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ABSORPTION OF VITAMIN B12 ADMINISTERED IN BUFFALO MILK: AN EXPERIMENTAL STUDY IN RATS

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Abstract

Background

Milk contains the natural form of vitamin B12, hydroxo-B12 (HO-B12), whereas vitamin pills contain the synthetic form, cyano-B12 (CN-B12). Here we compare the absorption of CN-B12 and HO-B12, dissolved in milk products or water.

Methods

Male Wistar rats (n = 42) were fed a B12-deficient diet for 10 days before given an oral dose of (^{57}Co) -labeled CN-B12 (only in water) or HO-B12 dissolved in Indian buffalo milk, Italian buffalo milk, bovine whey, bovine casein solution or water; (n = 7 in each group). Feces and urine were collected for 24 hours and measured for labeled B12 using a gamma counter. The amount of B12 absorbed was calculated as the B12 administered minus the B12 excreted in feces. Results are expressed as percentage of the given dose.

Results

Equal amounts of labeled B12 were excreted in the feces when supplying CN-B12 and HO-B12 in water, HO-B12 in Indian buffalo milk, bovine whey or bovine casein solution (~15% of the given dose within the first 24 hours). Only HO-B12 added to buffalo milk from Italy showed a higher fecal excretion as compared to CN-B12 in water (p = 0.038), possibly explained by the presence of a haptocorrin-like B12-binding protein in the milk. All groups excreted less than 1% of the administered B12 in the urine within the first 24 hours, yet with a significantly higher excretion of CN-B12 than HO-B12 (p = 0.016).

Conclusions

Judged from the presented data, B12 is absorbed from Indian buffalo milk to the same extent as from bovine dairy fractions and water. B12 added to Italian buffalo milk is bound to a B12-binding protein and absorbed slightly less efficiently.

Abbreviations

B12: Vitamin B12; CN-B12: Cyano-B12; HO-B12: Hydroxo-B12, cpm: Counts Per Minute

Introduction

Buffalo milk is popular in South Asia (India, Pakistan, and China] and Italy, but little is known about the bioavailability of vitamin B12 (B12) in buffalo milk.

Cow's milk is an important source of B12 [1-3], and is believed to have a higher bioavailability of B12 than other food items, such as fish, meat, and eggs [2,4]. One study in pigs even suggests that cow's milk, which contain the natural forms of B12, such as hydroxo-B12 (HO-B12) [5], is absorbed better than free cyano-B12 (CN-B12) in vitamin pills [6].
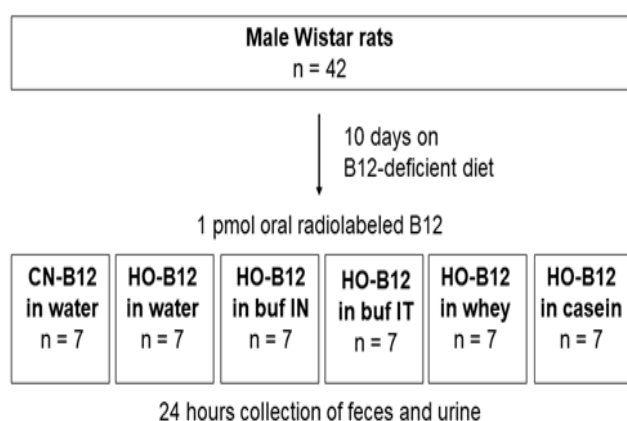
Figure 1: Study design. Rats (n = 42) were fed a B12-deficient diet for 10 days before given an oral dose of radioactive CN-B12 or HO-B12 in water or various milk solutions.

Absorption of B12 depends on degradation of the food matrix by gastric acid and a mixture of digestive enzymes. This step is crucial, as the vitamin needs to be liberated from the food proteins to be absorbed [7]. In cow's milk, B12 is bound partly to the B12-binding protein transcobalamin and partly to the casein fraction (ratio 50:50) [8,9]. Cow's milk is expected to be an unproblematic carrier of B12, because (i) transcobalamin easily releases B12 at the acid pH of the stomach [9,10], and (ii) transfer of B12 from its casein complex to the proteins of intestinal B12 transport is sufficiently fast at 37 °C [9]. Milk from other species, e.g. humans and pigs [11,12], contain haptocorrin, which is more resistant toward low pH [10], and to some extent also toward enzymatic degradation [13]. The binding patterns of B12 in buffalo milk are not known to date.

Employing a rat model, we have previously shown that HO-B12 added to cow's milk is absorbed as efficiently as free CN-B12 and HO-B12 [14,15]. Here we employ a B12-depleted rat model to explore the acute absorption of B12 from two types of buffalo milk and compare the results to the absorption of B12 added to water or the two fractions of dairy; whey and casein.

