels of AA and DHA in plasma

A & B: Ascorbic acid; C & D: DHA; E & F: DHA/TAA ratio; values are mean ± SEM; *denotes significantly different to placebo, p<0.05; #denotes significantly different to pre, p<0.05.
Results

Figure 6.9. Effect of supplementation on levels of AA and DHA in skeletal muscle

A & B: Ascorbic acid; C & D: DHA; E & F: DHA/TAA ratio; values are mean ± SEM; *denotes significantly different to placebo, p<0.05; #denotes significantly different to pre, p<0.05.
6.5. Oxidative stress, antioxidant levels and mitochondrial content

6.5.1. Blood measures

Whole blood GSSG required prior log transformation in order to approximate a normal Gaussian distribution. Vitamin C treatment had no effect on any blood-derived measures of oxidative stress including whole blood GSSG (Figure 6.10A), whole blood GSH/GSSG (Figure 6.10E) or plasma F₂ Isoprostanes (Figure 6.11A). Vitamin C treatment also had no effect on levels of whole blood TGSH (Figure 6.10B). A main effect of treatment was found for serum bilirubin \( (p=0.021) \), indicating decreased levels during vitamin C treatment versus placebo (Table 6.4). However the latter finding was possibly confounded by a trend towards a higher baseline value for bilirubin in the placebo group. Indeed, no significant treatment x time interaction was observed for serum bilirubin \( (p=0.759) \). Vitamin C treatment had no effect on any other blood-derived antioxidant measurements (Table 6.4). A single post-vitamin C data point was excluded from GSSG calculations due to gross sample oxidation and a single pre-vitamin C data point was missing from TGSH calculations due to accidental non-collection.
Results

Figure 6.10. Effect of supplementation on glutathione levels in basal whole blood

A & B: GSSG; C & D: Total GSH; E & F: GSH/GSSG ratio; A: data are geometric mean ± 95% CI; C & E: data are mean ± SEM; B, D & F: data are individual values.
**Results**

**Figure 6.11.** Effect of supplementation on basal F2-Isoprostane levels

A & B: plasma total F2-Isoprostanes; C & D: skeletal muscle total F2-Isoprostanes; A & C: data are mean ± SEM; B & D: data are individual values.

### 6.4.2. Muscle measures

A trend ($p=0.058$ for treatment x time interaction) was observed for basal muscle GSSG, reflecting a decrease with vitamin C supplementation and an increase with placebo supplementation (Figure 6.12A). Similar patterns of change were also found for basal muscle GSH/GSSG ($p=0.093$; Figure 6.12E) and basal muscle F2-Isoprostanes ($p=0.112$; Figure 6.11C). No significant effect of treatment was observed for muscle TGSH ($p=0.709$; Figure 6.12B). A significant ($p=0.006$) treatment x time interaction was found for total SOD activity in skeletal muscle. Post hoc analyses revealed that
Results

total SOD activity significantly increased during placebo supplementation but decreased during vitamin C supplementation (Table 6.4). No effects of vitamin C treatment on muscle GSH or uric acid concentrations were observed ($p>0.05$; Table 6.4).

DCFH oxidation was first investigated in insulin-stimulated muscle only. In this experiment, a main effect of treatment was found (vitamin C < placebo, $p=0.012$; data not shown). This finding was then confirmed in insulin-stimulated muscle in a second experiment (vitamin C < placebo, $p=0.012$; Figure 6.13A) that also involved an analysis of DCFH oxidation in basal muscle. In contrast to insulin-stimulated muscle, there was no significant effect of vitamin C supplementation on DCFH oxidation in basal muscle (main treatment effect $p=0.079$; Figure 6.13A). $\Delta$ DCFH oxidation (insulin-stimulated sample DCFH oxidation minus basal sample DCFH oxidation) was significantly decreased following vitamin C treatment compared with placebo ($p=0.006$; Figure 6.13C). When basal and insulin-stimulated samples were compared independently of treatment, DCFH oxidation was found to be significantly lower in insulin-stimulated muscle than in basal muscle ($p<0.001$; Figure 6.13A). Given the potential for hyperglycaemia to impact ROS/RNS levels, data was then corrected for the prevailing glucose level at the times of biopsy sampling. After this correction for glycaemia, DCFH oxidation was no longer different between basal and insulin-stimulated DCFH oxidation ($p=0.279$; data not shown); however, the effect of vitamin C treatment on DCFH oxidation in insulin-stimulated muscle remained statistically significant (main treatment effect $p=0.012$; data not shown).

Vitamin C supplementation had no significant effect on skeletal muscle citrate synthase activity ($p=0.17$ for treatment x time interaction; Figure 6.14).
### Table 6.4. Antioxidant levels in blood and skeletal muscle pre- and post-supplementation

<table>
<thead>
<tr>
<th></th>
<th>PLACEBO</th>
<th>VITAMIN C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH (μmol.l⁻¹)</td>
<td>747.5 ± 54.5</td>
<td>806.2 ± 41.0</td>
</tr>
<tr>
<td>Plasma Uric Acid (μmol.l⁻¹)</td>
<td>268.6 ± 24.2</td>
<td>264.3 ± 23.9</td>
</tr>
<tr>
<td>Serum Albumin (g.l⁻¹)</td>
<td>40.9 ± 1.1</td>
<td>42.0 ± 1.2</td>
</tr>
<tr>
<td>Serum Bilirubin (μmol.l⁻¹)</td>
<td>12.6 ± 2.3</td>
<td>14.9 ± 4.8</td>
</tr>
<tr>
<td><strong>Skeletal muscle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH (μmol.g⁻¹ protein)</td>
<td>10.2 ± 1.0</td>
<td>10.8 ± 1.0</td>
</tr>
<tr>
<td>Uric Acid (μg.g⁻¹ tissue)</td>
<td>7.8 ± 1.1</td>
<td>8.1 ± 0.6</td>
</tr>
<tr>
<td>SOD activity (U.mg⁻¹ protein)</td>
<td>11.9 ± 0.5</td>
<td>13.4 ± 0.8 * †</td>
</tr>
</tbody>
</table>

(Data are mean ± SEM; GSH – reduced glutathione; TR indicates main effect of treatment, p<0.05; * indicates significantly different to vitamin C, p<0.05; † indicates significantly different to pre, p<0.05)
Figure 6.12. Effect of supplementation on glutathione levels in basal skeletal muscle

A & B: GSSG; C & D Total GSH; E & F: GSH/GSSG ratio; A, C & E: data are mean ± SEM; B, D & F: data are individual values.
**Results**

*Figure 6.13. Effect of supplementation on skeletal muscle DCFH oxidation*

A & B: basal and clamp DCF fluorescence; C & D: Δ DCF Fluorescence (clamp minus basal DCF fluorescence); A & C: values are mean ± SEM; B & D: data are individual values; *indicates significantly different to placebo, p<0.05; PLA – placebo; VC – vitamin C; equiv.- equivalents.
Results

Figure 6.14. Effect of supplementation on skeletal muscle citrate synthase activity
A: data are mean ± SEM; B: data are individual values.
Results

6.6. Vitamin C transporter expression

6.6.1. SVCT2 protein expression

Vitamin C supplementation significantly increased SVCT2 protein expression in skeletal muscle compared with placebo supplementation ($p=0.008$ for treatment x time interaction; Figure 6.15). There was no effect of vitamin C supplementation on expression of the loading control GAPDH when compared to placebo ($p>0.05$; data not shown).

![Graph and Figure 6.15](image)

**Figure 6.15.** Effect of supplementation on muscle SVCT2 protein expression

A & B: SVCT2 protein expression normalized to GAPDH; C: a representative western blot including two participants (note: the order of samples has not been altered from the original membrane scan); A: data are mean ± SEM; B: data are individual values; *indicates significantly different to placebo, $p<0.05$; #indicates significantly different to pre, $p<0.05$. PLA: placebo; VC: vitamin C; T1: trial 1; T2: trial 2; PRE: pre supplementation, POST: post supplementation
6.7. Nitric oxide synthase levels

6.7.1. NOS activity

Data for basal NOS activity required log-transformation prior to parametric ANOVA analysis. A significant main effect of treatment was found for basal NOS activity \( (p=0.026) \), reflecting significantly higher levels during vitamin C supplementation compared with placebo (Figure 6.16A). However, this observed effect was possibly confounded by a higher pre-supplementation NOS activity in the vitamin C supplementation trial compared with placebo. Indeed, no significant treatment x time interaction \( (p=0.894) \) nor significant post-hoc comparisons were found, suggesting a lack of effect of vitamin C on total NOS activity. No significant effect of treatment was observed for total NOS activity in insulin-stimulated muscle (Figure 6.16B).

Figure 6.16. Effect of supplementation on total NOS activity in skeletal muscle

A: total NOS activity in basal skeletal muscle; B: total NOS activity in insulin-stimulated skeletal muscle; A: data are geometric mean ± 95 % CI; B: data are mean ± SEM.
Results

6.7.2. nNOS and eNOS protein expression

Basal and clamp nNOS and eNOS protein expression data required prior log-transformation after normalization to α-tubulin in order to approximate a Gaussian distribution. Vitamin C supplementation had no effect on skeletal muscle basal nNOS or eNOS protein expression when compared with placebo ($p>0.40$ for treatment x time interactions; Figure 6.17A & B). There was also no effect of vitamin C treatment on nNOS protein expression measured during the clamp ($p>0.99$ for treatment x time interaction; Figure 6.17D). However vitamin C supplementation significantly increased eNOS expression in insulin-stimulated muscle samples following vitamin C treatment compared with placebo ($p=0.022$ for treatment x time interaction; Figure 6.17E). There was no effect of vitamin C treatment on expression of the loading control α-tubulin in basal or clamp analyses for either nNOS or eNOS ($p>0.05$; data not shown). Basal nNOS and eNOS ANOVA analyses were made using only $n=5$ participants due to a lack of available tissue for two participants.
Figure 6.17. Effect of supplementation on NOS protein expression in skeletal muscle

A & B: basal nNOS and eNOS protein expression relative to α-tubulin expression; C: representative western blots for nNOS and eNOS with α-tubulin as the loading control (note: the order of samples from the original membrane scans have not been altered); D & E: clamp nNOS and eNOS protein expression relative to α-tubulin expression; data are geometric mean ± 95% CI; data analyses for basal nNOS and eNOS were for n=5 only due to a single randomly missing data point for two participants; pre: pre supplementation, post: post supplementation.
6.8. Cross sectional analysis of key measures based on muscle vitamin C concentration

