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Rapid induction of vitamin B12 deficiency in *Caenorhabditis elegans* cultured in axenic medium

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HIGHLIGHTS

- *C. elegans* grown in defined axenic medium without added B12 exhibit signs of deficiency in one generation.
- Worms grown in deficient media had retarded growth, reduced fertility, reduced quiescence and shortened lifespan.
- *C. elegans* cultured in a defined axenic medium is a suitable and rapid model for B12 deficiency study.

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ABSTRACT

Animal models of B_{12} deficiency have proven to be difficult due to storage of substantial amounts in the liver and the length of time required to maintain animals on a B_{12} deficient diet to induce deficiency. *Caenorhabditis elegans (C. elegans)*, due to its short lifespan, has recently emerged as an alternate model to investigate vitamin B12 (B12) deficiency. However, when *C. elegans* are maintained on bacterial diet, five generations of B12 deficient diet is required before the worms show signs of deficiency. Here we show that *C. elegans* grown in chemically defined axenic medium without added B12 exhibit signs of deficiency within one generation. Worms grown in deficient media had lower cobalt concentration, retarded growth, reduced fertility, increased motility, reduced quiescence and a shortened lifespan. In conclusion, *C. elegans* cultured in a defined axenic medium is a suitable and rapid model for studies on B12 deficiency.

1. Introduction

Research on vitamin B12 (B12) deficiency has long been constrained by lack of a suitable model organism due to the fact that animals store B12 in their liver for long term use [1], which prolongs the process of generating B12 deficient models. The commonly used model animals such as rodents require several months on a B_{12} deficient diet to deplete their body reserve before symptoms of deficiency become apparent.

The free-living soil nematode *Caenorhabditis elegans* is a microscopic (1 mm body length at its adult stage) and free-living organism with about 1000 cells. It needs only 3 days to complete its life cycle (L1 to adult) and its lifespan is 3–4 weeks. It has been well established that the nematode is an important model to study the functions of disease-related genes underpinning a number of major advances in the field of developmental biology and neurobiology. Watson, Olin-Sandoval [2] also reported that B12 deficient diets in *C. elegans* transcriptionally activate a B12-independent propionate breakdown shunt. Hence, these are believed to be important characteristics which back up the organism's strong potential to serve as a suitable model to study B12 deficiency.

Bito, Matsunaga [3] managed to induce B12 deficiency in *C. elegans* after feeding B12-deficient *E. coli* (0.2 μg/g wet weight) for five successive generations. According to their report, signs of B12 deficiency including reduced growth and fertility were not observed until the third generation. Here, we report that, in chemically defined axenic media without added B12, *C elegans* show signs of B12 deficiency including reduced growth, fertility and lifespan within one generation.

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2. Methods

2.1. Maintenance of C. elegans

Wild-type *C. elegans* strain (N2) and a dietary sensor strain (*Pacdh-1*::*GFP*) were acquired from *C. elegans* Genetics Center (CGC) and University of Massachusetts respectively and maintained on NGM agar medium with a lawn of *E. coli* OP50 bacteria as food. Prior to conducting all the experiments in CeHR medium, the worms were allowed to adapt to the axenic environment for three generations before use.

