British Journal of Pharmaceutical Research
2(2): 69-79, 2012

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Depletion in Serum Levels of Folic Acid, Antioxidant Vitamins and Trace Elements in Female Wistar Rats Treated with Sub-toxic Dose of Acetaminophen/Methionine Combination – A Chronic Study

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Research Article

Received 8th October 2011
Accepted 31st January 2012
Online Ready 27th March 2012

ABSTRACT

Context: Studies have identified that concurrent administration of methionine and acetaminophen (paracetamol) prevents tissue damage and both methionine and acetaminophen at high doses can induce oxidative stress. Antioxidants mediate against oxidative stress. Moreover, folic acid depletion has been identified to cause neural tube defects in neonates of affected female subjects.
Objective: The aim of this study is to investigate the impact of chronic exposure to sub-toxic dose of acetaminophen/methionine (ratio 5:1) on female Wistar rats, with emphasis on folic acid and antioxidant vitamins and minerals.
Material and Methods: Rats were divided into 3 groups with each group consisting of 8 rats and treated with acetaminophen/methionine, acetaminophen or saline daily by gastric gavage. The study lasted 30 days after which blood was obtained through retro-orbital bleeding.
Results: Results show that Wistar rats administered with 350 mg/kg BW (sub-toxic dose) of acetaminophen exhibited significant alteration ($p<0.05$) in levels of all trace elements (except Se) as well as vitamins (except vitamin A). Significant alterations in the levels of all vitamins (except riboflavin) and all minerals (except Cu, Mn, Se) ($p<0.05$) were also recorded in serum of rats administered with acetaminophen/methionine combination.
Discussion and Conclusion: Results of this study therefore suggest that chronic abuse of subtoxic dose of acetaminophen/methionine combination may induce alterations in

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levels of vital molecules, a situation which may increase an individual's risk to oxidative stress-induced diseases and her neonate to neural tube defects.

Keywords: Folic acid; antioxidant vitamins; female Wistar rats; acetaminophen; methionine.

1. INTRODUCTION

Acetaminophen, a standard analgesic and antipyretic agent, has wide acceptability because it is well tolerated at therapeutic level. In the developed World, its abuse at toxic level is for suicidal and para-suicidal purposes whereas in some parts of the developing World, acetaminophen abuse has been linked to poverty, illiteracy and medical ignorance among many other factors (Ucheya and Igwe, 2006; Agba et al., 2004), a situation in which differences in brand names are taken to mean differences in chemical composition, resulting in a subject using two or three APAP formulations at the same time culminating in sub-toxic abuse. Such abuse is possible because of the ease at which this drug is obtained being an over-the counter drug. This has led to the inclusion of an antidote (methionine) in many formulations. Acetaminophen taken at above the therapeutic level has been reported to induce oxidative stress; this study is embarked upon to investigate if such stress exists also with acetaminophen/methionine combination by using antioxidant vitamins and minerals as indices of study with emphasis on anti-oxidant minerals, vitamins A, E and C and folic acid. Vitamin C is the predominant plasma antioxidant while folic acid depletion has been linked to genome instability and neural tube defects.

Vitamins are organic compounds required in trace quantities from the diet, usually in micrograms to milligrams level per day, for health, growth and reproduction (Shenkin et al., 2006). These biomolecules play a number of physiologic roles. The physiological functions of ascorbic acid are generally related to its oxido-reduction properties. L-ascorbic acid is a co-factor for hydroxylases and monooxygenases, enzymes taking part in the synthesis of collagen, carnitine and neurotransmitters (Levin, 1986). It prevents scurvy, the disorder by which its deficiency is known (Olson, 1999). Adequate intake has been linked to reduced incidence of mortality from heart diseases, stroke and cancer (Carr and Frei, 1999a). Moreover, ascorbic acid hinders the oxidation of low density lipoprotein (LDL) primarily by scavenging the free radicals and other reactive oxygen species in the aqueous state (Frei et al., 1989). It is also important in wound repair and healing/regeneration process as it stimulates collagen synthesis.

