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Original Communication

Effect of a Klamath algae product ("AFA-B12") on blood levels of vitamin B12 and homocysteine in vegan subjects: a pilot study

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Abstract: Vitamin B12 is a critical nutrient that is often inadequate in a plant-based (vegan) diet, thus the inclusion of a reliable vitamin B12 source in a vegan diet is recommended as essential. Unfortunately, many natural sources of vitamin B12 have been proven to contain biologically inactive vitamin B12 analogues, inadequate for human supplementation. The aim of this non-randomized open trial was to determine whether supplementation with a natural Klamath algae-based product ("AFA-B12", *Aphanizomenon flos-aquae* algae plus a proprietary mix of enzymes) could favorably affect the vitamin B12 status of a group of 15 vegan subjects. By assessing blood concentration of vitamin B12, folate, and more importantly homocysteine (Hcy, a reliable marker in vegans of their B12 absorption), the vitamin B12 status of the participants at the end of the 3-month intervention period, while receiving the Klamath-algae supplement (T2), was compared with their vitamin B12 status at the end of the 3-month control period (T1), when they were not receiving any supplement, having stopped taking their usual vitamin B12 supplement at the beginning of the study (T0). Compared to the control period, in the intervention period participants improved their vitamin B12 status, significantly reducing Hcy blood concentration ($p=0.003$). In conclusion, the Klamath algae product AFA-B12 appears to be, in a preliminary study, an adequate and reliable source of vitamin B12 in humans.

Key words: Vegans, vitamin B12, cyanobacteria, Klamath algae, *Aphanizomenon flos-aquae*, homocysteine.

Introduction

Research conducted all over the world has established that a well-balanced, plant-based diet is a powerful tool in the prevention of many common Western diseases [1]. Meanwhile, to ensure the nutritional adequacy of a plant-based (vegan) diet, it is necessary to provide an adequate intake of some essential nutrients such as vitamin D, calcium, omega-3 fatty acids, and vitamin B12 [2]. Vitamin B12 is the only vitamin that humans cannot obtain from plant food in adequate amounts. Vitamin B12, which is produced exclusively by the bacteria of the soil, has traditionally entered the human food chain as a “pollutant” of animal and vegetable foods. However, while it cannot be removed from animal food, as it is stored in the animal tissues, it is totally removed from vegetable food during the common hygienic handling practices applied in civilized countries.

International nutritional research recommends supplementing a plant-based diet with enough vitamin B12 to assure an average daily absorption of 1.5 µg. This amount can be provided by the daily intake of 3 µg of vitamin B12 from different sources or 10 µg from one source, or from one weekly intake of 2000 µg [3]. Furthermore, the American Institute of Medicine has established that up to 30% of the older population (above 50 years of age), regardless of diet, loses the ability to assimilate vitamin B12 from food [4]. As a consequence, the latest American Dietary Guidelines recommend vitamin B12 supplementation to all people over the age of 50 [5].

The basic structure of vitamin B12, cobalamine, is a corrin linked to a Cobalt atom [6]. The human body can absorb vitamin B12 only in the form of cyanocobalamin, hydroxycobalamin, methylcobalamin, and adenosylcobalamin, which are its biologically active, or “true”, forms. All the cobalamins that cannot be converted into the active form of vitamin B12 are known as “B12 analogues”, and they are biologically inactive molecules, that cannot be utilized by the human body. One main reason for this is that, they contain adenine, a compound that is not recognized by the Intrinsic Factor, the enzyme complex that constitutes the major uptake route for B12 by humans. Unfortunately, many analytical methods cannot distinguish these analogues from true vitamin B12. As a consequence, the content of true vitamin B12 in food, as well as its blood concentration, can be overestimated.

How can vitamin B12 deficiency be prevented? Usually, vegans prefer to purchase vitamin B12 supplements produced by bacterial synthesis. These

supplements have proven to be biologically active in humans, and are also ethically compatible with veganism, given their non-animal origin. On the other hand, many vegetable foods considered to contain vitamin B12, such as some fermented products, are unreliable sources of vitamin B12, as they mainly contain biologically inactive, vitamin B12 non-cobalaminic analogues [7].