Stock solutions of salts, vitamins and growth factors, amino acids, nucleic acids, buffers were prepared separately with deionised water from Milli-Q system before assembling them with 20% UHT skim milk. All components of the media were filtered with 0.22 µm filter and stored in light protective containers. A mix of antibiotics (Nalidixic acid, 250 µg/mL; Streptomycin, 250 µg/mL and Tetracycline, 100 µg/mL) were also added after assembly to keep the medium sterile and axenic [4]. The pH was adjusted to 6.5 prior to culturing the worms. The media preparation steps were done as reported by Samuel, Sinclair [5]. Accordingly, the final concentrations of the ingredient in CeHR were as follows: Cyanocobalamine (2.77 µM), Biotin (15.35 µM), Folinate (Ca µM) (7.33 µM), Folic acid (16.99 µM), Niacinamide (61.41 µM), Pantetheine (6.76 µM), Pantothenate (Ca µM) (31.47 µM), Pyridoxal 5′-phosphate (15.18 µM), Pyridoxamine.2HCl (15.55 µM), Pyridoxine.HCl (36.48 µM), Niacine (60.92 µM), Riboflavine 5-PO4 (15.68 µM), Thiamine.HCl (22.24 µM), DL-6,8-Thioctic Acid (18.18 µM), Glucose (72.97 µM), HEPES Na salt (20 µM) and Cholestrol (12.93 µM), Adenosine 2'- & 3′-PO4 (1002.3 µM), Cytidine 5′-PO4 (1002.18 µM), Guanosine 5' - PO4 (999.45 µM), Uridine 5′-PO4 (999.73 µM), Thymine (999.05 µM), KH2PO4 (9 mM), Choline di-acid citrate (1.998 mM), i-Inositol (2.4 mM), Hemin Chloride (20 µM), MgCl2.6H2O (2016.62 µM), Sodium Citrate (986.06 µM), Potassium Citrate.H2O (1510.48 µM), CuCl2.2H2O (41.06 µM), MnCl2.4H2O (101.06 µM), ZnCl2 (73.37 µM), Fe(NH4)2(SO4)2.6H2O (153 µM), CaCl2.2H2O (136.04 µM), *N*-Acetylglucosamine (67.81 µM), DL-Alanine (168.35 µM) and *p*-Aminobenzoic Acid (54.69 µM). Additionally, 20 mL essential of Amino Acid mix (GIBCO 11130–051) and 10 mL of non-essential Amino Acid mix (GIBCO 11130–050) were added in 1L CeHR at the final media assembly stage.

2.2. B12 in individual worms

The B12 content of individual worms was determined by measuring their Cobalt (Co) concentration, using Inductively coupled plasma mass spectrometry (ICP-MS) as reported by Ganio, James [6]. Co concentration of ten independent biological replicates of individual *C. elegans* were analysed from those cultured in the absence of B12 and from the control group. Synchronous L1 *C. elegans* were cultured in the two groups until they reached mature adult age. They were then removed from the axenic media and transferred into 1.5 mL polypropylene test tubes containing 200 µL S-basal (5.85 g L^{-1} NaCl; 1 g L^{-1} KH_2PO_4; 6 g L^{-1} K_2HPO_4). The test tubes were gently inverted to clean the remnants of the culturing media attached with the bodies the worms. Samples were then washed three times with 200 µL S-basal, followed by three washes in 200 µL ultra-pure water to remove any remaining bacteria. To avoid sample loss, a stereomicroscope was used to monitor during aspirations between washes. Samples were then flash frozen in liquid nitrogen and lyophilized overnight. After lyophilisation, samples were digested in 20 µL of 65% HNO_3 for 12 h at room temperature and diluted 1:10 to a final volume of 200 µL using 1% HNO_3. To correct for any contamination that may occur during the digestion process, ten blanks were prepared parallel to each. Finally, Cobalt concentration along with other metals was quantified using NexION 350X ICP-MS (Perkin Elmer).

2.3. Growth, fertility and lifespan

Change in body length, the number of offspring produced by each adult worm and survival data were collected for each worm in both treatment and control group. Growth rate data was obtained from images collected every other day by measuring changes in length. Additionally, fertility assay was conducted by counting the number of progenies excluding unhatched eggs. Moreover, lifespan assay was also carried out by counting the number of dead animals every other day. In this study, instead of using Floxuridine (FuDR) to stop reproduction of worms, they were transferred to new plates with fresh media until they finished laying eggs. When the worms ceased laying eggs, they were left on the same plate and monitored for lifespan until all the worms died. Collecting lifespan data was started once most of the worms in each treatment became adults. For convenience in transferring and data collection, single worms were kept in individual wells of 24 or 48 well plates.

2.4. Acyl-CoA dehydrogenase expression

Acyl-CoA dehydrogenase *acdh-1* expression was determined by measuring the fluorescence of Pacdh-1:GFP strain grown and maintained in CeHR media with and without added B12. Adult worms were placed on 2% agar pads on mounting slides and covered with cover slides. Then the worms were immobilised by quick heat shock on a flame for 3–5 s to take fluorescence images using EVOS cell imaging system. Analysis of fluorescence images were done using the open access image and video analysis software imageJ where fluorescence intensity was measured based on image thresholding.