At any time point during the trial with or without the consideration of post vitamin C supplementation values, muscle vitamin C concentration correlated significantly with \( \Delta \text{Rd} \) \((r=0.554, p=0.002\) for all data and \(r=0.502, p=0.021\) for all data excluding post VC trial values), muscle F\(_2\)-Isoprostanes \((r=-0.483, p=0.009\) for all data and \(r=-0.613, p=0.003\) for all data excluding post VC trial values) and HbA1c \((r=-0.462, p=0.013\) for all data and \(r=-0.540, p=0.012\) for all data excluding post VC trial values). Furthermore, two-tailed unpaired t-tests were conducted for Rd, GSH/GSSG, F\(_2\)-Isoprostanes and HbA1c in which values corresponding to muscle vitamin C concentrations above and below the median vitamin C concentration (24.7 \(\mu g/g\)) were compared. It was found that when muscle vitamin C concentration exceeded 24.7 \(\mu g/g\), there was significantly higher insulin-stimulated Rd and GSH/GSSG levels but significantly lower muscle F\(_2\)-Isoprostanes and HbA1c (Figure 6.18).
Figure 6.18. Cross-sectional analysis of measures with respect to skeletal muscle vitamin C concentration

Cross sectional comparison of key measures grouped on the basis of the corresponding muscle total ascorbic acid (TAA) exceeding or falling below the median vitamin C concentration measured during the study. A: Rd measurements; B: Muscle GSH/GSSG; C: Muscle F₂-Isoprostanes and D: HbA₁c; data are mean ± SEM; * indicates p <0.05 vs. <24.7 μg/g tissue as determined using unpaired two-tailed t-tests. Data for <24 μg TAA/g tissue are for n=14; data for >24 μg TAA/g tissue are for n=14.
7.0. DISCUSSION

7.1. Effect of vitamin C supplementation on glucoregulatory measures

A major finding of the present study was that four months of high dose oral vitamin C supplementation significantly improved whole-body insulin-stimulated glucose disposal (Δ Rd) by more than 50% when compared with placebo supplementation in people with T2D. Skeletal muscle constitutes approximately 75-85% of whole-body glucose disposal during insulin-stimulated clamp conditions (25, 333) and is the primary defective site of peripheral insulin resistance in T2D (28). Thus, the improvement in Δ Rd likely represents predominantly an improvement in insulin-stimulated skeletal muscle glucose disposal.

This major finding lends support to a previous investigation in elderly people with T2D that similarly found improved whole-body insulin-stimulated glucose disposal following high dose oral vitamin C supplementation (169). Although a similar insulin infusion level was used in both trials (40mU/m²/min), the current study employed a hyperinsulinaemic, euglycaemic clamp while Paolisso et al. (169) used a hyperinsulinaemic, isoglycaemic clamp. The latter clamp method involves clamping glucose at fasting levels rather than at 5.5 mmol/l as was undertaken in this study. Thus, glucose disposal measured from euglycaemic clamps is not strictly comparable to glucose disposal measured from isoglycaemic clamps. Isoglycaemic clamps arguably represent a state more reflective of physiological conditions in people with T2D than do euglycaemic clamps. However, fluctuations in fasting glucose in individuals could potentially complicate measurements of changes in glucose disposal in individuals when using isoglycaemic rather than euglycaemic clamps. It has been shown that glucose MCR, a measure of glucose disposal per unit of blood
glucose, correlates highly between isoglycaemic and euglycaemic conditions in people with T2D (347). Given that a significant improvement in insulin-stimulated MCR (Δ MCR) was also found following vitamin C treatment, it is possible that we would have observed significant findings if an isoglycaemic rather than euglycaemic clamp were employed.

In further contrast to the study by Paolisso et al. (169), a glucose infusion containing 1 % tracer (i.e. “hot” glucose) was used during the hyperinsulinaemic clamp in the current study. It has been proposed that using a “hot” glucose infusion versus a non-labelled (or “cold”) glucose infusion can minimize potential errors associated with compartmental gradient effects and hepatic insulin sensitivity calculations (217). Indeed, only one participant displayed a negative value for EGP during the final hour of the euglycaemic clamp (-0.43 μmol/kg FFM/min) in this study. Consistent with findings of a previous study in T2D (323), EGP was not fully suppressed in participants during the final hour of the hyperinsulinaemic, euglycaemic clamp. The magnitude of suppression observed throughout the study (=74 % of basal EGP) is similar to levels previously reported by Powrie et al. (323) for T2D. This apparent hepatic insulin resistance was not altered by vitamin C supplementation. Basal EGP, which has been shown to be elevated in T2D (348), was also unaffected by vitamin C supplementation. The lack of effect of vitamin C supplementation on HOMA-IR further indicates a lack of effect of vitamin C on liver insulin resistance.

Another study (205) that investigated the effect of high dose vitamin C therapy (800 mg, 1 x/day) in people with T2D did not observe a change in whole-body insulin sensitivity. During their study, vitamin C intake for four weeks only partially replenished low baseline plasma vitamin C levels in participants (21 ± 3 μmol/l to 48 ± 6 μmol/l). In comparison to the study by Chen et al. (205), participants in the
present study did not have low baseline vitamin C levels (baseline vitamin C: 57 ± 5 μmol/l); and plasma vitamin C was saturated (225) in all participants in the present study following vitamin C treatment (post vitamin C: 89 ± 7 μmol/l). A failure to sufficiently elevate vitamin C levels could underlie the lack of effect of vitamin C on whole body insulin sensitivity in the study by Chen et al. (205). However, it is uncertain if the supplementation regimen used in the study by Chen et al. (205) was sufficient with respect to dose and duration in order to observe insulin-sensitizing effects of high dose vitamin C in people with T2D. Given the poor systemic bioavailability of high dose vitamin C (225), a single high daily dose might be less effective at increasing body vitamin C content than multiple smaller intakes throughout the day. Furthermore, considering firstly that exogenous vitamin C likely accumulates slower in tissues compared with plasma (228); and secondly, that impaired insulin sensitivity and oxidative stress are chronic impairments in patients; the duration of vitamin C intake might need to be prolonged for optimal anti-diabetic effects in both tissues and systemically. The necessity of a more prolonged high dose intake appears to be supported by the literature in which beneficial effects of vitamin C on glucose control and/or oxidative stress are found with supplemental dosages of \(\geq 1\) g ascorbic acid/day over \(\geq 6\)-weeks in people with T2D (169, 181, 182, 203-211).

Four months of high dose vitamin C supplementation did not alter levels of the secondary glucoregulatory outcome measures HbA1c, fasting glucose and fasting insulin. Some previous studies found improvements in these measures of glucose control in people with T2D following vitamin C supplementation (169, 209-211), although many other studies observed no effects (181, 203-208, 212, 214). There is a primary causal relationship between insulin resistance and glycaemic control in T2D (349). Therefore, a lack of improvement in HbA1c following vitamin C
supplementation is perhaps unexpected given the improvement in skeletal muscle insulin sensitivity. However, findings of improved skeletal muscle insulin sensitivity but unchanged HbA1c are not uncommon in studies using anti-diabetic treatments of exercise training and thiazolidinedione therapy in people with T2D (99, 350-352). This finding might reflect a lack of effect of vitamin C on factors other than skeletal muscle insulin sensitivity that can also affect glycaemic control such as liver glucose production and beta cell insulin secretory capacity.

It must be acknowledged that our study was underpowered to accurately investigate the effect of vitamin C specifically on changes in HbA1c (169). Some larger studies have indeed reported improvements in HbA1c following chronic high dose vitamin C supplementation in people with T2D (169, 209, 210). It therefore remains possible that if our study had had improved power (i.e. more participants) for the change in HbA1c and/or had involved a more prolonged period of supplementation, an improvement in glucose control measures would have also been observed. Based on findings of a previous study (169) that involved a similar design to the current study, a cross-over sample size of $n=57$ participants would be required to obtain 80% power for an improvement in fasting insulin of 2.8 uU/ml with vitamin C; while $n=121$ participants would be required to obtain 80% power for an improvement in HbA1c of 0.8% with vitamin C. Studies involving a considerably greater number of participants than the current study are therefore required to investigate these secondary outcome measures in response to regular high dose vitamin C supplementation.

Consistent with a previous investigation in people with T2D (169), high dose vitamin C supplementation had no effect on basal measures of whole-body oxidative or non-oxidative glucose metabolism; nor on insulin-stimulated oxidative glucose
metabolism. Unlike the previous study (169), a significant improvement in NOGM during the clamp following vitamin C treatment was not observed in this study. A trend ($p=0.065$) towards improvement in $\Delta$ NOGM following vitamin C supplementation was found however. Glycogen synthesis is believed to constitute the major cellular pathway of non-oxidative glucose metabolism (353). Thus, given the observed trend towards improved $\Delta$ NOGM following vitamin C supplementation, levels of glycogen in skeletal muscle samples were assessed in both basal and insulin-stimulated conditions. Vitamin C treatment did not significantly alter glycogen concentrations in either basal or insulin-stimulated muscle when compared with placebo. However, it should be noted that an insulin-stimulated period of 60 minutes did not produce a significant increase in muscle glycogen levels above basal conditions in participants when analysed independent of treatment. A 60-minute duration of insulin-stimulation prior to muscle sampling was initially chosen in order to measure insulin-stimulated effects on insulin signalling components in muscle (354, 355), but avoid potential anticipatory effects of the muscle biopsy in individuals on their blood glucose concentrations during the critical final hour of the clamp. A longer period of insulin infusion prior to muscle sampling might thus have proved more optimal in evaluating insulin-stimulated glycogen changes in muscle. On the other hand, it is uncertain based on previous findings (356, 357) that even 180 minutes of insulin stimulation during the hyperinsulinaemic, euglycaemic clamp would have yielded significantly elevated muscle glycogen levels in participants above basal levels.

