

EFFECTS OF ANTIBIOTIC-INDUCED VITAMIN B12 DEFICIENCY WITH AND
WITHOUT ORAL ADMINISTRATION OF VITAMIN B12

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BY

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Research Question

Does oral administration of vitamin B12 increase vitamin B12 and decrease methylmalonic acid, reticulocyte and homocysteine blood levels in long-term antibiotic-fed rats?

Null Hypothesis

Oral administration of vitamin B12 does not increase vitamin B12 and decrease methylmalonic acid, reticulocyte and homocysteine blood levels in long-term antibiotic-fed rats.

Background on which research question is based

1. Importance of Vitamin B12

Vitamin B12, also known as cobalamin, is an essential vitamin involved with erythropoiesis (red blood cell formation), nervous tissue synthesis, cellular repair, and DNA synthesis. The human body is incapable of synthesizing B12. We rely on the bacterial flora located in our gastrointestinal tract to synthesize vitamin B12, which can then be absorbed through our intestines by the aid of intrinsic factor and used elsewhere in the body (Stanislawski-Sachadyn et al., 2010). Intrinsic factor, also known as gastric intrinsic factor (GIF), is a glycoprotein produced by the parietal cells of the stomach. Intrinsic factor is necessary for the absorption of vitamin B12 in the small intestine. Deficiency of intrinsic factor results in the condition pernicious anemia. All B vitamins are water soluble, meaning that the body does not store them. Excess B12 is excreted in urine, so a constant supply of B12 is necessary to meet the needs of the body.

2. Vitamin B12 Deficiency

Vitamin B12 is an essential cofactor for methionine synthase, which is an enzyme that converts homocysteine to methionine (Figure 1). Deficiency of B12 results in scarcity of methionine and an accumulation of homocysteine (Ceyhan et al., 2010). Increased blood levels of homocysteine may indicate vitamin B12 deficiency (Savage et al., 1994; Stanislawski-Sachadyn et al., 2010; Martens et al., 2002). Vitamin B12 is an essential cofactor for methylmalonyl-CoA mutase which converts methylmalonyl-CoA to Succinyl-CoA (Figure 1). Deficiency of Vitamin B12 will lead to increased serum levels of methylmalonic acid or methylmalonyl-CoA and decreased serum levels of succinyl-CoA (Vashi et al., 2016; Stanislawski-Sachadyn et al., 2010). Vitamin B12 deficiency cannot be diagnosed solely on serum vitamin B12 levels. Both the hematological and the neurological manifestations of cobalamin deficiency may be subtle and therefore difficult to recognize. Normal or even high serum vitamin B12 levels can sometimes be seen in a B12 deficient state, and can therefore be misleading (Vashi et al., 2016).

Vitamin B12 deficiency will also lead to macrocytic anemia, also known as megaloblastic anemia, which is a blood disorder in which red blood cells are larger than normal and the number of red blood cells is lower than normal (Kaferle & Strzoda, 2009). When red blood cells are too large (Figure 2), they may not be able to exit the bone marrow to enter the bloodstream and deliver oxygen. Vitamin B12 and folic acid are important for the final maturation of red blood cells. Without B12, reticulocytes cannot differentiate into erythrocytes and an increased serum reticulocyte level will be present (Figure 3).

3. Vitamin B12 Synthesis

The lumen of the human gastrointestinal tract contains trillions of bacteria that are estimated to outnumber the cells of the human host by a factor of 10 (Ramakrishna, 2013). The gut microbiota produce a variety of nutrients including vitamin B12 and short-chain fatty acids (Ramakrishna, 2013). The human intestinal microbiota is essential to the health of the host and plays a role in nutrition, development, metabolism, pathogen resistance, and regulation of immune responses (Dethlefsen et al., 2008). Vitamin B12 biosynthesis is restricted to microorganisms (Martens et al., 2002; Raux et al., 2000). Vitamins are micronutrients that are essential for the metabolism of all living organisms and humans are incapable of synthesizing most vitamins (LeBlanc et al., 2011). Antibiotics may disrupt these coevolved interactions, leading to acute or chronic disease in some individuals (Dethlefsen et al., 2008).