2.5. Measurement of quiescence and motility

MBF WormLab software version 4.1 was used for video analysis. Motility of worms exposed to different B12 treatments were analysed. Parameters selected from the video analysis were track length (µm) and speed (µm/s) (See Fig. 1). Track length is the length of forward motion plus the length of reverse motion from mid-point in a total number of tracks. Whereas, speed represents the track length in every second.

2.6. Data collection and analysis

EVOS FL Auto Cell Imaging System was used to follow up growth, fertility and survival of worms. Growth and development of the worms were measured using Image J open access image analysis software. Image J open access image analysis software was also used for setting the scale of images and videos using based on scale bars. Video

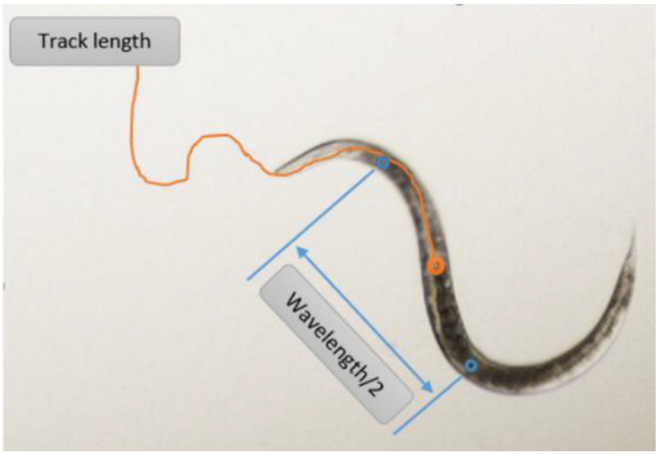

Fig. 1. Illustration of wavelength and track length measurements made by MBF-WormLab video analysis software.

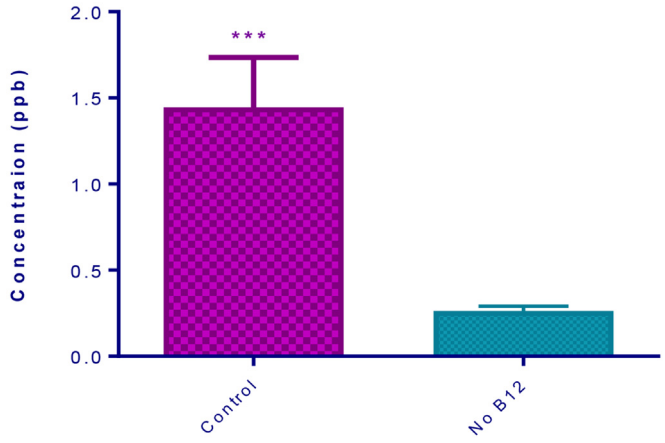

Fig. 2. Mean (SEM) Cobalt concentrations (ppb) in individual *C. elegans* cultured in the absence of B12 compared to the control group (added B12). n = 20 per treatment group.

acquisition was done on a Nikon Stereo microscope and MBF-WormLab video analysis software was used to analyze motility and morphologic measurements of worms cultured in B12 deficient media compared to control. All data were summarized on Excel and exported to GraphPad Prism 6 and IBM SPSS Statistics 24 for analysis and to generate graphs. Statistical analysis included ANOVA, independent *t*-test, survival analysis, post-hoc mean comparison and descriptive statistics. Observation values were reported in Mean, Median, Percentage and Standard Error of Mean (SEM).

3. Results

3.1. Single worm cobalt concentration to assess B12 status

Co concentrations in the control and B12 deficient worms is shown in Fig. 2 based on single worm ICPMS analysis. Accordingly, the Co concentration in individual worms decreased significantly *($p < 0.05$)* from 1.46 ppb in the control group to 0.26 ppb in the deficient group.

3.2. Effects of B12 deficiency on C. elegans Growth

Measurements on body length were collected from L1 to the adult stage. A substantial growth retardation was observed in B12 deprived worms compared to the control group (Fig. 3). At day two, B12 deficient worms had a mean (SEM) body length of 379 (20) μm, whereas the worms in control group were 1014 (33) μm long. At day four, the deficient worms were only 853 (40) μm compared to the control group that attained 1386 (26) μm. At day six, the deficient group attained mean (SEM) body length of 1280 (28) μm, while their counterparts in the control group reached 1652 (33) μm. Based on seven days data from L1, the mean body length attained by B12 depletion group (1291 μm) were significantly smaller than the control (1652 μm) ($p < 0.05$).