Vitamin A on the other hand, plays an important role as retinal in vision; it is known also to function in reproduction, growth and embryonic development as well as immune response (Shenkin et al., 2006). Vitamin E in addition plays an important role in reproduction (Shenkin et al., 2006). It is equally important in normal neurological function and prevention of red cell hemolysis. Inhibition of free-radical chain reactions of lipid peroxidation is the most well-defined of its roles, especially within the polyunsaturated fatty acids of membrane phospholipids. Vitamins contribute significantly to the plasma antioxidant capacity, with ascorbate contributing approximately 25%, vitamin E about 10% while the contribution of vitamin A is much lower compared with vitamin C (Shenkin et al., 2006). The essential trace elements; Zn, Cu, Se Mn are known for their antioxidant properties. Zinc, Cu, and Mn are co-factors for the enzymes superoxide dismutase and Se is a co-factor for glutathione peroxidase. These two enzymes have been reported to play a role in some of the processes

of acetaminophen- induced toxicity. On the other hand, despite the fact there is dearth of data on Mo, Co and Cr and acetaminophen-induced toxicity and these elements may not possess significant antioxidant properties, but they are known to play important roles in many biochemical reactions such that their alterations may derail vital metabolic processes.

Although folate has not been identified to possess significant antioxidant properties, it takes part in a wide spectrum of biochemical reactions; acts as a coenzyme in several single carbon transfers involved in biosynthesis of purine nucleotides and deoxythymidylic acid essential for DNA and RNA synthesis as well as provides one-carbon unit for methylation of a wide variety of biological substances including DNA, proteins, phospholipids, and neurotransmitters, thereby regulating their function (Wald and Oakley, 2007). For this reason, folate deficiency may impair de novo biosynthesis of purines and thymidylate thereby disrupting DNA and RNA metabolism, homocysteine remethylation, methionine biosynthesis, and subsequent formation of S-adenosylmethionine (the universal methyl donor) which ultimately may result in altered methylation reactions. Moreover, its deficiency has been linked with increase risk of various cardiovascular diseases, birth defects- in particular neural tube defects (NTDs), congenital heart defects, and possibly cancer.

An earlier study focused on the hepatic and renal effects of such abuse have shown that acetaminophen/methionine in the ratio of 5:1 (Neuvonen et al., 1985) confer protection on the hepatocytes, this study hopes to determine if such hepatic protection is also accompanied by non-alteration in levels of trace elements and vitamins. This is because depletion in folic acid level in the first trimester of life has been linked to neural tube defects and decrease in the levels of other vitamins may impair their anti-oxidative roles.

2. MATERIALS AND METHODS

2.1 Animals

Adult female Wistar rats weighing between 270–350g, bred by the animal house of the Department of Veterinary Physiology, University of Ibadan, were purchased and used for the study. The animals were given unrestricted access to water and standard laboratory rat pellets, produced by Ladokun Feed, Ibadan (Nigeria). The study was carried out in compliance with accepted principles for the use and care of laboratory animals as found in US guidelines (NIH publication/85-23, revised in 1985). The animals were divided into 3 groups with each group consisting of 8 rats.

2.2 Treatment and Chemical Analysis

Group 1 served as the control group and received 5 ml of physiologic saline supplied by Unique Pharmaceutical (Sango-Otta, Ogun State). Rats in treatment groups were administered with acetaminophen (350 mg/kg) or acetaminophen/methionine (350 mg/kg and 70 mg/kg) dissolved in physiologic saline, and are termed Groups 2 and 3 respectively (Abraham, 2004). The route of administration was by gastric gavage. In each case appropriate treatment option was administered every morning between 10:00 and 11:00 to each rats and the period of administration was for 30 days. On the thirty-first day, the study was terminated; about 5-8 ml of blood was collected between 09:00 and 11:30 by the retro-orbital method. The blood was left to clot and was centrifuged at 3000g for 10 minutes. The serum obtained was used for the estimations of vitamins (folic acid, riboflavin, vitamins A, C and E) as well as minerals (Zn, Cu, Se, Mn, Mo, Cr, Co, Fe). High Performance Liquid

Chromatographic (Waters® Corporation Milford, Massachusetts, USA) technique was used for the estimation of the vitamins while atomic absorption spectrometry (Buck® Scientific, East Norwalk, Connecticut, USA) was employed for the quantification of trace elements. Methionine and acetaminophen were supplied by Sigma-Aldrich Chemicals (St. Louis, MO).

2.3 Statistical Analysis

Data were subjected to statistical analysis using the Statistical Package for Social Sciences (SPSS) version 15. Student-t test was employed to establish the level of significant difference between of each of the treated group and the control group while analysis of variance (ANOVA) was used to detect significant difference among the three groups. Results are reported in Mean ± SD (standard deviation). P≤0.05 was considered significant.