Materials and Methods

Animals

Male Wistar rats (RjHan: WI) (n = 42) from Janvier Labs, France, were used for the experiment (seven weeks old upon arrival to the animal facility). The study was authorized by the Danish Animal Experimental Inspectorate in agreement with the EU directive 2010/63/EU on animal experiments (approval number: 2016-15-0201-00984) and conducted at the animal facility at Health Faculty, Aarhus University, Denmark. The institutional and national guidelines for care and use of animals were followed, and the rats were checked daily for any health and welfare problems.

The rats were housed in pairs in standard cages (Makrolon 1291 H type III H, 800 cm^2, Techni plast, Italy) in a

controlled environment (20.0 ± 0.5°C; 60% humidity) with a 12-hour light-dark cycle. Bedding material (asp chips, Tapvei, Finland) and soft paper wool (LBS biotech, United Kingdom) were changed daily. The rats had two weeks of acclimatization in the animal facility before initiation of the experiment. During this time, the rats were kept on a standard stock rat fodder (Altromin 1324, Brogaarden, Lynge, Denmark) containing 24 µg/kg CN-B12. All rats had free access to food and water before and during the study.

Study design

The design is outlined in Figure 1. Fourty-two male rats were fed a B12-deficient diet (Altromin C1024, Brogaarden, Denmark) for 10 days to obtain a suboptimal B12 status. At this point, the rats were randomly allocated to six groups (n = 7 in each), and given an oral dose of radioactive CN-B12 (^{57}Co) CN-B12) or HO-B12 (^{57}Co) HO-B12) either in water or in four different milk-derived solutions (see Figure 1 and below). This was done by gastric gavage using a 20-gauge needle. The rationale for choosing this technique for oral administration was to ensure that all rats received the exact same amount of labeled B12. Following the oral dose, the rats were transferred to separate metabolic cages (Scanbur 3700M061, Tecniplast, Italy), and 24-hours'feces and urine were collected, weighed, and stored a -80 until analysis. The contents of radioactivity in feces and urine were measured by gamma counting using the Wizard Automatic Gamma Counter (Perkin Elmer) and expressed as counts per minute (cpm) or as fraction of the administered dose.

Subgroups and solutions for oral administrations

For all groups, the rats received 1 pmol radiolabeledB12 (approx. 180000 cpm) in 0.5 ml water/milk solution. Commercially available (^{57}Co) CN-B12 (1.75 µCi/ml and 0.41 µCi/pmol CN-B12) (MP Biomedicals, Ohio, USA, catalogue no. 06B-430000) was used. (^{57}Co) HO-B12 was made from (^{57}Co) CN-B12 as previously described

[14]. Solutions of (^{57}Co) CN-B12 and (^{57}Co) HO-B12 was prepared 24 hours before the experiment. The labeled B12 was adjusted to the desired concentration by addition of unlabeled CN-B12 (cyanocobalamin, Sigma-Aldrich, Denmark) or HO-B12 (Vibeden, Sandoz, Denmark). Milk-bound (^{57}Co) HO-B12 was prepared by incubating (^{57}Co) HO-B12 with different milk solutions. To allow for the binding between (^{57}Co) HO-B12 and B12-binding components (if any are present), all mixtures were incubated for two hours at room temperature and then over-night at 4°C. The exact amount of radioactivity (in cpm) administered to each group was measured by gamma counting of a test aliquot (0.5 ml). To reduce the bias from potential inaccuracies in the oral administration procedure, the test aliquot was collected by imbibing 0.5 ml solution with the gastric gavage needle in the same manner as for oral administration in a rat and then transferring the content into a test tube.

The Indian buffalo milk was purchased from Chitale dairy, Pune, India, as a freeze-dried powder and redissolved in water to match the original volume. The Italian buffalo milk was collected from a commercial herd in the province of Macerata, Italy. The Italian buffalo milk samples were obtained from the morning milking, cooled at 4°C, and transported to our laboratory within three days. Both types of buffalo milk were used without dilution. The whey solution was made from a bovine whey protein concentrate (Arla Food Ingredients Group P/S, Denmark) with a high content of bovine transcobalamin fully saturated with endogenous B12 but did not contain casein and with a reduced content of alpha-lactalbumin (as stated by the manufacturer). The powder (1g) was dissolved in 10 ml water and further diluted 1:2 with water prior to use. The casein solution was made by subjecting 80 ml of skim milk from a Danish supermarket to ultracentrifugation at 100000 x g for 1 hour at 4°C. The obtained pellet contained the casein fraction at the top and milk fat globules at the bottom. The top fraction of caseins was suspended in 50 mMNaCl (48 Ml) and heated at 65ºC for 30 minutes. The preparation was briefly centrifuged (5 minutes at

5,000 x g), and the concentration of protein in the turbid supernatant was measured after a strong dilution (1:100) with 1M NaCl, 0.1 M Tris pH 7.5, where the micelle was dissolved. The absorbance coefficient of E280 = 0.866 AU·ml·mg-1 was used [9]. Presence of the high molecular aggregate of caseins was confirmed by gel filtration (not shown) as described earlier [9]. The solution (40 mg/ml micelle-dissolved casein) was diluted 100 times prior to usage.