Algae and cyanobacteria have long been considered to be reliable natural sources of vitamin B12 [8]. However, different kinds of algae (i.e. Nori), and cyanobacteria (i.e., Spirulina) have been shown to contain mainly inactive analogues of vitamin B12 [9,10]. The single-celled algae Coccolithophorid (*Pleurochrysis carterae*) and Chlorella have been identified as possible sources of true vitamin B12 [11]. Nevertheless, research results are still inconclusive, and the variation of vitamin B12 content in these foods suggests caution in recommending them as sources of reliable vitamin B12.

The cyanobacterium *Aphanizomenon flos-aquae* Ralfs ex Born. & Flah. var. *flos aquae* (Klamath Lake AFA) has also been proposed as a source of true vitamin B12. Since this alga is a wild plant food, it may be a more acceptable alternative to synthetic or technologically produced vitamin B12 for many people. Previous unpublished laboratory tests found very high levels of vitamin B12 in Klamath algae. However, these tests did not identify the specific corrinoid content of the algae, and no human study has ever been performed.

A recent study by Miyamoto *et al.* [12] confirmed this very high content of vitamin B12 in Klamath algae (approximately 6 µg/g vs. 1–2 µg/g for both Spirulina and Chlorella); yet, it found that when tested through the intrinsic factor (IF)-based chemiluminescence method, Klamath’s algae content of true B12 is reduced to 0.32 µg/g.

Even assuming that such results were indeed reliable, 3 g of algae per day would still give 64% of the UK-recommended daily intake of 1.5 µg, a relevant amount. However, the IF-based chemiluminescence test is generally rejected by both researchers and vegans/vegetarians as inconclusive and inconsistent. For instance, in the same Miyamoto *et al.* study, the true B12 content of Spirulina was found to be only around 5% of its overall B12 content; yet, in a previous study done by the same group of researchers and with the same method, Spirulina’s content of true B12 was found to be 17%. [13]. If we apply the same degree of variability to Klamath algae, its content of true B12 could vary from 0.320 µg/g to 1.088 µg/g, the latter being such a significant amount that 3 grams of

algae would give a high percentage of any of the different recommended daily intake (RDA) standards: 200 % of the UK RDA of 1.5 µg/day; or in any case 100 % of the RDA of 3 µg/day of countries like France or Germany. Even if the actual amount were somewhere in the middle, it would still probably be able to provide an average daily absorption of 1.5 µg, when consumed regularly.

The only reliable way to establish the true vitamin B12 content of a food is the *in vivo* method: by consuming the investigated food in the context of a plant-based diet (virtually vitamin B12-free) and subsequently by evaluating the vitamin B12 status in the subjects [14].

Owing to the unreliability of the plasma levels of vitamin B12 as a marker of the actual absorption of the vitamin, due to the potential inclusion in the assessment of vitamin B12 analogues as well as to the reduced absorbability of the vitamin used, it has been suggested that homocysteine (Hcy) can be used as a better marker of the body total content of metabolically active vitamin B12, at least in vegans. According to this, Hcy concentrations above 15 µmol/L should be considered a reliable marker of vitamin B12 deficiency. In fact, methylcobalamin (an active form of vitamin B12) is necessary for the enzymatic conversion of Hcy to methionine, so a deficiency of vitamin B12 blocks this pathway, raising Hcy concentration. It is true that there are other conditions that can raise Hcy concentration, such as a deficiency of vitamin B6 and/or folate, but these are very rare in a well-balanced vegan diet, and usually occurs only in a situation of general malnutrition [15]. Indeed, deficiency of B6 or folic acid have never been reported for vegans, and even a recent study on the B6 status of German vegans reported that only 4 % of them had an insufficient intake of B6, while the rest had a high total intake of vitamin B6 [16]. The American Dietetic Association (ADA) confirms this by not including B6 among the deficiencies of vegans [1]. Moreover, the content of B6 in Klamath algae is so negligible that it cannot affect the B6 status of the subjects. As to folates, ADA reports that all vegetarians have a high intake of folates, so any high Hcy levels in vegans cannot be attributed to low folate intake [1]. As we shall see, this is confirmed also by our data.

Therefore, as applied to vegans, Hcy concentration appears to be a good marker of true vitamin B12 status. In fact, giving us information about the actual absorption and functional efficacy of the vitamin, it is a much better marker than the mere plasma level of B12.