3.3. Effects of B12 deficiency on C. elegans Fertility

The worms grown in the absence of B12 were found to hatch significantly smaller number of offspring compared to the control group ($p < 0.05$; Fig. 4B). The mean (SEM) number of offspring in the control group was 166 (6) while in the B12 deficient group it was 80 (5). Time to lay the first egg was delayed by three days in the B12 deficient worms. (Fig. 4A).

3.4. Effects of B12 deficiency on C. elegans Quiescence and Activity

The activities of the worms deprived of B12 and the control groups were compared based on locomotion parameters from a video analysis of adult *C. elegans* cultured in CeHR with and without added B12 (Fig. 5A). Accordingly, the speed of B12 deprived worms were significantly higher than their control counterparts *($P < 0.05$)*.

Moreover, Fig. 5B illustrates that the average quiescence duration of the control group was longer (10 s) than B12 deficient counterparts (2 s) based on analysis of 10 min long videos. As illustrated in Fig. 5B, different quiescence durations were observed. Hence, percentage indicates how often each quiescence duration was observed.

3.5. Effect of B12 deficiency on lifespan

As shown in Fig. 6, a significant difference in survival was observed between B12 deficient worms and the control group. *C. elegans* culture in the medium with B12 (control) showed significantly higher ($p < 0.05$) median survival (21 days) compared to the deficient group (17 days).

3.6. Pacdh-1:GFP expression in B12 deficient worms

Fluorescence intensity (Fig. 7A) measured from images of the worms cultured in medium without added B12 was found to be significantly higher than the control (2.77μM B12) at *($P < 0.05$)*. Fig. 7B and C also show visual differences where GFP expression was up-regulated in worms grown in no added B12 medium (7 B) than in control (7C).

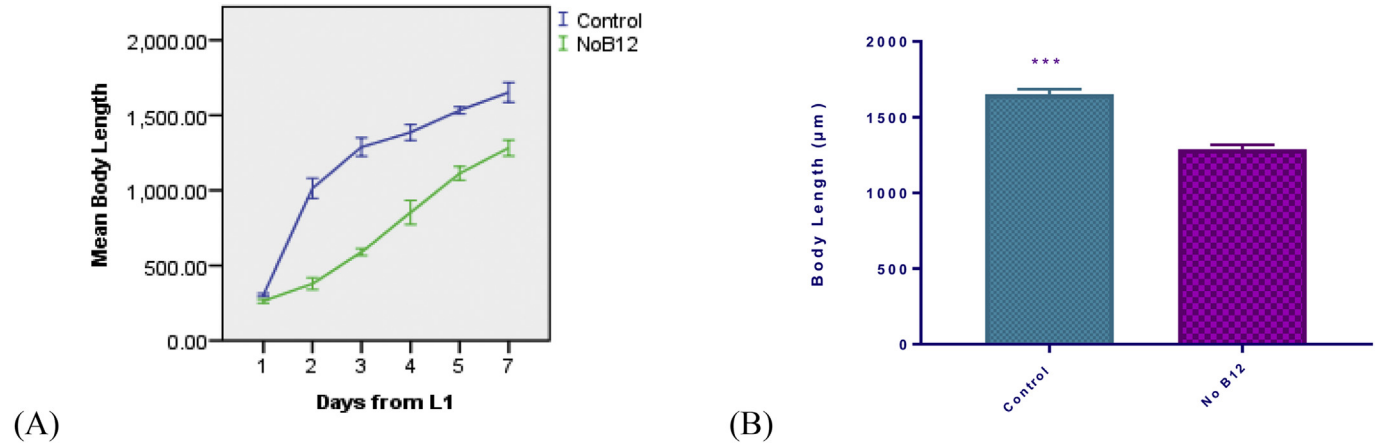

Fig. 3. (A) Growth curve of the worms deprived of B12 compared to the control. (B) Mature body length of N2 in a culturing media lacking B12 compared to control. n = 24 per treatment group.

