The impact of B vitamins on the functioning of methylation cycle in the liver and the kidneys of hyper- and hypothyroid rats

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Hyperhomocysteinemia is a risk factor for endothelial dysfunction and, consequently, for cardiovascular disease and multiple other conditions. Impairment of homocysteine metabolism is known to occur in thyroid dysfunction. In particular, patients with hypothyroidism have significantly higher homocysteine levels than healthy people. Metabolism of homocysteine occurs in methylation cycle (whose normal functioning is dependent on tissue pools of vitamins B_9, B_{12} and betaine), and also in reactions of trans-sulfonation, where pyridoxal phosphate (a pyridoxine derivative) acts as a coenzyme.

***The aim** of this study was to perform an experimental feasibility assessment of using pyridoxine, betaine, folic acid and cyanocobalamin to correct the methionine and homocysteine metabolism impaired by hyper- and hypothyroidism.*

***Material and methods.** Prolonged hyperthyroidism and hypothyroidism were modeled in experimental rats by dosing the animals with L-thyroxine and thiamazole, respectively, for 21 days.*

***Results.** Prolonged hyper- and hypothyroidism was found to cause oppositely directional changes in homocysteine metabolism. Hyperthyroidism was causing a significant increase in activity of S-adenosyl-methionine synthase, betaine-homocysteine methyltransferase and S-adenosylhomocysteine hydrolase in the liver and kidneys compared to control group of animals. Such directionality of changes in activities of above mentioned enzymes has led to a reduction in serum homocysteine levels. Hypothyroidism inhibited the activity of S-adenosyl-methionine synthase, betaine-homocysteine methyltransferase and S-adenosylhomocysteine hydrolase in the liver and in the kidneys of rats compared to controls. Betaine partially prevented impaired betaine-homocysteine methyltransferase activity in hyper- and hypothyroidism. Folic acid, cyanocobalamin and pyridoxine significantly reduced homocysteine levels in the blood of animals with hypothyroidism.*

***Conclusions.** A conclusion was made that the above agents could be effective factors to prevent endothelial dysfunction in hypothyroidism.*

Key words: *hyperthyroidism, hypothyroidism, remethylation cycle, homocysteine, vitamins B_9, B_{12} and B_6, betaine*

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Wpływ witaminy B na funkcjonowanie cyklu metylacyjnego w wątrobie i nerkach szczurów z niedoczynnością i nadczynnością tarczycy

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Hiperhomocysteinemia jest czynnikiem rozwoju dysfunkcji śródbłonka, a w konsekwencji chorób sercowo-naczyniowych i wielu innych chorób. Wiadomo, że zaburzony metabolizm homocysteiny obserwuje się w zaburzeniach funkcjonowania tarczycy. Szczególnie chorzy na niedoczynność tarczycy mają znacznie zwiększone stężenie homocysteiny niż osoby zdrowe. Metabolizm homocysteiny zachodzi w cyklu metylacji, którego normalne funkcjonowanie jest uwarunkowane zaopatrzeniem tkanek w witaminy B_9, B_{12} i betainę, a także w reakcjach transsiarczania, w których koenzym jest pochodną pirydoksyny – fosforanem pirydoksalu.

***Celem** tego badania było zbadanie w eksperymencie możliwości korekty metabolizmu metioniny i homocysteiny przez metabolity metioniny i homocysteiny, na które wpływa nad- i niedoczynność tarczycy, przy użyciu pirydoksyny i betainy, kwasu foliowego i cyjanokobalaminy.*

***Materiał i metody.** Stan przedłużonej nadczynności tarczycy i niedoczynności tarczycy u szczurów doświadczalnych symulowano przez podawanie zwierzętom odpowiednio L-tyroksyny i merkazolilu przez 21 dni.*

***Wyniki.** Wykazano, że przedłużona nadczynność i niedoczynność tarczycy powodują liczne zmiany w metabolizmie homocysteiny. Nadczynność tarczycy doprowadziła do znacznego zwiększenia aktywności syntetazy S-adenozylometioniny, metylotransferazy betainy-homocysteiny i hydrolazy S-adenozylohomocysteiny w wątrobie i nerkach w porównaniu z grupą kontrolną zwierząt. Ten kierunek zmian aktywności powyższych enzymów doprowadził do obniżenia stężenia homocysteiny w surowicy. Niedoczynność tarczycy hamowała aktywność syntetazy S-adenozylometioniny, metylotransferazy homocysteiny betainy i hydrolazy S-adenozylohomocysteiny u szczurów i nerek w porównaniu z grupą kontrolną. Betaina częściowo zapobiegała zaburzeniu aktywności metylotransferazy homocysteiny betainy w nadczynności i niedoczynności tarczycy. Kwas foliowy, cyjanokobalamina i pirydoksyna znacznie zmniejszyły zawartość homocysteiny we krwi zwierząt z niedoczynnością tarczycy.*