In our study, we investigated whether Klamath algae can represent an adequate source of true vitamin B12 in humans by evaluating its actual ability to consistently prevent and correct vitamin B12 deficiency in healthy subjects that follow a diet free from other sources of this vitamin. To our knowledge, this is the first study on Klamath algae as a potential source of B12 performed *in vivo*.

Subjects and Methods

Subjects

Fifteen subjects, aged 19 to 56 years (mean±SD: 37.1±10.6) were recruited after giving their informed consent. All subjects had been following a plant-food (vegan) diet for more than two years. Though all subjects stated that they were taking vitamin B12 supplements in adequate amounts (3–10 µg/day or 2000 µg/week), at baseline (T0) 4 subjects (27 %) had blood vitamin B12 concentrations under 200 pg/mL, and 2 had Hcy concentrations above 15 µmol/L. Two other subjects had Hcy blood concentrations above 15 µmol/L, although they had vitamin B12 blood concentrations above 300 pg/mL. All subjects stopped taking their usual vitamin B12 supplement at the beginning of the study. The study protocol was in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Study Design

All participants were evaluated at three time points:

- T0: 3 months before the beginning of the supplementation with the Klamath-algae product, when all participants stopped taking their usual vitamin B12 supplement (beginning of the control period);
- T1: at the onset of the intervention period, when all participants were still without any form of vitamin B12 supplementation, but immediately before starting the consumption of the AFA-B12 Klamath algae product;
- T2: 3 months after the subjects had begun consuming AFA-B12 (end of the intervention period).

In the intervention period, all subjects took 6 capsules per day of AFA-B12, also containing a proprietary mix of digestive enzymes (Enzy-B®) to improve the absorption of vitamin B12. The enzymes used are of fungal origin, so are fully consistent with a vegan diet.

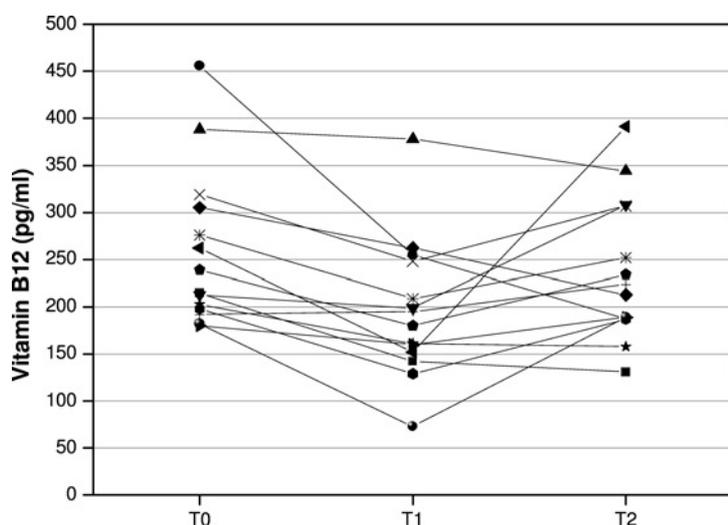


Figure 1: Individual vitamin B12 blood concentrations at T0, T1, and T2 (n=14).

As they are purified vegetable enzymes, they are wholly deprived of any vitamins, so they cannot be considered in any way causally related to the physiological changes in vitamin B12 status. AFA-B12 was kindly provided by the manufacturer (Nutratec s.r.l., Urbino, Italy).

Laboratory Analyses

Blood folate, iron, transferrin, ferritin, homocysteine, and blood cell count were evaluated in all participants. Vitamin B12 concentration was evaluated in all subjects but one (n=14), owing to technical problems. Biohumoral variables were assessed in the local chemistry laboratory where the study was performed, using a Bayer ADVIA 120 Haematology Analyzer, a Bayer ADVIA 1650 Chemistry Analyzer, and a Bayer ADVIA Centaur Immunoassay System.

Statistical Analyses

Biohumoral variable concentrations at each time point were summarized as mean \pm SD. Due to small sample size, comparisons between repeated measures were performed by Wilcoxon non-parametric test at $p < 0.05$ [17]. All analyses were done using SPSS 14.0 for Windows.