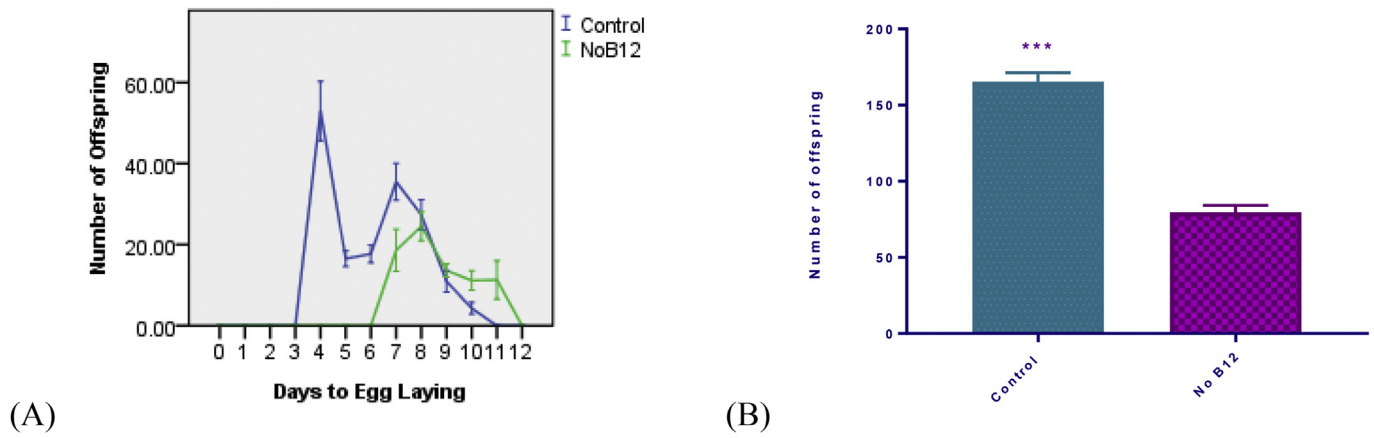

Fig. 4. (A) Mean (SEM) number of offspring against number of days spent for egg laying. N = 24 per treatment group; (B) Mean (SEM) Number of Offspring in *C. elegans* cultured in the absence of B12 compared to control group where the culturing medium contains B12. N = 24 per treatment group.

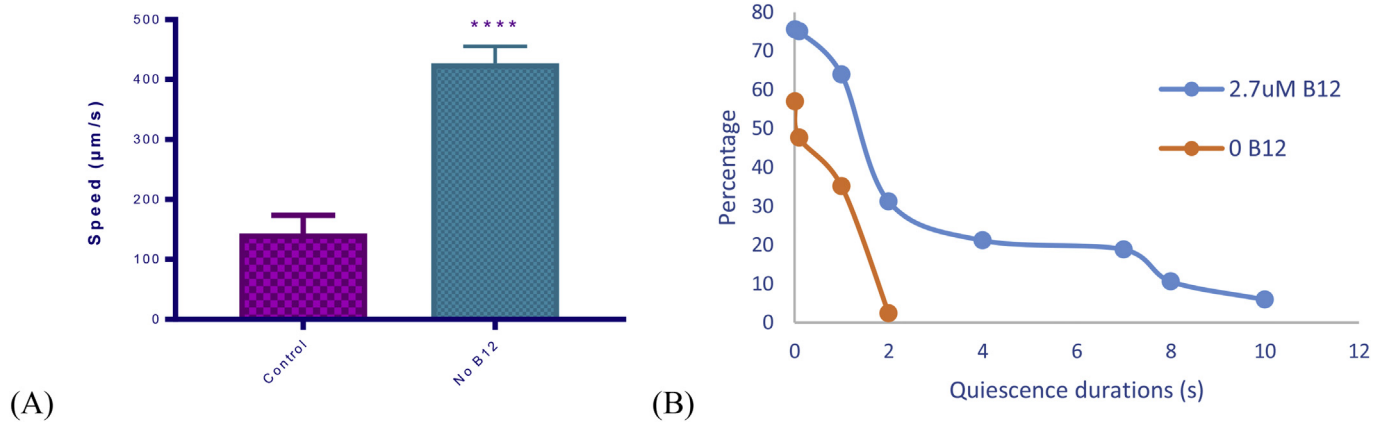

Fig. 5. Activity of *C. elegans* cultured in B12 deficiency condition compared to control. (A) Speed (um/s) [peristaltic track length per second]. (B) Duration in a state of quiescence (s) and percentage (%) of occurrence of each duration in *C. elegans* cultured in media with no added B12 compared to control group.