3. RESULTS

Results in Table 1 shows that administration of female Wistar rats with sub-toxic dose of acetaminophen resulted in significant alteration in the serum levels of many of the vitamins and minerals. Specifically riboflavin, folic acid, vitamin E and vitamin C are significantly decreased ($p<0.05$) in acetaminophen administered rats than control but vitamin A is not significantly different ($p>0.05$). In the same set of rats zinc, copper, manganese, iron are significantly decreased ($p<0.05$) compared with controls whereas molybdenum, chromium and cobalt are significantly increased ($p<0.05$) but selenium is not significantly different ($p>0.05$) (Table 2).

Table 1. Serum levels of antioxidant vitamins in acetaminophen and acetaminophen/methionine-exposed Wistar rats at sub-toxic level

	Riboflavin (nmol/L)	Folic (nmol/L)	Vitamin C (µmol/L)	Vitamin A (µmol/L)	Vitamin E (µmol/L)
Control X ± SD	784.43±276.37	16.94±2.58	41.45±6.81	2.50±0.24	19.26±2.55
350mg/kg X ± SD (P)	468.43±57.72*	9.92±1.95*	28.96±5.68*	2.32±0.29	11.83±2.78*
350mg/kg X ± SD (P and M)	714.48±114.65	14.04±0.52*	19.31±2.27*	1.94±10.46*	13.22±0.18*
F-value	2.13	24.44	32.43	5.32	11.33
p-value	0.14	0.01**	0.01**	0.02**	0.02**

*Results are expressed as mean ± standard deviation; *p <0.05 is significant when compared with control using Student 't' test. **P<0.05 is significant using ANOVA.*

Table 2. Serum trace element levels in acetaminophen and acetaminophen/methionine-exposed Wistar rats at sub-toxic level

	Zn (µmlo/L)	Cu (µmol/L)	Mn (nmol/L)	Se (µmol/L)	Mo (nmol/L)	Co (nmol/L)	Fe (µg/dl)	Cr (nmol/L)
X ± SD (controls)	14.39±0.81	21.48±0.92	10.32±1.09	1.26±0.09	0.97±0.32	0.51±0.08	146.73±6.26	4.03±0.58
350mg/kg X ± SD (P)	9.83±0.72*	14.31±2.17*	5.67±0.81*	1.28±0.12	1.32±0.32*	0.68±0.05*	70.72±26.44*	8.65±1.15*
350mg/kg X ± SD (P and M)	11.38±1.33*	17.87±0.92	10.09±0.87	1.28±0.13	1.48±0.25*	0.74±0.050*	78.31±11.51*	4.23±0.56
F-value	43.58	6.18	63.39	0.06	6.19	29.16	48.34	1.09
P-value	0.01**	0.03**	0.01**	0.94	0.04**	0.02**	0.01**	0.35

*Results are expressed as mean ± standard deviation; *p <0.05 is significant when compared with control using Student 't' test. **P<0.05 is significant using ANOVA.*

On the other hand, rats administered with acetaminophen/methionine showed significant decrease ($p<0.05$) in the levels of all water and fat soluble vitamins except riboflavin which was found not to be significantly different ($P>0.05$) compared with control (Table 1). Some serum trace element levels in acetaminophen/methionine treated-rats are significantly altered, zinc and iron are significantly decreased while Mo and Co are significantly increased compared with controls, although copper, manganese and chromium are not significantly different ($p>0.05$) (Table 2). Using ANOVA, only riboflavin was not significantly different among the vitamins while only Se and Cr were not significantly different of all the elements estimated; Zn, Cu, Mn, Mo, Fe and Co were significantly different.