Other pathways of NOGM in addition to glycogen synthesis cannot be ignored in the interpretation of NOGM findings. These pathways include de novo lipogenesis, nonoxidative glycolysis to lactate and the pentose phosphate pathway (358). In
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accordance with previous interpretations (358), lipid synthesis can likely be ignored since RER did not exceed 1.0 in our participants in basal or insulin-stimulated conditions; and the pentose phosphate pathway is negligible in skeletal muscle (359). Non-oxidative glycolysis to lactate might therefore partially explain any observed changes in NOGM independent of glycogen synthesis. Plasma lactate levels were unaltered by vitamin C supplementation in this study. Furthermore, plasma lactate levels decreased in response to euglycaemic hyperinsulinaemia, while in contrast, NOGM increased in response to euglycaemic hyperinsulinaemia. Thus, non-oxidative glycolysis likely does not explain our trend for altered Δ NOGM findings.
Discussion

7.2. Effect of vitamin C supplementation on skeletal muscle vitamin C concentration

This study reports for the first time the effect of oral vitamin C supplementation on skeletal muscle vitamin C concentrations in people with T2D. Vitamin C supplementation significantly increased levels of reduced and total ascorbic acid in skeletal muscle by 71% and 36% respectively. Given the capacity for transport of vitamin C into and out of cells (184), skeletal muscle vitamin C could serve as an important reservoir of vitamin C for both intramuscular and systemic antioxidant defence. Skeletal muscle vitamin C concentration could also potentially serve as a good marker of total body vitamin C content since it contains the bulk ($\approx 65\%$) of total body vitamin C (219-221, 234). Thus, these findings suggest both an enhanced reserve of ascorbic acid available for antioxidant scavenging of ROS/RNS in skeletal muscle as well as a marked increase in total body vitamin C content following vitamin C supplementation.

A cross-sectional analysis of pooled data from all trials independent of treatment and time found statistically significant correlations between muscle vitamin C concentration and both $\Delta$ Rd (positive association) and HbA1c (negative association) when either including or excluding post vitamin C supplementation values. Furthermore, when Rd and HbA1c values were compared on the basis of their corresponding muscle vitamin C level exceeding or falling below the median vitamin C concentration measured (24.7 $\mu$g/g tissue) irrespective of treatment, it was found that insulin-stimulated Rd and HbA1c were significantly improved at levels above 24.7 $\mu$g/g tissue total ascorbic acid. These findings suggest that establishing a sufficient absolute muscle concentration of vitamin C might be an important requisite for healthy insulin action in muscle irrespective of nutritional means. Based on
findings of the current study, it appears that for most people with T2D, long-term and high dose vitamin C supplementation would be an effective means of elevating skeletal muscle vitamin C concentrations.

Vitamin C supplementation also significantly increased plasma levels of reduced and total ascorbic acid by 62 % and 59 % respectively. However, unlike skeletal muscle vitamin C concentration, plasma vitamin C concentration did not correlate with \( \Delta \text{Rd} \) or HbA1c. These different findings in muscle and plasma raise the possibility that tissue and plasma vitamin C concentrations are not interchangeable measures of vitamin C status in people with T2D. Different associations between leukocyte and plasma vitamin C concentrations were also reported in people with T2D by Yamada et al. (227). In that study, it was found that both uncomplicated patients with T2D and patients with complicated T2D had similar levels of plasma AA compared with healthy controls. However, in the same study, leukocyte levels of AA were lower in people with uncomplicated T2D when compared with healthy controls; and lower still in people with complicated T2D when compared to people with uncomplicated T2D (227). Thus, a lack of investigation of tissue vitamin C levels in addition to plasma vitamin C concentration could potentially overlook associations of vitamin C status with disease indicators.

A measurement of vitamin C levels in plasma only without regard for tissue vitamin C status might inadequately gauge the effectiveness of a given vitamin C supplementation regimen to increase body vitamin C concentrations. It is rather clear from the studies summarized in Table 1.2 (on page 37) that vitamin C supplementation was effective at increasing plasma vitamin C concentrations in people with T2D, seemingly independent of dose or duration of supplementation.
However, only studies that incorporated a supplementation regimen of ≥ 1 g vitamin C/day for ≥ 6 weeks appeared to be effective at improving glucoregulatory and/or oxidative stress measures in people with T2D. Unfortunately the majority of studies outlined in Table 1.2 (on page 37) lack data on tissue levels of vitamin C in addition to plasma levels to further verify that the regimen used was effective at augmenting body vitamin C status.
7.3. Effect of vitamin C supplementation on levels of reduced versus oxidized vitamin C in skeletal muscle

As already discussed, vitamin C supplementation caused a significant increase in levels of reduced ascorbic acid in both skeletal muscle and plasma. A recent bioavailability study in otherwise healthy males with low baseline vitamin C levels reported an increase in skeletal muscle ascorbic acid levels ≈ 3.5 fold over baseline following 42-days of supplementation with 53 or 212 mg of vitamin C (as kiwi-fruit) (233). The relative increase reported by Carr et al. (233) was greater than the relative increase in skeletal muscle ascorbic acid above pre-supplementation levels found in this study (≈ 1.7 fold) despite the use of a considerably higher dose and duration of supplementation in this study. The lower baseline ascorbic acid concentration in their study appears to account for much of this difference however (≈ 3 μg/g tissue versus ≈ 6 μg/g tissue in this study), since post supplementation ascorbic acid levels were similar in both studies (≈ 12μg/g tissue versus ≈ 11 μg/g tissue). It should be noted that the bioavailability of ascorbate in kiwi fruit does not differ in skeletal muscle when compared with synthetic ascorbic acid found in supplements (360) thus comparisons between studies would appear to be valid. The relatively lower but significant increase in ascorbic acid observed in this study might reflect the capacity to further increase muscle ascorbic acid levels with high dose supplementation despite being mostly on the flatter portion of the skeletal muscle ascorbic acid saturation curve. This is likely in contrast to participants in the Carr et al. (233) study who might have been positioned mostly on the steep portion of the tissue saturation curve and thus sensitive to lower levels of intake. However it still remains possible that some of the difference in the relative change observed between studies was due to differences in skeletal muscle vitamin C bioavailability in people with T2D.
compared with healthy individuals. Future studies comparing the effects of the same vitamin C supplementation regimen in matched diabetic and healthy participants is required to investigate this issue further.

In the present study ascorbic acid constituted the majority of TAA in plasma (≈ 97%), while skeletal muscle was considerably more oxidized (≈ 70% DHA). It is widely believed that vitamin C is found predominantly in its reduced, ascorbic acid form intracellularly (221, 223, 243). However, experimental studies in animal models have produced mixed findings in relation to the oxidative status of vitamin C in healthy tissues. In guinea pigs, who like humans cannot synthesize ascorbic acid endogenously, the majority of vitamin C in the adrenal glands, brain, testis and liver after being fed a diet adequate in vitamin C was reported to be in the reduced, ascorbic acid form (246, 247). On the other hand, the majority of vitamin C in the heart, spleen, thymus and skeletal muscle was found to be in the oxidized DHA form (246, 247).

Differing methods of measurement and sampling such as HPLC versus enzymatic assays; or post mortem tissue analysis versus muscle biopsy sampling might account for some of the differences in reported DHA and AA levels in studies. However, improper treatment of samples prior to analysis has been suggested to result in increased oxidation of AA to DHA and diketogulonic acid, thus leading to artifactually increased DHA levels and a potential irreversible loss of vitamin C (243). In this study muscle samples were blotted free of blood and visible fat and snap frozen in liquid nitrogen within 30 seconds of sampling. Additionally, frozen samples were homogenized on ice in an acidic buffer containing a metal chelator (EDTA) and then analysed in nitrogen-flushed amber vials within 30 minutes of removal from liquid
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nitrogen. Therefore, given these precautions taken, it is perhaps unlikely that any substantial artifactual sample oxidation occurred after the time the sample was removed from the biopsy needle. The oxidation status of ascorbic acid in human diabetic skeletal muscle is currently unknown. Thus these findings report for the first time that vitamin C treatment was unable to significantly alter the vitamin C redox status (DHA/TAA) of skeletal muscle in people with T2D. Vitamin C was also unable to modulate the relatively oxidized status of skeletal muscle vitamin C in people with T2D, despite causing a significant increase in both reduced ascorbic acid and total vitamin C content.
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7.4. Effect of vitamin C supplementation on skeletal muscle oxidative stress and mitochondrial content

Vitamin C treatment significantly decreased insulin-stimulated DCFH oxidation. The average post vitamin C treatment DCFH oxidation was approximately 36 % lower when compared with placebo treatment during the hyperinsulinaemic, euglycaemic clamp. These findings implicate improved insulin-stimulated antioxidant scavenging of reactive species in muscle following vitamin C treatment. An increased production of reactive species including $\text{O}_2^\cdot -$ and $\text{H}_2\text{O}_2$ in skeletal muscle have been implicated in impairments in insulin signalling pathways responsible for normal insulin-stimulated glucose transport (64, 126). In particular, increased serine phosphorylation of insulin signalling components such as IRS-1 via ROS/RNS- induced stress signalling pathways including JNK activation have been shown to be important in impaired insulin sensitivity in skeletal muscle (128, 129, 361). Effects of vitamin C supplementation on activation levels of insulin signalling or stress signalling pathways in skeletal muscle were not investigated in this study. However, it is possible that by reducing ROS/RNS levels during insulin-stimulated conditions, high dose vitamin C supplementation was able to attenuate ROS/RNS-induced activation of stress signalling pathways and their subsequent impairment on insulin signalling in skeletal muscle. This could have then facilitated an improved insulin-stimulated glucose disposal via improved insulin-mediated glucose transport.

It is uncertain why vitamin C’s effects on DCFH oxidation were observed in insulin-stimulated conditions but not in basal conditions. It could be speculated that insulin exerted a permissive role on vitamin C, such that hyperinsulinaemia was required for vitamin C to act effectively as an antioxidant. However, insulin has itself been shown to exert intracellular antioxidant effects in vivo (334, 335). For example, 60 minutes
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of insulin infusion (40mU/m²/min) during a hyperinsulinaemic, euglycaemic clamp was shown to increase erythrocyte GSH/GSSG levels via an increase in GSH and a decrease in GSSG in people with T2D (334, 335). Consistent with these findings, it was observed in the current study that DCFH oxidation decreased in skeletal muscle after 60 minutes of insulin infusion when compared with basal levels, independent of treatment. It is possible that the glucose lowering effect of insulin underlies its apparent antioxidant effect in people with T2D. There is evidence to suggest that hyperglycaemia induces oxidative stress in skeletal muscle and systemically (125, 362). Moreover, this hyperglycaemia-induced oxidative stress can be attenuated by acute insulin treatment (125). Interestingly, after correcting for the prevailing glycaemia at the time of muscle biopsy sampling in the current study, the attenuative effect of hyperinsulinaemia on DCFH oxidation disappeared. However, the decreased DCFH oxidation following vitamin C treatment remained statistically significant after this correction. These findings suggest that the combined effects of acute hyperinsulinaemia and elevated muscle vitamin C concentration could have acted in a synergistic manner to significantly improve skeletal muscle oxidative stress and insulin sensitivity. A combined effect of vitamin C supplementation and insulin therapy was shown in a study by Evans et al. (181) in people with T2D. In that study, oral vitamin C supplementation potentiated the beneficial effects of chronic insulin therapy on endothelial function and systemic oxidative stress in people with T2D (181).