4. Discussion

Co is the central ion of cyanocobalamin, the form of B12 used in this study. Given that the two exclusive sources of Co in the chemically defined medium, CeHR are skim milk and added cyanocobalamin, and exactly same amount of skim milk was added to both control and deficient media, it is reasonable to consider that added cyanocobalamin would be the only source of variation in Co concentration between worms cultured in the two media. Hence, Co concentration can safely serve as a proportional indicator of B12 status of the worms.

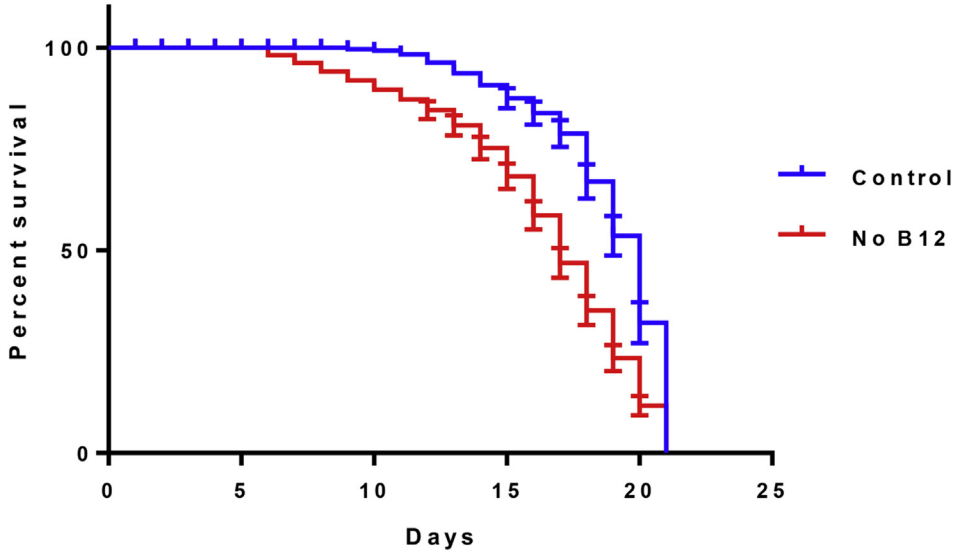

Fig. 6. Lifespan of *C. elegans* cultured in the absence of B12 compared to control. n = 24 per treatment group.