Results

Vitamin B12. Table 1 shows a significant difference in vitamin B12 levels at 3 months after the withdrawal of any form of vitamin B12 supplementation and im-

mediately before the beginning of AFA-B12 supplementation (T1). Mean concentration fell with respect to baseline (T1 vs. T0 196 ± 74 vs. 259 ± 83 , $p = 0.001$). Figure 1 shows vitamin B12 concentration at the 3 time points for each participant: in 9 (64%) of the subjects, vitamin B12 concentration increased after Klamath-algae supplementation, with a range of improvement from 15% to 159%. Of the remaining 5 subjects, in 2 (14%) the level of vitamin B12 remained approximately the same, without further decreasing; while in 3 (21%) it continued to decrease, but at a rate (8% to 27%) significantly lower than the previous decrease at T1. **Homocysteine.** Table 1 shows a significant difference in homocysteine levels after supplementation. In fact three months after the beginning of Klamath-algae supplementation (T2), mean concentration fell with respect to T1 (T2 vs. T1 12.0 ± 4.7 vs. 15.2 ± 5.8 , $p = 0.003$). Figure 2 shows Hcy concentrations at each of the 3 time points for each participant: after AFA-B12 supplementation, Hcy concentration increased in one subject (+28%) and remained stable in another subject at around $10 \mu\text{mol/L}$. In the remaining 13 subjects (87%), Hcy concentrations decreased by up to 57% (range 4–57%).

Other biohumoral variables. No other biohumoral variable showed significant changes during the 6-month study period (Table 1).

Discussion

Recommended daily intakes of vitamin B12 vary, but are typically 1–3 μg : for example, 2.4 μg in North America and 2 μg in Italy for adults [5,18]. Vitamin B12 absorption depends on various factors such as the presence of the gastric intrinsic factor, the ability to

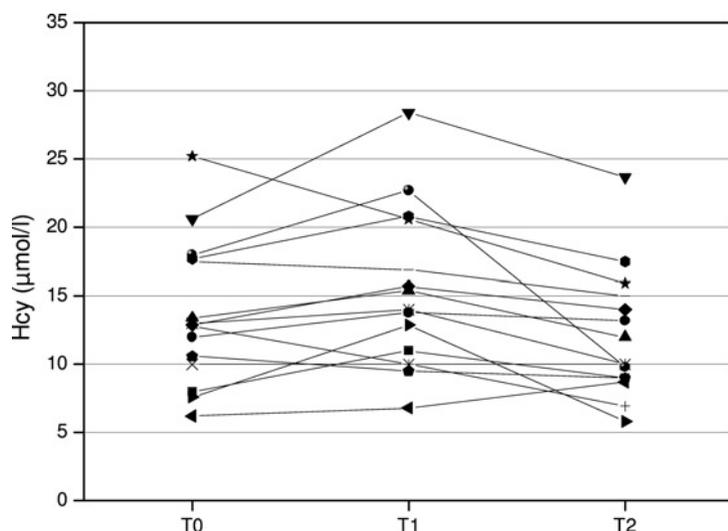


Figure 2: Individual homocysteine blood concentrations at T0, T1, and T2 (n=15).

Table 1: Blood concentration of the biohumoral variables at T0 (3 months before the beginning of the supplementation with the Klamath algae product), T1 (onset of the intervention period), and T2 (3 months after the beginning of the supplementation with the AFA-B12 Klamath algae product) (n=15, mean±SD).

Biohumoral variables	T0	T1	T2
Vitamin B12 (pg/mL) (1 missing)	259±83*	196±74*	237±75
Hcy (μmol/L)	13.7±5.2	15.2±5.8**	12.0±4.7**
Folate (ng/mL)	11.0±4.1	10.9±4.7	12.5±7.7
Iron (μg/dL)	87.6±26.8	93.5±33.9	100.4±46.8
Ferritin (ng/mL)	33.8±19.2	36.1±21.0	36.6±25.5
Transferrin (mg/dL)	273.6±26.8	268.5±39.1	268.9±43.6
MCV (fL)	92.1±2.9	92.0±2.9	92.9±3.4
Erythrocytes (10 ⁶ /μL)	4.63±0.36	4.60±0.32	4.59±0.35
Hemoglobin (g/dL)	14.4±1.4	14.3±1.3	14.3±1.5

* T1 vs. T0 p=0.001 (Wilcoxon matched-pairs signed-ranks test)

** T2 vs. T1 p=0.003 (Wilcoxon matched-pairs signed-ranks test)

absorb the food-bound vitamin, interaction with some antacid products, and on the amount of vitamin B12 consumed through food and supplements and the frequency of their intake. Most studies have shown that vitamin B12 from algae is mainly comprised of analogues without true biological activity in humans. Klamath algae has been shown to have a very high content of vitamin B12, yet *in vitro* studies have questioned that such content is indeed constituted by adequate true B12. Nevertheless, such *in vitro* studies, like the IF chemiluminescence test, are not reliable, and their usefulness tends to be questioned. On the other hand, no *in vivo* study has ever been done on Klamath algae to prove its efficacy as a natural source of vitamin B12.