4. Antibiotic Effects on Gut Microbiota

The average child in the United States and other developed countries has received 10-20 courses of antibiotics by the time he or she is 18 years old (Blaser, 2011). Antibiotics kill the bacteria we do want, as well as the bacteria we do not want (Blaser, 2011). A short course of the antibiotic ciprofloxacin reduces the diversity of the intestinal microbiota, with significant effects on roughly one-third of the bacterial species (Dethlefsen, 2008). An oral or injectible antibiotic diffuses through the bloodstream and affects targeted pathogen and residential microbiota alike (Blaser, 2011).

5. Animal Studies on Vitamin B12

Orally administered antimicrobial agents, while interfering with the life cycle of pathogenic bacteria, are known to cause disturbances in the normal resident intestinal microbial

communities in the rat model (Upreti et al., 2008). In rats, the gut microbiota is considered to have great impact on host health through either direct interaction with host cells or through production of metabolites such as vitamins and short chain fatty acids (Tulstrup et al., 2015). In a study conducted by Scalabrino and his colleagues in 1998, TGX-rats were found to have a fall in serum levels of methylmalonic acid and homocysteine after repeated vitamin B12 injections (Scalabrino et al., 1998). After total gastrectomy the level of vitamin B12, in the serum of rats fell more rapidly than that in the liver of neomycin, streptomycin and erythromycin treated rats (Williams & Spray, 1970). Before starting antibiotics, the rats on the deficient diet excreted much more methylmalonic acid than those on the supplemented diet (Williams & Spray, 1970). On the other hand, the deficient group treated with the mixture of antibiotics excreted more methylmalonic acid than their controls (Williams & Spray, 1970).

Explanation of how background led to your research question

It has been suggested that oral vitamin B12 therapy may be an effective therapy for treating vitamin B12 deficiencies related to food-cobalamin malabsorption. However, the duration of this treatment was not determined (Andrès et al., 2003). Individuals taking long-term antibiotic treatments are prone to developing vitamin deficiencies including vitamin B12 deficiency (Shirakawa et al., 1990).

As stated above, vitamin B12 is an essential vitamin involved in erythropoiesis (red blood cell formation). Without vitamin B12, reticulocytes cannot mature into red blood cells. This will result in an increased serum level of reticulocytes. If the reticulocyte does differentiate into an erythrocyte, they will become macrocytic (enlarged).

Vitamin B12 is also a cofactor involved in the process of converting methylmalonyl-CoA into succinyl-CoA and homocysteine into methionine (Vashi et al., 2016 and Stanislawska-Sachadyn et al., 2010). Without cobalamin as a cofactor, homocysteine and methylmalonic acid serum levels will increase. My research question will address whether oral administration of vitamin B12 will increase vitamin B12 and decrease methylmalonic acid, reticulocyte, and homocysteine blood levels in rats taking long-term antibiotic treatments. This is something that has never been seen in rats.

Anticipated results of the research project

Due to similar approaches, vitamin B12, Methylmalonic acid, and homocysteine serum concentration control data has been obtained from Scalabrino et al. in 1998. Reticulocyte count control data has been obtained from Bor-Kucukatay et al. in 2000.

Table 1. Mean \pm SEM vitamin B12, methylmalonic acid, and homocysteine levels expressed in pg/mL, nmol/L, umol/L and % of rat serum post-treatment respectively.

	Control Rats	Rats with Augmentin	Rats with Vitamin B12	Rats with Augmentin and Vitamin B12
Vitamin B12 (pg/ml)	744.2 \pm 67.1 (n=6)	577.1 \pm 58.3* (n=6)	804.5 \pm 71.2 (n=6)	715.3 \pm 57.3 (n=6)
Methylmalonic acid (nmol/L)	682.2 \pm 55.2 (n=6)	991.5 \pm 90.4* (n=6)	674.3 \pm 45.7 (n=6)	691.8 \pm 56.2 (n=6)
Homocysteine (umol/L)	9.2 \pm 0.9 (n=6)	28.3 \pm 5.4* (n=6)	8.9 \pm 0.7 (n=6)	9.4 \pm 0.8 (n=6)
Reticulocyte (%)	2.42 \pm 0.23 (n=15)	6.41 \pm 0.47* (n=6)	2.14 \pm 0.51 (n=6)	2.63 \pm 0.43 (n=6)

Data marked with * show statistical significance (p<0.05).