Statistical methods

The number of animals in each group (n = 7) was based on power calculations using a multiple linear regression model showing a statistical power of 80% (α = 0.05). The calculations were based on an earlier study showing 22% accumulation of (^{57}Co) CN-B12 out of the given dose in feces within the first 24 hours after oral administration [14], and a wish to detect mean differences (increase or decrease) ≥ 15% in the groups receiving (^{57}Co) HO-B12 in water or various milk fractions.

Results are given as the percentage of the cpm of the total administered dose per rat. The number of rats in each group did not allow for determination if data followed the Gaussian distribution. For this reason, differences in means between groups were determined using the non-parametric Mann Whitney test. Values of p ≤ 0.05 were accepted as statistical significant. The data analysis was performed using the statistical software available in Graph Pad Prism version 7.03.

Results

We explored the 24-hour fecal excretion after orally administered (^{57}Co) CN-B12 (in water) and (^{57}Co) HO-B12 (in water or milk solutions)using a B12-depleted rat model. There was no weight difference between the six groups of rats (data not shown) that all weighed 309 (278-360) g (median and) range)) (n = 42) at the end of the experiment.

Fecal excretion of CN-B12 and HO-B12 administered in water and milk solutions

We measured the amount of radioactivity recovered in the feces 24 hours after oral administration of labeled B12 to rats (Figure 2). No difference was observed betweenCN-B12 and HO-B12 dissolved in water, or when HO-B12 was administered in Indian buffalo milk, a whey matrix or a casein solution. Only B12 in Italian buffalo milk showed a higher 24-hour fecal excretion (Figure 2).

Urinary excretion of CN-B12 and HO-B12 administered in water and milk solutions

Less than 1% of the administered labeled B12 was excreted in the urine within 24 hours. Compared to CN-B12 dissolved in water; all HO-B12 groups showed a tendency to excrete lower amounts of radioactivity in the urine (irrespective of the supplementation solution). This difference was, however, only significant for two out of five HO-B12 groups (Figure 2).

Discussion

Here we investigated the 24-hour fecal excretion of synthetic CN-B12 (as in vitamin pills) and natural HO-B12 (as in food items); dissolved either in buffalo milk, bovine dairy fractions or water. Our results show that the absorption of B12 from Indian buffalo milk compares to the absorption of B12 added to bovine dairy fractions or water. In contrast, B12 seems to be absorbed slightly less efficient from Italian buffalo milk.

The amount of B12 excreted in feces represents the part of B12 not absorbed into the body. Thus, our data show that approximately 85% of the administered dose is absorbed. This value is higher than in a comparable rat study, where ~78% of the given dose was absorbed [14]. The difference may be driven by the fact that our rats were on a B12-deficient diet, and because of that, the dietary B12 did not interfere with the absorption of the administered dose.
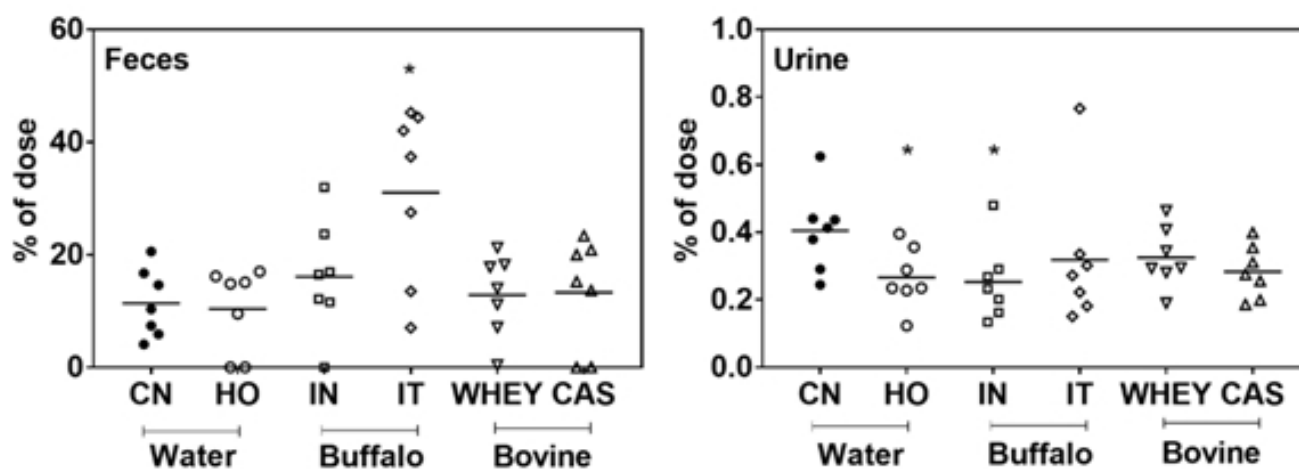
Figure 1: Excretion of [57Co] CN-B12 (full symbols) and [57Co]HO-B12 (open symbols) in feces and urine 24 hours after oral administration