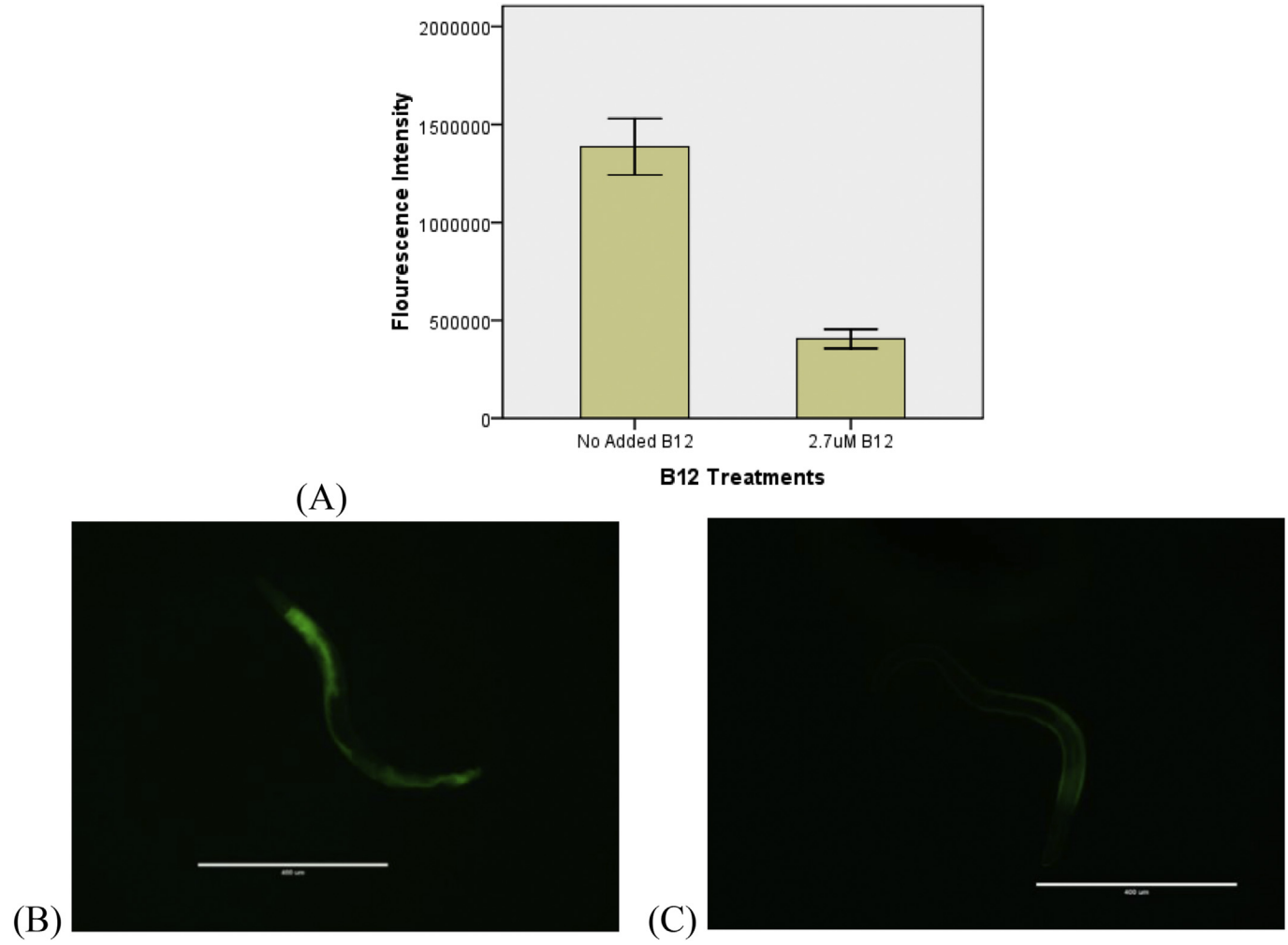

Fig. 7. (A) Mean (SEM) fluorescence intensity of Pacdh-1:GFP *C. elegans* strain cultured in the absence of B12 compared to control. n = 10 per treatment group. (B) GFP expression of the stain cultured in the absence of B12 and (C) Control. n = 10 per treatment group.

The results of this study showed that by using axenic culture, effects of B12 deficiency can be clearly detected within the first generation of B12 depletion in *C. elegans*. Previously, Bito, Matsunaga [3] reported induction of B12 deficient worms after five successive generation of feeding *E coli* grown under B12 deficient conditions. The concentration of B12 in the worms fed the B12 deficient *E coli* decreased gradually from 75% of the control group in the first generation to 4% of the control group in the fifth generation. In our study, cobalt content of worms decreased to 14% of the control within one generation indicating rapid depletion of B12 stores in axenic media without added B12. This illustrates that substantial amount of B12 was consumed by the worms in the medium with added B12 in comparison to their deficient counterpart. It is also interesting to note that skim milk, making up 20% of the CeHR medium which is a rich source of B12 (0.5 μg/100 g), failed to sustain B12 concentration in the worm. We recently reported that *C elegans* are unable to access nutrients in solution and require particulate matter for delivery of nutrients to the worm [7]. Results of the present study suggest that B12 in skim milk is a poor source of B12 to *C. elegans*.

Our observation of a significant decline in fertility is consistent with previous reports of reduced egg laying in *C. elegans* [3] and fetal loss and infertility in humans with B12 deficiency [8,9]. B12 plays its major roles involving its two forms: methylcobalamin, and deoxyadenosylcobalamin. The former serves as a cofactor in the process of methyl transfer from methyltetrahydrofolate (MTHF) to methionine. Additionally, the amino acid methionine is the building block for the universal methyl donor, S-adenosylmethionine. Hence, methylcobalamin plays substantial roles in DNA methylation and DNA replication [10,11]. DNA replication is also known for its critical role in biological processes during multiplication of cells. This, in turn, is the basis in fertilization and development of the fetus.