Control data sources: Scalabrino, Buccellato, Tredici. Methylmalonic Acid As A Marker For Cobalamin Deficiency: Fact Or Fantasy? Elucidations From The Cobalamin-Deficient Rat. British Journal of Haematology. 1998;100(3):615–616.

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Significance of Project

Vitamin B12 plays essential roles in red blood cell formation as well as being a cofactor used by enzymes present in the body. Humans cannot synthesize vitamin B12, so we rely on our gut microbiota to synthesize vitamin B12 in our gastrointestinal tract for human usage (Stanislawski-Sachadyn et al., 2010, LeBlanc et al., 2011, Martens et al., 2002 & Raux et al., 2000).

Antibiotics kill the bacteria in our gastrointestinal tract, as well as the bacteria we do not want (Blaser, 2011). This project will provide insight to individuals taking long-term antibiotic treatments as to whether oral administration of vitamin B12 will increase vitamin B12 and decrease homocysteine, methylmalonic acid and reticulocyte serum concentrations in individuals taking long-term antibiotics.

Contemplated method of approach to problem

1. Rationale for approach to be used:

As discussed above, the bacteria in the human gut has essential roles for maintaining the health of an individual. For the purpose of this study, we will treat rats that contain no known bacterial infections with Augmentin, which is a broad spectrum penicillin antibiotic. These antibiotics will destroy the bacteria located in the gastrointestinal tract of the rat. This study will be done using an animal rat model due to the unwanted long-term effects of the antibiotic, Augmentin, towards the microflora of the human gastrointestinal tract. This study will give us a better understanding of the effects of long-term antibiotic treatment with and without oral

administration of vitamin B12 and may lead to a new treatment strategy for individuals with pneumonia that are receiving long-term antibiotic treatment.

2. Experimental Design

Male Lewis rats of similar weight (approximately 375 – 425g) will be obtained, separated into four groups of six, labeled Control, Augmentin-fed, Vitamin B12-fed, and Augmentin & Vitamin B12-fed, and housed under identical living conditions with a 12 hour light/ dark cycle, allowing free access to food and water. After approximately 2 months, an overnight fast will occur following the final treatment administration. Blood samples will be taken, processed, and homocysteine, vitamin B12, methylmalonic acid and reticulocyte serum concentrations will be determined and compared between groups.

3. Statistical analysis of data:

All data will be expressed as Mean \pm SEM and significance will be determined at $p < 0.05$. One-way analysis of variance (ANOVA) will be used to compare variables between all four groups (Leblanc, 2004). Data between experimental and control groups will be compared using a Tukey's test for each variable using Minitab software and expressed in pg/mL, nmol/L and $\mu\text{mol/L}$ of blood serum (Leblanc, 2004).

4. Experimental methodology

One Augmentin chewable tablet, containing 125 mg of amoxicillin in 5 mL and 31.25 mg of clavulanate potassium in 5 mL, will be given to each rat daily except for the control and vitamin B12-only rats. Vitamin B12 will be added to the water source of the vitamin B12-only group and Augmentin & Vitamin B12 group. Water source of the control group and Augmentin group will contain only water. After two months of feeding, perform an overnight fast for both

groups. Obtain blood samples from each rat the following morning by cutting and milking the tail.

Vitamin B12, methylmalonic acid, homocysteine and reticulocyte serum concentrations can be determined using spectrophotometric assays using biochemical kits and complete blood counts. Vitamin B12 medium assay kit is obtained from Sigma-Aldrich. Methylmalonic acid ELISA kit is obtained from MyBioSource. Homocysteine enzymatic assay kit is obtained from Bioo Scientific. Complete blood count kit is obtained from Walk-In-Lab. Blood smears can be used in lab to look for abnormally large erythrocytes.

5. Anticipated problems:

Anticipated problems include funding for biochemical kits and complete blood counts. Other potential problems include rats not wanting to drink the vitamin B12 from the water source. If this occurs, we can inject vitamin B12 intramuscularly, or add glucose to the water source to make it sweet. If rats do not want to eat the chewable Augmentin tablets, we could administer the antibiotic via oral gavage.

6. Ethical aspects of the proposed research project:

Research was approved by the Animal Care and Use Committee of Ball State University of the Internal Review Board of Ball State. All animals will be treated humanely and all measures to minimize pain and discomfort will be taken.

7. Acknowledgments

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