A limitation of the DCFH probe is that it is sensitive to a diverse range of ROS including H₂O₂, HO· and ROO·; as well as to RNS including NO and ONOO⁻ (306). Therefore it is uncertain which (if any) particular reactive species might have been selectively attenuated in response to vitamin C treatment. Furthermore, DCFH oxidation was
assessed in muscle homogenates rather than in subcellular compartments, thus making it difficult to discern the cellular origins of the reactive species that were decreased. In basal conditions cellular ROS is believed to be predominantly of mitochondrial origin (103). In addition, evidence from experimental studies implicates mitochondria as a key cellular source of excess ROS production in high fat diet-induced insulin resistance (64, 126). Despite these findings, there is a lack of convincing evidence showing increased levels of mitochondrial ROS production in skeletal muscle of people with T2D (97, 137, 138). Furthermore, recent experimental investigations in high fat-fed C57BL/6 mice (122, 123) were unable to demonstrate improved insulin sensitivity following treatments with mitochondrial-targeted antioxidants despite improvements in skeletal muscle oxidative stress. Sources of excess non-mitochondrial-derived reactive species have also been implicated in T2D. In particular, excess ROS produced during endoplasmic reticulum stress, by NADPH-oxidases and by xanthine oxidases have also been found to be important sites of excess ROS production in diabetes (124, 125, 363). It is therefore possible that the reactive species observed to be decreased in muscle homogenates following vitamin C supplementation originated from mitochondrial and/or non-mitochondrial cellular locations.

Despite the observed findings using the DCFH probe, no statistically significant changes in levels of other markers of oxidative stress in skeletal muscle or in whole blood or plasma were observed following vitamin C treatment. There were trends toward improvements in basal muscle oxidative stress markers GSSG, GSH/GSSG and F$_2$-Isoprostanes during vitamin C treatment compared with their worsening during placebo treatment. Given the relatively small number of participants that completed the trial in full compliance, it remains possible that this study lacked statistical power
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to fully realize a significant effect of treatment on these markers of oxidative stress in muscle. However, it should also be noted that these oxidative stress measures were performed in basal muscle samples only, and thus unlike for DCFH oxidation, an insulin-stimulated effect of treatment on these oxidative stress measures was not explored. Unfortunately there was a lack of sufficient sample remaining to investigate GSH/GSSG or F₂-Isoprostanes levels in insulin-stimulated muscle also.

Despite the lack of statistically significant improvements for muscle GSH/GSSG and F₂-Isoprostanes following vitamin C treatment, cross-sectional analyses were undertaken to explore relationships between vitamin C concentration and oxidative stress measures independent of treatment and time. When GSH/GSSG and F₂-Isoprostanes data were compared on the basis of their corresponding muscle vitamin C concentration exceeding or falling below the median muscle vitamin C concentration measured (24.7 μg/g tissue), both GSH/GSSG and F₂-Isoprostanes levels were significantly improved at vitamin C concentrations > 24.7 μg/g tissue. This suggests that achieving a sufficient skeletal muscle vitamin C concentration regardless of nutritional means might be important in improving skeletal muscle oxidative stress in people with T2D.

Interestingly, total SOD activity in skeletal muscle was marginally, but significantly lower post vitamin C treatment compared with placebo treatment. The relative decrease in SOD activity found following vitamin C treatment might reflect an improved overall antioxidant scavenging of ·O₂⁻ in muscle due to exogenous ascorbic acid and a compensatory reduction in endogenous SOD activity. SOD activity and SOD isoform protein expression show mixed relationships with oxidative stress in diabetic skeletal muscle. Studies have shown SOD levels to be reduced (364), unaltered (365), or increased (366) in association with increased oxidative stress in
diabetic skeletal muscle. In theory, decreased SOD levels could contribute to oxidative stress in T2D due to a reduced capacity for scavenging of \( \cdot \text{O}_2^- \) produced, while on the other hand increased levels could represent a compensatory response to increased levels of \( \cdot \text{O}_2^- \) and oxidative stress. The case for SOD activity better reflecting the latter of these theoretical situations is supported by the very similar patterns of change found for muscle DCFH oxidation, muscle GSSG and muscle F\textsubscript{2}-Isoprostanes when compared with SOD activity during both vitamin C and placebo supplementation. In all of these measurements, levels tended to decrease following vitamin C treatment but increase following placebo treatment.

Vitamin C treatment did not significantly alter levels of citrate synthase activity in skeletal muscle. Citrate synthase has been shown to be highly associated with skeletal muscle mitochondrial content in humans (298). Decreased skeletal muscle mitochondrial biogenesis has been implicated as a cause of impaired insulin sensitivity in people with T2D (367), although findings are not consistent (101, 136). A lack of change in citrate synthase activity with vitamin C supplementation suggests that the observed change in insulin sensitivity was independent of skeletal muscle mitochondrial content. Mitochondrial content and insulin sensitivity were also not likely influenced by a change in physical activity of participants (97), since a crude measure of usual physical energy expenditure (a 7-day recall adapted from the Australian National Nutrition Survey) found no significant difference in physical activity between vitamin C and placebo treatments. Overall, these findings suggest that the observed change in skeletal muscle insulin sensitivity following vitamin C supplementation was likely not a result of altered physical activity or mitochondrial content of participants.
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7.5. Effect of vitamin C supplementation on skeletal muscle SVCT2 levels

Chronic high dose vitamin C supplementation significantly increased SVCT2 protein expression in skeletal muscle of people with T2D. SVCT2 protein expression increased approximately two-fold post vitamin C supplementation when compared with placebo. It was originally hypothesised that skeletal muscle SVCT2 protein expression would decrease following vitamin C supplementation in response to increased intramuscular vitamin C content and/or decreased skeletal muscle oxidative stress. This hypothesis was based on studies in various human non-muscle cell types indicating that SVCT2 expression is inversely regulated by intracellular ascorbic acid concentration (259, 262). Moreover, SVCT2 was found to be redox-sensitive in skeletal muscle cells (236). In particular, treatment of C2C12 myotubes with the antioxidant lipoic acid decreased, while treatment with H$_2$O$_2$ increased, both SVCT2 mRNA and protein levels (236). Thus, it could be argued that interventions which aim to increase vitamin C concentrations and/or decrease oxidative stress in tissues would decrease tissue SVCT2 expression and thus attenuate vitamin C accumulation in accordance with intracellular vitamin C content and/or redox status.

In mice who are capable of producing vitamin C endogenously, oral vitamin C supplementation is largely ineffective at increasing tissue vitamin C concentrations to high levels due to strict regulatory control exerted by SVCT transporters (294). However, as our current findings suggest, this situation could be different in humans, who unlike mice are incapable of producing vitamin C endogenously. The current findings, coupled with findings in healthy humans (Chapter 2 on page 71), suggest that SVCT2 expression is augmented in skeletal muscle by high dose vitamin C supplementation despite the prevailing tissue vitamin C concentration. It is possible
that SVCT2 expression responds differently to high dose vitamin C supplementation in different tissues as a function of relative tissue vitamin C concentration (294, 312). On a gram per wet weight basis, skeletal muscle vitamin C concentrations are relatively low compared with many other human tissues (221). Thus, SVCT2 transport capacity might not be nearly as saturated by ascorbic acid in skeletal muscle as it might be in other human tissues such as the adrenal glands, brain and eye lens after high dose vitamin C supplementation.

The present study did not investigate transport activity of SVCT2. Studies investigating ascorbic acid transport into cells and tissues have typically used radiolabelled ascorbic acid such as [14C] ascorbic acid to trace its uptake in vitro and in vivo e.g. (229, 368, 369). These studies have rarely been undertaken in humans however (219, 370). Recent in vitro studies investigated SVCT2 expression in C2C12 mouse cells using time-dependent and concentration-dependent experiments (236, 260). These studies observed a matching of ascorbic acid transport with total protein and gene expression levels of SVCT2, such that increased and decreased SVCT2 expression was associated with increased and decreased ascorbic acid transport respectively (236, 260). Thus, total SVCT2 protein expression appears to be a good indicator of SVCT2 transporter activity in skeletal muscle. Nonetheless, it is possible that additional post translational or cellular redistribution regulation of SVCT2 occurred (371). For instance, SVCT2 has been shown to undergo translocation from the cytosol to the plasma membrane in response to extracellular ascorbic acid in cultured striatal rat neuronal cells (372) and in response to prostaglandin E2 in MC3T3-3T preosteoblastic mouse cells (368). Furthermore, a dominant negative short SVCT2 isoform has been identified in a large number of human cell types (345). This short SVCT2 isoform can act to decrease normal SVCT2 transport activity via
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protein-protein interactions in human kidney 293T cells (345). Therefore, further investigations of SVCT2 expression should additionally focus on levels of SVCT2 in membrane versus cytosolic fractions, and on the relative expression levels of the normal SVCT2 isoform versus the short SVCT2 isoform.