4. DISCUSSION

This study recorded significant alteration in the levels of many of the vitamins and minerals that were studied. Such alterations had earlier been identified in male rats (Iyanda et al., 2011). In male rats, significant decrease in levels of vitamins such as vitamins A, E and riboflavin was linked to a probable antioxidant role, since acetaminophen-induced toxicity is associated with free radical generation. Moreover, many of these agents (vitamins/minerals) are antioxidants and have been used in animal experiment to combat acetaminophen-induced toxicity. For example, zinc and vitamin E have been used successfully in ameliorating side effect/oxidative effect of some therapeutic drugs; Ekam and Ebong (2007) as well as El Sheikh (2008) have observed the hepato-protective effects of vitamins A and E as well as vitamin C respectively in paracetamol treated rats. Whereas Woo et al. (1995), by using zinc sulphate, were able to reduce the quantity of malondialdehyde, an index of lipid peroxidation generated after acetaminophen intoxication. Kroger et al. (1996), on the other hand, have also reported a probable antioxidant role of nicotinic acid amide since it inhibited acetaminophen-induced injury in mice kept on standard laboratory diet. Apart from these an assortment of phytochemicals/nutraceuticals can also serve as much more powerful agents in preventing acetaminophen-induced injuries (Ray et al., 2006). The anti-oxidant role of these bio-molecules therefore in the female Wistar rats can also be postulated.

Methionine is incorporated in acetaminophen formulation to prevent hepatic damage in cases of over-dosage, and a study by Iyanda et al. (2010), has shown that sub-toxic abuse did not result in hepatic damage especially if these two agents are concurrently administered. That study made a submission of its effectiveness as an antidote as well as its safety using hepatic indices as focus of study and therein laid the danger. This is because in spite of lack of tissue damage a significant alteration in the levels of many of these antioxidant minerals and vitamins may result in a variety of oxidative-stress induced diseases. This study carried out in female rats which has shown a significant decrease ($p<0.05$) in the levels of folic acid for the APAP/methionine group compared with controls may be an indication that women within the reproductive stage who abuse this combination at subtoxic level may be at risks of having babies predispose to neural tube defects, maternal folic acid deficiency is a cause of neural tube defects.

Even for such female themselves increase in incidence of some other diseases may not be ruled out; especially as folate deficiency has been associated with increased risk of various cancers and cardiovascular diseases. And since abnormality in folate status is common in a number of patho-physiological conditions such as inflammatory bowel disease, cancer, alcoholism, pregnancy, neonatal growth, and during administration of some drugs (Wani et al., 2008) and abnormality in folate-dependent intracellular metabolism can lead to several key pathologies, such as megaloblastic anemia, homocysteinemia, cardiovascular disease, congenital heart defects, and possibly cancer, sub-toxic abuse of this combination for a

prolonged period of time may prone an individual to some of these conditions. Studies in other mammals have suggested that folate deficiency may also impair implantation and early embryogenesis in mice (Heid et al., 1992).

Although the mechanism by which folate is linked to NTD is not clear, especially as a complex interactions of genetics, metabolic status and environment combine to result in NTD but Gelineau-van Waes et al. (2008), have highlighted the critical importance of folate transport in chorioallantoic fusion, hematopoiesis and the development of neural tube, limbs, lungs, heart, and skin. Apart from the possibility of an increase risk for neural tube defects, sub-toxic abuse of this combination may be especially dangerous for some categories of women especially those with conditions such as stress, smoking, alcoholism, fever, viral infections; conditions that have been reported to lead to a rapid decline in blood levels of ascorbic acid and when in combination with sub-toxic abuse of acetaminophen/methionine may aggravate these conditions. Moreover, depletion in serum levels of vitamin C has been linked to increased incidence in mortality from heart diseases, stroke and cancer (Carr and Frei, 1999b). Ascorbic acid was recorded to be significantly decreased ($p<0.05$) in rats administered with this combination.

This significant difference may also affect some of the basic but essential functions of ascorbic acid, such as synthesis of muscle carnitine; its role as a co-factor in carnitine synthesis (Hulse et al., 1978) as well as the enzyme dopamine-β-hydroxylase, which catalyzes the conversion of neurotransmitter dopamine to norepinephrine, making it an essential ingredient for synthesis of catecholamines. Moreover, chronic exposure to this combination may also predispose an individual to modulation of some enzymatic reactions; reactions such as those involving amidation necessary for maximal activity of hormones oxytocin, vasopressin, cholecystokinin and alpha-melanotripin (Cameron and Pauling, 1973) as well as modulation of microsomal 7 α-hydroxylation important in the transformation of cholesterol to bile acids; when this reaction is hindered, accumulation of cholesterol in liver, hypercholesterolemia and formation of cholesterol gall stones (Ginter et al., 1982) cannot also not be ruled out.