In this *in vivo* pilot study, we found that in 15 vegan subjects, vitamin B12 plasma concentration fell during the first 3-month period, when all vitamin B12 supplementation was stopped. If Klamath algae contained mainly inactive vitamin B12, we should have found a further continued decrease in blood vitamin B12 concentration during the period of algal supplementation. On the contrary, we found a relevant increase in plasma vitamin B12 concentration during the AFA-B12 supplementation period, and more importantly, we found a significant parallel decrease in Hcy concentration.

In vegans, when vitamin B12 status is inadequate, Hcy concentration increases, as we have seen occurring at T1 in our subjects, although the small group size didn't allow detection of statistical significance;

while Hcy concentration significantly decreased after the 3-month period of algal supplementation. The fall in vitamin B12 concentration is the only plausible cause of the higher Hcy concentration in our healthy vegan subjects at T1. As clarified above, in vegan subjects the status of folate and vitamin B6 are ordinarily normal, as confirmed also by our vegan participants, in whom folate concentration remained stable throughout the different phases of the study. Therefore, the decrease in Hcy concentration can be explained only by the parallel increase in the levels of vitamin B12 at T2.

The fact that the Hcy levels at T2, after 3 months of AFA-B12 supplementation, were lower than those recorded at T0, when the subjects were regularly consuming a B12 supplement, indicates that the algal supplementation not only increased the plasma levels of vitamin B12, but more importantly it improved, with regard to the B12 supplementation before the baseline, its absorption and physiological utilization.

Here it begins to emerge clearly the superiority, in vegans, of the Hcy data in establishing not just how much B12 transits the blood, but if that circulating B12 is actually absorbed and utilized physiologically. In turn, this sheds a different light on the issue of how many people responded to the AFA-B12 supplementation.

In looking at the plasma levels of B12, we see that 9 subjects responded with an increase in plasma vitamin B12 concentration during the 3-month period of AFA-B12 supplementation; 2 responded partially by maintaining the levels of plasma B12 acquired at T1, indirectly indicating that the algal supplementation was partially effective; while 3 had a further decrease in their plasma levels of B12, although the decrease itself was proportionally lower than that obtained at T1. Applying a strict reading of these data, although the result is still positive, with a significant majority responding to the AFA-B12 supplementation, there would still be a sizable portion of non-responders.

However, if we then look at the variation of Hcy concentration, except for one single subject in whom Hcy had a slight increase, in 13 subjects (87%) Hcy concentration decreased by up to 57%, and in one more subject it remained stable at the optimal level of around 10 $\mu\text{mol/L}$. Therefore, 14 out of 15 subjects had positive results relative to their Hcy status, and given that this is the only actual marker (in vegans) of the physiological absorption and utilization of B12, we can actually conclude that almost everyone responded to the AFA-B12 supplementation.

As to the gap between the plasma B12 and Hcy trends, it could be explained, paradoxically, by the

fact that the subjects with a lower reading of plasma B12 did actually absorb and utilize the vitamin faster and more efficiently, so that little of it was left in the plasma when their blood was taken. This would be in line with the fact that these same subjects had their Hcy status modified, and as we have explained before, this change in Hcy in vegan subjects can be attributed only to the actual absorption and physiological utilization of B12. Though this is certainly a point to be further analyzed and deepened in future studies, we can confidently claim that, since Hcy level in vegans is the only reliable marker of the physiological status of B12, our data show that vitamin B12 status improved, after AFA-B12 supplementation, in more than 90% of the subjects.

In conclusion, the fact that most subjects responded positively to the AFA-B12 supplementation seems to suggest that this product's vitamin B12 can be well absorbed and is biologically active in humans. Therefore, the Klamath algae "AFA-B12" product may represent a food source of natural vitamin B12, and the current preliminary study warrants further, larger, and longer-term randomized trials to confirm such preliminary conclusions.

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