The present study did not measure changes in expression levels of the DHA transporters GLUT1 and GLUT4 (266, 270) in skeletal muscle. Given the non-specificity of these transporters for DHA, the significance of changes in their expression in response to vitamin C supplementation without evaluation of their DHA transport activities is unclear in people with T2D. Moreover, the physiological significance of GLUT transport of DHA in cellular vitamin C accumulation is uncertain (266).
7.6. Effect of vitamin C supplementation on skeletal muscle NOS levels

In this study, vitamin C supplementation was found to significantly increase total eNOS protein abundance in insulin-stimulated skeletal muscle of people with T2D. Skeletal muscle and adipose tissue eNOS protein abundance has been found to be decreased in states of obesity and insulin resistance (373). In particular, eNOS protein expression was found to be decreased in skeletal muscle of relatively insulin-resistant, obese humans when compared with relatively insulin-sensitive, non-obese humans (374). In another study, eNOS protein expression was decreased in gastrocnemius muscle in association with insulin resistance in male Sprague Dawley rats after chronic high-fat, high-sugar feeding (375). Furthermore, co-treatment of these high-fat, high-sugar rats with the antioxidant SOD mimetic Tempol was shown to improve diet-induced impairments in both glucose uptake and eNOS protein expression in skeletal muscle (376). These findings suggest that eNOS expression is modulated in association with skeletal muscle insulin sensitivity. However, direct effects of insulin sensitizing treatments on eNOS expression in skeletal muscle are also possible. Indeed, anti-diabetic thiazalodinedione drugs have been shown to robustly increase gene and protein expression of eNOS in endothelial cells (373). Thus, the observed findings of increased eNOS protein abundance in skeletal muscle following vitamin C supplementation could be a consequence of improved skeletal muscle insulin sensitivity and/or a direct effect of vitamin C on eNOS transcription and/or translation.

eNOS has been localized to mitochondrial, sarcolemmal and vascular endothelial sites in skeletal muscle (377). In particular, decreased endothelial eNOS activation has been implicated in impaired insulin-stimulated glucose uptake by skeletal muscle.
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(378). eNOS activation was shown to play an important role in enhancing insulin-stimulated capillary recruitment and insulin delivery to skeletal muscle; thus leading to improved glucose uptake by muscle (378). Unfortunately eNOS activity was not specifically investigated in response to vitamin C supplementation in this study. Therefore, it is uncertain if the increased eNOS protein levels observed were concordant with increased eNOS activity in skeletal muscle. The lack of change observed in total NOS activity following vitamin C supplementation would appear to suggest that this was not the case. However, nNOS is the dominant NOS isoform in skeletal muscle (379); and a lack of change in total NOS activity following vitamin C supplementation might more closely reflect the lack of change in nNOS protein abundance than eNOS protein abundance. Indeed, vitamin C supplementation was shown to potentiate the effects of L-arginine and BH₄ supplementation on total eNOS protein expression in skeletal muscle of ischaemic rats without significantly altering total calcium-dependent NOS activity (291).

It needs to be acknowledged that an alteration in NOS protein abundance might not fully reflect changes in NOS activity. For example, it is known that both eNOS and nNOS undergo post-translational phosphorylation in response to insulin stimulation (379). This in turn can affect their activation levels (379). eNOS dimerization is another important post-translational mechanism involved in the regulation of eNOS activity (380). Additional investigations are therefore required to expand on the significance of the change in total eNOS protein given the potential of complex post-translational regulation of eNOS activity.

A proposed in vivo function of ascorbic acid is to maintain BH₄ in its reduced state (288). Impaired BH₄ levels due to its oxidation by ONOO⁻ (381) can promote eNOS “uncoupling” in endothelial cells through a mechanism that promotes the transfer of
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electrons to molecular oxygen rather than to arginine (292). This leads to the preferential formation of ·O$_2^-$ rather than NO. BH$_4$ levels in skeletal muscle were not measured in this study. Thus, it is uncertain if vitamin C treatment promoted increased BH$_4$ levels in skeletal muscle. Additional studies are required to further investigate the effects of ascorbic acid on BH$_4$ levels in diabetic skeletal muscle.

Finally, it should be noted that iNOS protein could not be detected in a selection of skeletal muscle samples, despite its previous report in diabetic skeletal muscle (142, 274). Therefore the effect of vitamin C supplementation on iNOS activity in skeletal muscle was not specifically investigated. Given iNOS is not constitutively expressed in skeletal muscle (273), the possibility that iNOS contributed to total NOS activity in some but not all skeletal muscle samples cannot be ruled out.
7.7. Comparison of people with T2D and healthy individuals

It is possible to make some tentative comparisons of vitamin C effects in skeletal muscle of people with T2D in the major project and healthy individuals in the study outlined in Chapter 2 (on page 71). However, it needs to be emphasized that comparisons are tentative at best when considering, firstly, that the participants in the two studies were not matched for subject characteristics; and secondly, that the duration of vitamin C supplementation was different between studies. On the other hand, the supplemental regimen of both studies was identical in terms of daily dosage and supplement products used. Furthermore, there was considerable overlap in terms of the analytical methods used in sample analyses. Thus, a brief discussion is now provided in relation to effects of vitamin C supplementation on vitamin C concentration, redox status, and SVCT2 transporter expression in people with T2D compared with healthy individuals.

7.7.1. Vitamin C concentrations

There is some evidence to suggest that human blood and rodent liver vitamin C concentrations are lower and more oxidized in T2D compared with healthy individuals (194, 250, 253). Prior to vitamin C supplementation, skeletal muscle TAA concentration was lower in the type 2 diabetic participants of the current study (≈22 μg/g tissue) when compared with age-matched cadavers with deaths of miscellaneous cause (≈30 μg/g tissue) (234); and also healthy males of the study in Chapter 2 (≈30 μg/g tissue). However, after vitamin C supplementation, muscle concentrations in the people with T2D were comparable with these previous values.
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(≈30 μg/g tissue). It might therefore be speculated that vitamin C supplementation normalised the decreased skeletal muscle vitamin C concentrations in people with T2D. Furthermore, considering the significant cross-sectional correlation found in the diabetic study between muscle TAA and insulin-stimulated Rd, it might be further speculated that a normalization of impaired skeletal muscle vitamin C level was an underlying factor in the improvement in Δ Rd found following vitamin C supplementation.

Despite an apparent difference in baseline skeletal muscle vitamin C concentrations when comparing between the study in people with T2D and the study in Chapter 2 (on page 85), there was no obvious difference between the studies in either the absolute increase (mean: T2D +7.6 μg/g tissue vs. healthy +8.6 μg/g tissue) or the relative increase (mean: T2D +36.0 % vs. healthy +31.1 %) in muscle TAA concentrations above baseline following vitamin C supplementation. In contrast to skeletal muscle, baseline plasma vitamin C levels were similar in both studies (mean: T2D 53.8 μmol/l vs. healthy 61.0 μmol/l). However, the absolute increase (mean: T2D +32.2 μmol/l vs. healthy +50.4 μmol/l) and relative increase (mean: T2D +59.4 % vs. healthy +95.1 %) in plasma vitamin C concentration above baseline were greater in the healthy individuals after vitamin C supplementation. These apparent differences in vitamin C status in skeletal muscle versus plasma in people with T2D and healthy individuals lends further support to the notion that is is important to assess both tissue and plasma levels of vitamin C to establish vitamin C status.

7.7.2. Redox status

Vitamin C supplementation was able to improve skeletal muscle DCFH oxidation in people with T2D (albeit only in insulin-stimulated muscle) but had no effect on DCFH
Discussion

oxidation in healthy individuals in Chapter 2 (on page 89). A lack of effect in healthy individuals is perhaps not surprising considering that pathological oxidative stress is unlikely to be present. Moreover, if skeletal muscle vitamin C levels are already at sufficient levels in healthy individuals, it is unlikely that further enhancement of vitamin C concentrations is going to greatly augment its antioxidant effects. Previously, antioxidant supplementation was shown to be relatively more effective in people with T2D compared with matched healthy individuals. In people with T2D, four months of vitamin E supplementation (900 mg/day) was shown to produce relatively greater increases in insulin-mediated WBGD and NOGM; and relatively greater reductions in plasma \( \cdot {O}_2^- \), HbA1c and oxidative stress than in non-diabetics (167). In addition, in elderly people with T2D, an acute infusion of high dose vitamin C (0.9 mmol/l) produced a greater relative improvement in WBGD and NOGM; and relatively greater reductions in plasma \( \cdot {O}_2^- \) and oxidative stress than in non-diabetics (168). Finally, eight weeks of high dose antioxidant supplementation (24 mg \( \beta \) carotene + 1 g ascorbate + 1000 IU \( \alpha \)-tocopherol) was found to decrease LDL-oxidation and lipid peroxidation markers in men with T2D when compared with healthy men (382). Thus, the different findings in people with T2D and healthy individuals for DCFH oxidation coupled with these previous findings collectively suggest that antioxidant supplementation is of benefit for modulating redox status in individuals with oxidative stress.

The only other common redox-related measure undertaken shown to have different outcomes following vitamin C supplementation in people with T2D compared with the healthy individuals was total SOD activity. As discussed above, increased SOD levels could be a compensatory response to increased levels of ROS in skeletal muscle (366). Therefore, a decrease in total SOD activity in people with T2D when compared
Discussion

with no change in healthy individuals following vitamin C supplementation could indirectly indicate an improvement in elevated ROS levels in people with T2D. This remains somewhat speculative however, since findings are mixed in relation to altered SOD levels in diabetic skeletal muscle (364, 365). Further investigations looking more specifically at particular SOD isoform activities and expression levels might be required to more thoroughly evaluate the significance of these different outcomes with respect to total SOD activity.