Results are given as the percentage of cpm of the given dose of [57Co]-B12 per rat (n = 7 in each group). Horizontal lines show mean values.

Our study has some limitations. The experiment was performed only once, and there is a low probability that our findings are caused by inter-animal variation. However, previous [14,15] and ongoing studies, performed in similar settings, do not suggest any major change of the outcome upon repetition of the studies. The uptake of B12 was estimated by addition of labeled B12 to different milk solutions. For this reason, we can only trace the uptake of added B12 and not of endogenous milk B12. For a few rats (six out of 42), less than five fecal droppings were collected from the fecal compartment of their metabolic cages after 24 hours. In general, rats have a daily fecal deposition of 43 (28-59) (median (range)) droppings [16]. Because of the low amount of feces collected, the total amount of fecal radioactivity (calculated by adding the cpm in all fecal droppings collected) was low in these six animals compared with the other rats. As the rat is an obligate coprophagic species [16,17], known to digest stool directly from the anus [18], we speculate that the low amount of fecal droppings collected from these animals is the result of coprophagia. We do not believe

that these few outliers have shifted the outcome or made a major impact on the key conclusions of the study, since all the groups are expected to be similarly affected by this phenomenon.

In accordance with previous studies [14,15], we find no difference in absorption of free CN-B12 and free HO-B12 in the rat. Our most important result is that B12 in Indian buffalo milk is absorbed just as efficiently as the vitamin dissolved in water or fractions of bovine dairy, while the absorption of B12 from Italian buffalo milk is absorbed to a slightly lesser degree. A speculative explanation for this difference is that B12 added to Italian buffalo milk binds to a haptocorrin-like protein, while this is not the case for B12 added to Indian buffalo milk. It has previously been shown that various types of milk contain different B12-binding proteins including the observation that the major B12-binding protein present in cow's milk is transcobalamin whereas the major B12-binding protein in milk from humans and pigs is haptocorrin [11,12]. A possible negative role of haptocorrin-like proteins

in relation to the bioavailability of B12 is in accord with studies on other food items. In fish, B12 is bound by a haptocorrin-like protein that is resistant toward enzymatic degradation and low pH [10], leading to a relative poor bioavailability of B12 ≈ 40% [17]. The same is the case for eggs, where a haptocorrin-like protein has been found to reduce absorption of B12 [18]. Further studies are needed to investigate whether Italian buffalo milk contains haptocorrin or a yet unidentified B12-binding protein, and if the absorption of endogenous B12 from Italian buffalo milk is impaired as a results hereof.

The finding that B12 added to Indian buffalo milk is efficiently absorbed is concordant with a study showing that daily intake of 600 ml non-enriched Indian buffalo milk rescues a low B12 status in B12-deficient individuals [19]. Our study also suggests that B12 added to Indian buffalo milk and to cow's milk is absorbed equally well. However, intervention studies in a clinical setting are needed to investigate whether these two types of milk provide the same supply of B12.

Interestingly, the presence of a milk matrix, which theoretically should stimulate gastric function and secretion of intrinsic factor, did not affect the absorption of B12, as judged from the fact that HO-B12 in whole milk or the whey and casein fraction is absorbed comparable to HO-B12 in water.

We examined the urinary excretion of absorbed B12 and found a slightly higher excretion of CN-B12 than HO-B12. This observation is in agreement with our previous rat studies suggesting that HO-B12 homes for the liver, while CN-B12 homes for the kidneys [14,15]. The results indicate that HO-B12 is retained slightly better in the body than CN-B12, which is in accord with old studies on humans [20,21].

Conclusion

We report that B12 added to Indian buffalo milk is absorbed as efficient as B12 added to water or fractions of bovine dairy, while B12 added to Italian buffalo milk is absorbed to a slightly lesser degree. Further studies are needed in order to characterize the B12-binding proteins in buffalo milk and to explore the impact of such proteins for the bioavailability of B12.

Acknowledgements

Conflict of Interest

Marie S. Ostenfeld is an employee at Arla Food Ingredients Group P/S that produces the whey powder used in the study.

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