The proteins which packages the DNA in the nucleus, histone, also undertakes methylation where B12 plays its vital role as cofactor during transfer of the methyl groups [12]. A gradual drop in fertility of *C. elegans* was reported as a result of deletion of histone H3 lysine 4 dimethyl (H3K4me2) demethylase, signifying the role of histone, H3 in regulating the fertility of the worms [13]. Hence, linking mechanism for B12 deficiency and decline in fertility might also be histone methylation.

Watson, MacNeil [14] reported that the mechanism through which vitamin B12 accelerates development in *C. elegans* is due to the role of B12 as a cofactor in S-Adenosylmethionine (SAM) cycle. To reach to this conclusion, they measured the growth rate in mutants and genes involved in the B12 dependent SAM cycle (Δ*metr-1* and Δ*sams-1*). The growth rate of Δ*sams-1* mutant animals, which are impaired in converting methionine to SAM was not enhanced by methionine supplementation, implying that the developmental acceleration by methionine depends on its conversion to SAM. Further to this, the study also testified that propionic acid accumulation as a result of B12 deficiency is toxic to *C. elegans* and causes growth retardation, which can be corrected to some extent by B12 supplementation.

Prior to this, Bito, Matsunaga [15] suggested that B12 deficiency

reduces the activity of methylmalonyl-CoA mutase (MCM) and causes accumulation of methylmalonic acid (MMA). Elevated MMA obstructs activity of succinate dehydrogenase, which in turn inhibits mitochondrial respiration. Mitochondrial respiration is known to be the major biochemical process in cells through which energy from macronutrients is converted to ATP. Therefore, it is sensible to relate the interruption of this process with disorders such as growth retardation.

Our observation of the increased GFP expression of Pacdh-1:GFP worms under B12 deficiency conditions is in agreement with previous reports on increased Pachdh-1 expression in *C elegans* fed *E. coli* grown in low B12 media (Watson, MacNeil et al., 2014). Acyl CoA dehydrogenase is a mitochondrial enzyme that catalyses the initial steps of beta oxidation [16]. Recently [2], reported that, under B12 deficient conditions, *C elegans* transcriptionally activates an alternate pathway for the breakdown of propionate. Accordingly, expression of several genes including *acdh-1* is increased in response to accumulation of propionate. It was consequently proposed that this alternate propionate shunt that enables *C. elegans* to survive B12 deficient diet they may encounter in the wild. Interestingly, this alternate propionate shunt is also present in humans.

It has been documented that satiated animals tend to stop eating, decrease exploring and fall asleep in a phenomenon known as 'behavioral sequence of satiety'. Worms, according to You, Kim [17], when they are satisfied, they stop eating and moving, named as 'satiety quiescence'. The study was executed based on OP50 E. *coli* compared to a higher quality Comamonas food. Previously, Watson, MacNeil [14] reported that Comamonas supports growth and reproduction of the nematode better than OP50 E. *coli*, where the metabolites that made the difference was found to be B12. The findings in this report, which illustrated significantly longer quiescence shown by worms grown in the presence of B12 compared to those grown in the absence of B12, can relate to satiety quiescence. Supporting this suggestion, vital roles of B12 as a cofactor in metabolic processes and in utilizing macromolecules for normal functioning it well documented [18]. Hence, it is rational to consider that lower quiescence of B12 deficient worms represents "metabolic hunger" induced by the deficiency of B12.

In conclusion, our results show that *C. elegans* cultures in defined axenic media is a rapid and useful model for studies on B12 deficiency.

Conflicts of interest

All authors confirm that there was no conflict of interest identified.

Acknowledgments

Authors Contribution: S.M.T. and M.J. designed the experiments, S.M.T. executed the experiments as a piece of PhD candidature, M.R.F. assisted in media preparation and maintenance of *C. elegans* strains, D.L.C. and D.B. assisted in ICPMS analysis. All authors were involved in manuscript preparation.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jnim.2018.08.001.

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