7.7.3. SVCT2 protein expression

The finding of increased SVCT2 expression in skeletal muscle of people with T2D following high dose vitamin C supplementation supports the earlier novel finding of increased SVCT2 protein expression in response to vitamin C supplementation in healthy individuals in Chapter 2 (on page 86). Collectively, findings from the two studies provide an original insight into the regulation of ascorbic acid transport in skeletal muscle in humans \textit{in vivo}. Interestingly, in both studies it was hypothesised that SVCT2 expression would be decreased in a compensatory manner following vitamin C supplementation in response to increased vitamin C concentrations and/or improved antioxidant status. However, none of these hypotheses held true. Thus, further investigations that focus on human \textit{in vivo} regulation of SVCT2 expression could be required to better assess its biological responses to modulation of vitamin C levels and oxidative stress than the current \textit{in vitro} or non-human \textit{in vivo} models of investigation (383).
Discussion

7.8. Safety of high dose oral vitamin C

Vitamin C supplementation only produced short term (1-2 weeks) and minor gastrointestinal discomfort in one participant during supplementation. In comparison, placebo supplementation produced no adverse events in any participants. Gastrointestinal side effects are considered one of the few adverse events associated with high dose oral vitamin C supplementation (189). A comprehensive battery of renal and hepatic function tests that included estimated age-adjusted glomerular filtration rate, urea, creatinine, GGT, ALT, AST (Table 6.2 on page 155) and serum electrolytes (data not shown), was undertaken during the study in people with T2D, for which vitamin C supplementation produced no detrimental effects. Of potential concern during high dose vitamin C supplementation is the formation of oxalate from ascorbic acid that could crystallize in the urine, forming kidney stones in susceptible individuals (171). No participants reported on this adverse event. Some studies have found modestly increased urinary oxalate with doses of 1-2 g/day in both stone-forming individuals and non-stone-forming individuals (197-200), leading to suggested recommendations of <2 g vitamin C/day (197, 198) in individuals who are known stone formers. However, most studies in humans with normal renal function have not reported significantly elevated levels of urinary oxalate following high dose vitamin C (189, 384). Greater uncertainty exists in relation to a safe vitamin C dose that avoids worsening of existing hyperoxaluria and an increased risk of kidney stones in individuals with end stage renal disease (384). Individuals with kidney disease or impaired renal and/or hepatic function were excluded from the study in people with T2D.
Discussion

It has been argued that high dose vitamin C supplementation should be avoided in patients with haemochromatosis due to potential toxic pro-oxidant effects associated with iron overload (385). It has thus been recommended that individuals with haemochromatosis limit intake of vitamin C to 500 mg/day from supplements (386). However, there is a lack of data investigating the safety profile of vitamin C supplementation in these potentially susceptible individuals \textit{in vivo} (189). Moreover, empirical evidence for \textit{in vivo} pro-oxidant effects of high dose oral vitamin C are lacking (387). Nonetheless, individuals with haemochromatosis were excluded from the study in people with T2D.
The present thesis investigated effects of chronic high dose vitamin C supplementation on insulin sensitivity, oxidative stress, vitamin C concentration, NOS activity, vitamin C redox state and SVCT2 transporter expression in skeletal muscle of people with T2D. Each key investigation undertaken provides novel insights into in vivo biological effects of vitamin C supplementation in people with T2D. A separate study in healthy individuals was also undertaken to evaluate effects of a similar dose of vitamin C in healthy skeletal muscle.

Vitamin C supplementation significantly improved insulin-stimulated glucose disposal and oxidative stress in skeletal muscle of people with T2D. The finding of improved insulin-stimulated oxidative stress in skeletal muscle is of mechanistic relevance to the improved insulin-stimulated glucose disposal in skeletal muscle. Through an improvement in ROS/RNS levels in skeletal muscle, insulin- and stress-related signalling pathways could have been favourably modulated by chronic vitamin C ingestion to enhance insulin-stimulated glucose uptake in muscle. Targetted mechanistic studies are now required to further elucidate the specific stress-signalling pathways and insulin-signalling targets that vitamin C might modulate in diabetic skeletal muscle.

This thesis also reports for the first time that high dose vitamin C supplementation significantly increases vitamin C levels in skeletal muscle of people with T2D. High dose vitamin C supplementation similarly enhanced vitamin C content of skeletal muscle in healthy young individuals. An increased skeletal muscle vitamin C concentration following vitamin C supplementation in people with T2D could underly
the improvement in insulin-stimulated oxidative stress found in skeletal muscle. The increased total ascorbic acid concentrations of skeletal muscle and plasma found following vitamin C supplementation in people with T2D was largely accounted for by an increased content of reduced ascorbic acid rather than dehydroascorbic acid. However, no significant effect of vitamin C supplementation on vitamin C redox status in either muscle or plasma was observed.

High dose vitamin C supplementation significantly increased SVCT2 protein levels in both diabetic and healthy human skeletal muscle. These findings were contrary to originally hypothesized outcomes, that included the supposition that vitamin C would decrease SVCT2 expression in accordance with intracellular ascorbic acid and/or tissue oxidation status. However, there is a paucity of studies that have investigated effects of vitamin C supplementation on SVCT2 levels in humans in vivo. Moreover, typical human in vitro and non-human in vivo investigations of vitamin C transport kinetics could be of limited relevance to the human condition in vivo. Thus, these novel findings warrant future research into substrate regulation of SVCT2 in humans in vivo.

NOS activity has previously been found to be impaired in skeletal muscle of people with T2D. Mechanistically, vitamin C may play a role in improving NOS levels through stabilization of the NOS cofactor, BH₄. Vitamin C supplementation did not change total NOS activity in skeletal muscle when compared with placebo, although it increased eNOS protein expression. The implications of the latter finding are unclear however, and further investigations into the direct (or indirect) modulation of NO metabolism by vitamin C are required. In particular, the effects of vitamin C supplementation on levels of BH₄, eNOS activity and eNOS enzyme phosphorylation
Conclusion

status are required to better understand the significance of these findings and how they might relate to the improvement in insulin sensitivity observed.

Several factors implicate the applicability of these findings to the clinical field of T2D treatment. Vitamin C supplements were well tolerated by participants, thus offering further evidence of the safety of high dose vitamin C intake. In addition, use of an over-the-counter vitamin C supplement with a daily dosage equivalent to both the prudent upper limit of intake in Australia and a common recommended intake with vitamin C supplements was used in the current investigations. This suggests that such a supplementation regimen could be a practical approach to improving skeletal muscle insulin resistance. Furthermore, vitamin C supplementation was delivered as an add-on therapy to usual medically prescribed diabetes treatments in the people with T2D. This suggests that vitamin C supplementation could potentially serve as an effective complimentary approach to treating skeletal muscle insulin resistance within the domain of mainstream medical prescriptions for T2D.

Overall, these findings provide a biological basis for undertaking a larger clinical trial in people with T2D that is focussed on improving measures of glycaemic control with long-term, high dose oral vitamin C supplementation. Moreover, these findings provide evidence for the potential application of high dose ascorbic acid as a safe adjunct therapy in the treatment of skeletal muscle insulin resistance in people with T2D.
9.0. REFERENCES


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References


References


References


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References


References


References


References


References


References


**Appendix 10.1.** Summary of individual characteristics of participants and screened individuals

**Table 10.1.** Summary data for participants and screenees involved in clinical trial

<table>
<thead>
<tr>
<th>Participant</th>
<th>General Characteristics</th>
<th>Blood Pressure</th>
<th>Plasma Vitamin C</th>
<th>Glucose Control</th>
<th>Lipid Profile</th>
<th>Renal Function</th>
<th>Liver Function</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Participant</th>
<th>General Characteristics</th>
<th>Blood Pressure</th>
<th>Plasma Vitamin C</th>
<th>Glucose Control</th>
<th>Lipid Profile</th>
<th>Renal Function</th>
<th>Liver Function</th>
</tr>
</thead>
<tbody>
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<td></td>
</tr>
</tbody>
</table>

**Note:**
- MEAN ± SD
- Significance level: p < 0.05
- Statistical analysis performed by the

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**Reason for non-inclusion:**
- Pulled out, BP high
- Pulled out, impaired renal function
- HbA1c low
- Pulled out
- HbA1c low, taking vitamin D
- HbA1c high, BP high
- HbA1c low
- newly diagnosed, HbA1c low
- smokes occasionally
- HbA1c low, newly diagnosed
- HbA1c low, TG high, taking vitamin D

---

**Key:**
- met - metformin
- AP - aspirin
- A - Asian/Pacific Islander
- sulf - sulfonylurea
- AT - asthma
- C - Caucasian
- dpp - dpp-4 inhibitor
- BP - blood pressure
- diet - diet-controlled
- CHOL - cholesterol
- G - glaucoma
- OA - osteoarthritis
- P - psychoactive
- U - stomach ulcer
- GD - gord
- AF - atrial fibrillation
- AL - allergies
Appendix 10.2. Diet and physical activity self-reports

7-day diet checklist & Physical activity recall

1. The following diet checklist is to be completed at the end of each day for seven (7) consecutive days. There are three pages for each day to be completed. An example day’s completion is provided at the beginning of the booklet.

2. Please tick (✓) the appropriate serving size box that best approximates your intake (refer to the reference serving size guides and photographs attached).

3. If you consume a listed item more than once during the day or if you consume an amount exceeding the largest serving size indicated, add an extra tick to the appropriate box in the same row.

4. Where indicated, please also list the type and brand name of the food item. Also, where indicated, please note whether the food item consumed was fresh, canned or packaged (including frozen), or if it was a take-away food item.

5. If you do not consume a listed item, do not tick a box for that item.

6. If you would like to explain your intake of a listed food item in more detail, some extra space is provided at the end of each day.