In addition, its role in boosting immune system by enhancing T-cell proliferation in response to infection, causing production of large quantities of cytokines and aiding B cells to synthesize immunoglobulins to control inflammatory reactions, as well as blocking pathways that lead to apoptosis of T-cells and thus stimulating or maintaining T cell proliferation so as to attack the infection may also be affected (Campbell et al., 1999). The result outcome of this study (chronic) also seems to be capable of raising the possibility of an increase risk of cervical cancer in females who abuse this combination at sub-toxic level for an extended period of time; low levels of ascorbic acid and some of other vitamins have been linked with an increased risk of cervical cancer (Brock et al., 1988; Verreault et al., 1989; Potischman and Brinton, 1996; Rock et al., 2000). Moreover, vitamins E and A (retinoic) which act individually or in synergy with ascorbic acid to impart a protective effect against various cancers were also significantly decreased ($p<0.05$) in these rats.

Moreover, the role of vitamin C as antioxidant in scavenging/neutralizing an array of reactive oxygen species viz., hydroxyl, alkoxyl, peroxyl, superoxide anion, hydroperoxyl radicals and reactive nitrogen radicals such as nitrogen dioxide, nitroxide, peroxynitrite even at very low concentrations can also be compromised (Carr and Frei, 1999a). This may cause a significant increase in degree of oxidative damage to macromolecules such as lipids, DNA and proteins which have been implicated in chronic diseases such as cardiovascular disease, stroke, cancer, neurodegenerative diseases and cataractogenesis (Halliwell and

Gutteridge, 1999). Moreover, the role of vitamin C in regenerating other antioxidants such as α-tocopheroxyl, urate and β-carotene radical cation from their radical species (Halliwell and Gutteridge, 1999) may also be compromised.

That sub-toxic abuse of this combination may be capable of inducing or aggravating some of the above clinical conditions or altering many of the biochemical processes mentioned above is evident by a body of evidence which exists in relation to drug usage and nutrient depletions. Some of the drugs which studies have recognized to cause vitamins and mineral depletions include cholestyramine, colestipol, biguanide, biphosphonate and corticosteroids. Others are calcium channel blocking drugs (amlodipine and felodipine); antibiotics (penicillin and amoxicillin) as well as tricyclic antidepressants (amitriptyline and clomipramine). Cholestyramine and colestipol which have been recognized to deplete nutrients such as vitamin A, vitamin E and folic acid have been linked with disorders such as poor vision, cataract, anemia and birth defects (Levy et al., 2011; Smith and Whitehall, 2009; Wen et al., 2008). Although the metabolism of acetaminophen/methionine by Wistar rats is close to that of human subjects, the possibility of slight changes in presentation in human subject cannot be ruled out, and caution may be therefore be exercised in extrapolating these data to human subjects.

Moreover, the significant difference in the levels of Mo, Co, Fe and Cr is capable of enhancing these negative effects and inducing many pathological signs for which iron depletion and Mo, Co and Cr excesses are noted for. For example, iron depletion has been reported to induce anemia while significant increase in the levels of Mo, Co and Cr may induce oxidative stress. Although the mechanism by which zinc, vitamins A and E depletion can be linked to increased utilization because of their anti-oxidant role in combating a number of reactive species produced as a result of acetaminophen abuse, the increase in the levels of Mo, Co and Cr may not be unassociated with increased levels of different protein fraction which has been reported to occur post-paracetamol administration (Merrick et al. (2006). Many of the elements are protein bound and their general metabolism can be generally influence by protein levels.

While Masubuchi et al. (2011), have demonstrated that sex difference in susceptibility to acetaminophen hepatotoxicity can be reversed using buthionine sulfoximine and Chandrasekaran et al. (2011), have revealed that 17β-estradiol protects against acetaminophen-overdose-induced acute oxidative hepatic damage and increases the survival rate in mice, the results of our study showed that alterations in the levels of some vitamins and minerals occurred in these female rats.

5. CONCLUSION

The idea of incorporating methionine with acetaminophen is to raise its degree of safety in cases of accidental and intentional overdose, but the significant alterations ($p<0.05$) in the levels of many of these bio-molecules and elements point to the danger associated with its chronic abuse at subtoxic level which raises the possibility of an increased risk in oxidative stress-induced diseases and neural tube defects. Being an over-the-counter drug contained in several brands, consumers may not be aware of overdosing, therefore they should be encouraged if possible read the labels or consult a health care professional in cases of doubts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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