7. At the end of day 7 only, please complete the attached physical activity questionnaire. This questionnaire relates to physical activity over the previous week.
<table>
<thead>
<tr>
<th>Food Category</th>
<th>Serving size:</th>
<th>Small</th>
<th>Med</th>
<th>Large</th>
<th>XL</th>
<th>Reference serving sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Beverages, cereals, meat</strong> (indicate type &amp; brand name if known)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit juice, fruit drink (indicate if freshly prepared)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/2 cup or 125ml</td>
</tr>
<tr>
<td><strong>Type:</strong> Orange Juice</td>
<td><strong>Brand:</strong> Berri</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 cup or 250ml</td>
</tr>
<tr>
<td>Vegetable juice, tomato juice (indicate if freshly prepared)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/2 cup or 125ml</td>
</tr>
<tr>
<td><strong>Type:</strong> Carrot Juice</td>
<td><strong>Brand:</strong> Extra-large (XL) serve: 2 cups or 500ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit-based smoothie drink or fruit-based cordial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/2 cup or 125ml</td>
</tr>
<tr>
<td><strong>Type:</strong> Freshly prepared</td>
<td><strong>Brand:</strong> Extra-large (XL) serve: 2 cups or 500ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy drink or vitamin-fortified supplement drink</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/2 cup or 125ml</td>
</tr>
<tr>
<td><strong>Type:</strong> Mother energy drink</td>
<td><strong>Brand:</strong> Coca cola</td>
<td>Extra-large (XL) serve: 2 cups or 500ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breakfast cereal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/2 cup (any cereal)</td>
</tr>
<tr>
<td><strong>Type:</strong> Cornflakes</td>
<td><strong>Brand:</strong> Kellogg’s</td>
<td>1 cup (any cereal)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pie with fruit or vegetables</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/2 slice pie (about 50g)</td>
</tr>
<tr>
<td><strong>Type:</strong> Berri and Apple pie</td>
<td><strong>Brand:</strong> Sara-Lee</td>
<td>1 slice pie (about 100g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organ meats &amp; pâté (including liver, kidney, heart, brain)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/3 cup meat cooked or 4 tablespoons pâté</td>
</tr>
<tr>
<td><strong>Type:</strong> Lamb liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2/3 cup cooked meat or 2/3 cups pâté</td>
</tr>
<tr>
<td><strong>FRUITS</strong> (indicate fresh “FS” or canned/packaged “CP” beside the food item)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 cup cooked meat or 1 cup pâté</td>
</tr>
<tr>
<td>Apple or Pear</td>
<td>small &lt;8cm dia; large &gt;8cm dia</td>
<td>1/2 piece or 1/2 cup</td>
<td>1 small or 1 cup chopped</td>
<td>1 large or 1 1/2 cups chopped</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orange</td>
<td>small &lt;8cm dia; large &gt;8cm dia</td>
<td>1/2 orange or 5 slices</td>
<td>1 small or 8 slices</td>
<td>1 large or 1 cup sections</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mandarin</td>
<td>small &lt;6cm dia; large &gt;6cm dia</td>
<td>1 small</td>
<td>1 large or 1/2 cup slices</td>
<td>2 (any size) or 1 cup slices</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grapefruit</td>
<td>small &lt;11cm dia; large &gt;11cm dia</td>
<td>1/2 small or 1/2 cup</td>
<td>1 small, 1/2 large, or 1 cup</td>
<td>1 large or 1 1/2 cups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food Category</td>
<td>Small</td>
<td>Med</td>
<td>Large</td>
<td>Small</td>
<td>Medium</td>
<td>Large</td>
</tr>
<tr>
<td>----------------------</td>
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<td>-----</td>
<td>-------</td>
<td>-------</td>
<td>--------</td>
<td>-------</td>
</tr>
<tr>
<td>Guava (any type)</td>
<td></td>
<td></td>
<td></td>
<td>1 common or 1/2 cup</td>
<td>2 common or 1 cup</td>
<td>3 common or 1 1/2 cup</td>
</tr>
<tr>
<td>Kiwi Fruit</td>
<td>✔</td>
<td></td>
<td></td>
<td>1 kiwi fruit or 1/2 cup</td>
<td>2 kiwi fruit or 1 cup</td>
<td>3 kiwi fruit or 1 1/2 cup</td>
</tr>
<tr>
<td>Mango</td>
<td></td>
<td></td>
<td></td>
<td>1/2 mango or 1/2 cup sliced</td>
<td>1 mango or 1 cup sliced</td>
<td>1 1/2 mango or 1 1/2 cup sliced</td>
</tr>
<tr>
<td>Paw-paw</td>
<td></td>
<td></td>
<td></td>
<td>1/2 cup diced or 1/4 fruit</td>
<td>1 cup diced or 1/2 fruit</td>
<td>1 1/2 cup diced or 3/4 fruit</td>
</tr>
<tr>
<td>Pineapple</td>
<td></td>
<td></td>
<td></td>
<td>1/2 cup diced or 1 thin slice</td>
<td>1 cup diced or 1 thick slice</td>
<td>1 1/2 cup diced; 1/2 pineapple</td>
</tr>
<tr>
<td>Banana (small &lt;17cm long; large &gt;17cm long)</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td>1/2 banana or 1/2 cup sliced</td>
<td>1 small banana</td>
<td>1 large or 1 cup sliced</td>
</tr>
<tr>
<td>Grapes (any type)</td>
<td></td>
<td></td>
<td></td>
<td>1/2 cup (about 15 grapes)</td>
<td>1 cup (about 30 grapes)</td>
<td>1 1/2 cups</td>
</tr>
<tr>
<td>Cherries</td>
<td></td>
<td></td>
<td></td>
<td>1/2 cup (about 10 cherries)</td>
<td>1 cup (about 20 cherries)</td>
<td>1 1/2 cups</td>
</tr>
<tr>
<td>Strawberries (small:&lt;3cm dia; large: &gt;3cm dia)</td>
<td></td>
<td></td>
<td></td>
<td>1/2 cup or 6 small/4 large</td>
<td>1 cup or 11 small/7 large</td>
<td>1 1/2 cups or 16 small/11 large</td>
</tr>
<tr>
<td>Other berries (indicate type)</td>
<td></td>
<td></td>
<td></td>
<td>1/2 cup</td>
<td>1 cup</td>
<td>1 1/2 cups</td>
</tr>
<tr>
<td>Plum or Apricot</td>
<td></td>
<td></td>
<td></td>
<td>1 piece or 1/3 cup sliced</td>
<td>2 pieces or 2/3 cup sliced</td>
<td>3 pieces or 1 cup sliced</td>
</tr>
<tr>
<td>Peach or Nectarine (small &lt;5.5cm dia; large &gt;5.5cm dia)</td>
<td></td>
<td></td>
<td></td>
<td>1 small or 1/2 cup sliced</td>
<td>1 large or 1 cup sliced</td>
<td>2 small or 1 1/2 cup sliced</td>
</tr>
<tr>
<td>Rockmelon</td>
<td></td>
<td></td>
<td></td>
<td>1/2 cup diced or 2 slices</td>
<td>1 cup diced or 1/4 melon</td>
<td>1 1/2 cup diced</td>
</tr>
<tr>
<td>Watermelon</td>
<td></td>
<td></td>
<td></td>
<td>1/2 cup diced</td>
<td>1 cup diced</td>
<td>1 1/2 cups or 1/16th melon</td>
</tr>
<tr>
<td>Honey-dew melon</td>
<td></td>
<td></td>
<td></td>
<td>1/2 cup diced</td>
<td>1 cup diced</td>
<td>1 1/2 cup diced or 1/4 melon</td>
</tr>
<tr>
<td>Fruit salad (combination of fruits)</td>
<td></td>
<td></td>
<td></td>
<td>1/2 cup diced</td>
<td>1 cup diced</td>
<td>1 1/2 cup diced</td>
</tr>
</tbody>
</table>

List any other fruits consumed *e.g. lychees, lemon, quince, tangelo, custard apple, passionfruit, rambutan etc.*
*(indicate type, amount & if fresh or canned/packaged)*

2 passionfruit, FS
1/2 custard apple, FS
<table>
<thead>
<tr>
<th>Food Category</th>
<th>Serving size:</th>
<th>Small</th>
<th>Med</th>
<th>Large</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VEGETABLES (indicate if freshly prepared - &quot;FS&quot; or if canned/ packaged/takeaway products - &quot;CP&quot;)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green/mixed salad (including lettuce, tomato, onion etc.) in a sandwich/as a side salad/with a meal</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stir-fried or mixed vegetables</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coleslaw or potato salad</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mixed dish: casserole, stew, curry or stir-fry with vegetables; ratatouille; paella etc.</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>type: Chicken curry with mixed vegetables</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soup with vegetables</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>type: Pumpkin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>VEGETABLES (excluding their use in the above dishes)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potatoes (small potato: 4-6cm dia; medium: 6-8cm dia; large: 8-11cm dia)</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Were they boiled, baked or mashed?</td>
<td>Mashed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hot potato chips/gems/wedges</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Were they homemade, cooked from frozen or takeaway?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potato crisps (any flavor)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Pumpkin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Was it boiled, baked or mashed?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweet potato</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Was it boiled, baked, fried or mashed?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green peas or green beans</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snow Peas</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silverbeet or spinach</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Broccoli</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cauliflower or broccoflower</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brussels sprout</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cabbage (all types)</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carrots</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swede or Turnip</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food Category</td>
<td>Serving size</td>
<td>Small</td>
<td>Med</td>
<td>Large</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------</td>
<td>-------</td>
<td>-----</td>
<td>-------</td>
</tr>
<tr>
<td>Zucchini</td>
<td>(small &lt;16cm long; large &gt;16cm long)</td>
<td>1/2 small or 1/4 cup</td>
<td>1 small or 1/2 cup</td>
<td>2/3 large or 1 cup chopped</td>
</tr>
<tr>
<td>Squash</td>
<td></td>
<td>1 1/2 button or 1/4 cup</td>
<td>3 button or 1/2 cup</td>
<td>6 button squash or 1 cup chopped or 1 capsule</td>
</tr>
<tr>
<td>Capsicum (any colour)</td>
<td></td>
<td>1/4 cup or 1/4 capsicum</td>
<td>1/2 cup or 1/2 capsicum</td>
<td>1 cup</td>
</tr>
<tr>
<td>Sweetcorn/corn on cob</td>
<td>(small cob &lt;8cm long; med cob 13cm; large cob &gt;20cm long)</td>
<td>small cob or 1/4 cup</td>
<td>med cob or 1/2 cup</td>
<td>large cob or 1 cup</td>
</tr>
<tr>
<td>Tomatoes</td>
<td>(small tomato &lt;6cm dia; large tomato &gt;6cm dia)</td>
<td>1/2 small or 3 thin slices</td>
<td>1 small or 1/2 cup</td>
<td>1 large tomato or 1 cup chopped</td>
</tr>
<tr>
<td>Cherry Tomato</td>
<td></td>
<td>2 tomatoes or 1/4 cup</td>
<td>5 tomatoes or 1/2 cup</td>
<td>9 tomatoes or 1 cup</td>
</tr>
<tr>
<td>Parsley</td>
<td></td>
<td>5 sprigs or 1 tablespoon</td>
<td>10 sprigs or 2 tablespoons</td>
<td>20 sprigs or 3 tablespoons</td>
</tr>
<tr>
<td>Avocado</td>
<td></td>
<td>1/4 cup or 4 slices</td>
<td>1/2 cup or 1/2 avocado</td>
<td>1 cup or 1 avocado</td>
</tr>
<tr>
<td>Lettuce (any type)</td>
<td></td>
<td>1/2 cup</td>
<td></td>
<td>2 cups</td>
</tr>
<tr>
<td>Cucumber (any type)</td>
<td></td>
<td>1/4 cup or 3-4 slices</td>
<td>1/2 cup chopped</td>
<td>1 cup chopped or 1/2 whole</td>
</tr>
<tr>
<td>Chilies (any type)</td>
<td>(small: 5cm long; medium: 11cm long)</td>
<td>4 small chili or 1/4 cup</td>
<td>2 med chili or 1/2 cup</td>
<td>1 cup chili</td>
</tr>
<tr>
<td>Onions (any type)</td>
<td></td>
<td>4 thin slices or 1/4 cup</td>
<td>1/2 cup chopped</td>
<td>1 cup</td>
</tr>
<tr>
<td>Other beans (indicate type)</td>
<td>Type:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

List other vegetables consumed e.g. Okra, watercress, leeks, shallots, choko, asparagus, artichoke, radish, alfalfa sprouts
(indicate type, amount & if fresh, frozen, canned, cooked or raw):

Additional notes you have on your intake of the foods above:

________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
Physical activity recall

A. DURING THE LAST WEEK how many times did you walk continuously, for at least 10 minutes, for recreation/exercise or to get to or from places? ______ times

What do you estimate was the total time that you spent walking in this way? _______ mins

B. DURING THE LAST WEEK how many times did you do any vigorous physical activity which made you breathe harder or puff and pant? (e.g. jogging, cycling, aerobics, competitive tennis, etc.) ______ times

What do you estimate was the total time that you spent doing this vigorous physical activity? _______ mins

C. DURING THE LAST WEEK how many times did you do any other more moderate physical activity that you haven’t already mentioned? (e.g. gentle swimming, social tennis, golf) ______ times

What do you estimate was the total time that you spent doing these moderate physical activities? _______ mins
Photographs of sample serving sizes

Beverages:

Juice/Fruit drink:

small serve (125ml)                   medium serve (250ml)               large serve (375ml)

small serve (125ml popper)        medium serve (250ml popper)    extra-large serve (500ml)

Energy drink/vitamin fortified drinks

small serve (1-2 small “shots”)             medium serve (250ml drinks)

Large energy drinks (approx.375ml)      Extra-large energy drink (approx.500ml)
Cereals:

*Breakfast cereal*

- Small serve (1/2 cup)
- Medium serve (1 cup)
- Large serve (1 ½ cup)

**Pie**

- Small serve (about 50g)
- Medium serve (about 100g)
- Large serve (about 200g)

**Organ Meats:**

*Liver*

- Small serve (1/3 cup)
- Medium serve (2/3 cup)
- Large serve (1 cup)
Fruit (examples):

Rockmelon

small serve (1/2 cup)       medium serve (1 cup or ¼ melon)          large serve (1 ½ cup)

Pineapple

small serve (1/2 cup)                 medium serve (1 cup)                    large serve (1 ½ cup)

Grapes

small serve (1/2 cup)                  medium serve (1 cup)                     large serve (1 ½ cup)

Fruit salad

small serve (1/2 cup)                medium serve (1 cup)               large serve (1 ½ cup)
Small fruit serve examples:

1 apricot
1 kiwi fruit
1 small mandarin

Medium fruit serve examples:

1 small apple
1 small orange
1 cup cherries
1 large peach or nectarine
1 small pear
1 mango

Large fruit serve examples:

3 plums
11 large strawberries
1 large banana (about 20cm long)
Vegetables (examples):

Boiled broccoli

Boiled pumpkin

Green Beans

Mixed cooked vegetables
**Corn**

1 small cob or \( \frac{1}{2} \) med cob (8cm)  
medium serve: 1 med cob (13cm)  
large serve: 1 large cob (20cm)

**Boiled Swede**

small serve (1/4 cup chopped)  
medium serve (1/2 cup chopped)  
large serve (1 cup chopped)

**Onions**

small serve (1/4 cup diced)  
medium serve (1/2 cup diced)  
large serve (1 cup diced)

**Tomatoes**

small serve (1/4 cup chopped)  
medium serve (1/2 cup chopped)  
large serve (1 cup chopped)
Mixed salad (including lettuce, tomatoes, onion etc.)
small serve (1/2 cup)  medium serve (1 cup)  large serve (2 cups)

Parsley (on small plate)
small serve (5g or 5 sprigs)  medium serve (10g or 10 sprigs)  large serve (20g or 20 sprigs)

Vegetable soup
small serve (1/2 cup)  medium serve (1 cup)  large serve (2 cups)

Stew with vegetables
small serve (1/2 cup)  medium serve (1 cup)  large serve (2 cups)
Boiled potato

1 small potato (or about 90g)  
1 medium potato (or about 120g)  
1 large potato (or about 180g)

Mashed potato

small serve (1/2 cup)  
medium serve (3/4 cup)  
large serve (1 cup)

Hot potato chips

small serve (1/2 cup)  
medium serve (3/4 cup)  
large serve (1 cup)

Baked potato

small serve: 1 small potato  
medium serve: 1 medium potato  
large serve: 1 large potato

(some photographs from (388))
Appendix 10.3. Calculations of glucose metabolic measurements from GC/MS-derived data and indirect calorimetry

Rate of glucose appearance, Ra (µmol.kg FFM⁻¹.min⁻¹) =
\[\frac{E_b}{E_p(t)} - \frac{p \times V \times G(t)}{E_p(t)} \left(\frac{dE_p(t)}{dt}\right) + \frac{E_{var} \times I_{var(t)}}{E_p(t)}\]

Rate of glucose disappearance, Rd (µmol.kg.FFM⁻¹.min⁻¹) =
\[\frac{E_b}{E_p(t)} - \frac{E_b}{100} - \frac{p \times V \times G(t)}{E_p(t)} \left(\frac{dE_p(t)}{dt}\right) + \frac{E_{var} \times I_{var(t)}}{E_p(t)} - \frac{p \times V \times dG(t)}{dt}\]

where

- \(E_b\) = enrichment of basal continuous 6, 6-D₂ glucose in moles per cent excess
- \(E_p(t)\) = plasma MPE, as average of two consecutive samples
- \(E_{var}\) = MPE of glucose infusate
- \(I_b\) = continuous tracer infusion rate (µmol.kg⁻¹.min⁻¹)
  \((Note: I_b = 0.22 \text{ µmol.kg}^{-1}.\text{min}^{-1} \text{ in this study})\)
- \(P\) = pool fraction assumed to be 0.65
- \(V\) = glucose space assumed to be 25% of body weight
- \(G(t)\) = plasma glucose (µmol.l⁻¹), as average of two consecutive samples
- \(I_{var}\) = exogenous glucose infusion rate (µmol.kg⁻¹.min⁻¹)

Rate of glucose disappearance/plasma insulin, Rd/l (l.kg.FFM⁻¹.min⁻¹) =
\[\frac{Rd}{\text{plasma insulin (in µmol.l}^{-1})}\]

Metabolic clearance rate, MCR (ml.kg⁻¹.FFM⁻¹.min⁻¹) =
\[\frac{Rd}{\text{plasma glucose (in µmol.ml}^{-1})}\]

Endogenous glucose production rate, EGP (µmol.kg·FFM⁻¹.min⁻¹) =
\[Ra – \text{glucose infusion rate (in µmol.kg·FFM}^{-1}.\text{min}^{-1})\]

Non-oxidative glucose metabolism, NOGM (µmol.kg·FFM⁻¹.min⁻¹) =
\[Rd – \text{carbohydrate oxidation rate (in µmol.kg·FFM}^{-1}.\text{min}^{-1})\]

Carbohydrate oxidation rate (g.min⁻¹) =
\[4.55 \times VCO₂ - 3.21 \times VO₂ - 2.87 \times N \text{ (where N is assumed to be 0.009 g.min}^{-1})\]

Fat oxidation rate (g.min⁻¹) =
\[1.67 \times VO₂ - 1.67 \times VCO₂ - 1.92 \times N \text{ (where N is assumed to be 0.009 g.min}^{-1})\]

(Calculations above are from (323), (326) & (327))
Appendix 10.4. Optimization of GSH/GSSG assay

**Figure 10.1.** Protein optimization of muscle GSH assay

An optimal range of protein concentration was determined as 0.0625-0.5 mg/ml, with a concentration of 0.125mg/ml used in assays.

![Graph showing absorbance over time for different protein concentrations](image)

**Figure 10.2.** Protein optimization of muscle GSSG assay

An optimal range of protein concentration was determined as 0.5-1.0 mg/ml, with a concentration of 0.6mg/ml used in assays.

![Graph showing absorbance over time for different protein concentrations](image)

**Table 10.2.** Muscle GSH optimization

<table>
<thead>
<tr>
<th>Protein concentration (mg/ml)</th>
<th>Concentration GSH (μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.37</td>
</tr>
<tr>
<td>1</td>
<td>3.25</td>
</tr>
<tr>
<td>0.5</td>
<td>2.18</td>
</tr>
<tr>
<td>0.25</td>
<td>1.27</td>
</tr>
<tr>
<td>0.125</td>
<td>0.69</td>
</tr>
<tr>
<td>0.0625</td>
<td>0.37</td>
</tr>
</tbody>
</table>

**Table 10.3.** Muscle GSSG optimization

<table>
<thead>
<tr>
<th>Protein concentration (mg/ml)</th>
<th>Concentration GSSG (μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.63</td>
</tr>
<tr>
<td>2</td>
<td>2.12</td>
</tr>
<tr>
<td>1</td>
<td>1.23</td>
</tr>
<tr>
<td>0.5</td>
<td>0.59</td>
</tr>
<tr>
<td>0.25</td>
<td>0.36</td>
</tr>
<tr>
<td>0.125</td>
<td>0.24</td>
</tr>
</tbody>
</table>
Figure 10.3. Control samples run on skeletal muscle GSH and GSSG assay plates

Demonstration of linearity of sample protein concentration in assays for (A) GSH and (B) GSSG in skeletal muscle. Data are n=1.
Appendix 10.5. Optimization of SOD assay

Figure 10.4. Lysis buffer optimization for SOD assay in skeletal muscle

A comparison of SOD activity for samples homogenized in different lysis buffers. CS buffer contained 0.175 mol/l KCl and 2 mmol/l EDTA (pH 7.4). SOD buffer contained 100 mmol/l NaH₂PO₄ (pH 7.0) with 1 mmol/l EDTA and 0.5 % Triton-X100. WB buffer contained 50 mmol/l Tris (pH 7.5), 1 mmol/l EDTA, 10 % vol/vol Glycerol, 1 % vol/vol Triton X, 50 mmol/l NaF, 5 mmol/l NaPP, 1 mmol/l DTT, 1 mmol/l PMSF, and 5 μl/ml protease inhibitor cocktail (#P8340, Sigma-Aldrich). Data are mean ± SEM; *and # indicate significantly different to unlabelled bars using a one-way repeated measures ANOVA (n=4). The SOD buffer produced the highest SOD activity and was chosen as the sample lysis buffer.

Figure 10.5. Control samples run on skeletal muscle SOD assay plate

Demonstration of linearity of sample protein concentration used in SOD assay (0.2 mg/ml). Data are n=1.