***Wnioski.** Stwierdzono, że wyżej wymienione środki mogą być skutecznymi czynnikami w zapobieganiu dysfunkcji śródbłonka w niedoczynności tarczycy.*

Słowa kluczowe*: nadczynność tarczycy, niedoczynność tarczycy, cykl remetialny, homocysteina, witaminy B_9, B_{12}, B_6, betaina*

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Homocysteine (HCy), a sulfur-containing amino acid, is an intermediate product in biosynthesis of such amino acids as methionine and cysteine. HCy is recognized as an independent risk factor of cardiovascular diseases that lead to impaired quality of life and are the leading cause of death in the world, accounting for 45.1% of mortality [5,12,13]. Immune-mediated activation and inflammation may cause hyperhomocysteinemia (HHCy). Recent research has demonstrated a positive relationship between HCy levels and some of the biological and humoral markers of inflammation, such as levels of circulating receptors to various cytokines or C-reactive protein.

Disorders of HCy metabolism are known to occur in thyroid dysfunction. Specifically, patients with iatrogenic hypothyroidism (*Hashimoto's* disease) have substantially higher HCy levels compared to patients without *Hashimoto* thyroiditis. Graves' disease is a common type of autoimmune thyroid disease to cause hyperthyroidism. Patients with *Graves'* disease are at an increased risk for cardiovascular disease; however, the relationship between this immune-mediated inflammatory thyroid condition and HCy levels remains unclear [9]. Previously, we have demonstrated that experimental modeling of hyperthyroidism leads to a reduction in HCy levels, while hypothyroidism, conversely, increases HCy levels in the blood. These changes are associated with changes in activities of methionine- and cysteine-metabolizing enzymes [14]. Since HCy is synthesized in methylation cycle (whose normal functioning is dependent on tissue pools of vitamin B_9, vitamin B_{12} and betaine) and since pyridoxal phosphate (a pyridoxine derivative) is a coenzyme of HCy trans-sulfonation enzymes, it was interesting to explore the possibility of using B vitamins and betaine for correction of methionine and HCy metabolism, the latter being impaired in hypo- and hyperthyroidism.

The objective of this work was to evaluate the influence of folic acid, cyanocobalamin, pyridoxine and betaine on the functioning of methylation cycle in animals with hyper- and hypothyroidism.

MATERIALS AND METHODS

The study has used 110 mongrel male rats weighing 150-180 g and kept on a standard diet. All animals were divided into 11 groups: Group 1: intact rats (intragastric administration of a 1% starch solution); Group 2: animals with hyperthyroidism receiving daily intragastric L-thyroxine for 21 days (200 μg/kg [per 1 kg of body weight]); Group 3: hyperthyroid rats receiving daily pyridoxine (10 mg/kg*day); Group 4: hyperthyroid rats receiving daily betaine (20 mg/kg*day); Group 5: hyperthyroid rats receiving daily folic acid (2 mg/kg*day) and cyanocobalamin (0.2 mg/kg*day); Group 6: hyperthyroid rats receiving daily folic acid, cyanocobalamin, pyridoxine and betaine; Group 7: animals with hypothyroidism receiving daily thiamazole for 21 days (10 mg/kg*day); Group 8: hypothyroid rats receiving daily pyridoxine (10 mg/kg*day); Group 9: hypothyroid rats receiving daily betaine (20 mg/kg*day); Group 10: hypothyroid rats receiving daily folic acid (2 mg/kg*day) and cyanocobalamin (0.2 mg/kg*day); Group 11: hypothyroid rats receiving daily dosing with a combination of folic acid, cyanocobalamin, pyridoxine and betaine. The animals were sacrificed on Day 22 with cervical dislocation. Serum, hepatic tissue and renal tissue were used for the tests. The study was performed in accordance with the general ethical guidelines of animal experimentation.

Total serum HCy content was obtained with immunoenzymometric assay using test kits manufactured by Axis-Shield (United Kingdom). The liver and the kidneys were perfused with cold 1.15% potassium chloride solution and homogenized at 3000 rpm in 1.15% potassium chloride (at 1:3 ratio). The homogenates were centrifuged for 30 minutes at 1500 g and +4°C. Activities of S-adenosyl-methionine synthase (S-AMS, EC 2.5.1.6) in liver and kidney homogenates were determined based on increases in inorganic phosphate formed in ATP hydrolysis [1]. The activity of S-adenosylhomocysteine hydrolase (S-AHH, EC 3.3.1.1) was assessed in the reaction of S-adenosylhomocysteine hydrolysis based on increases in sulfhydryl groups [8]. The activity of betaine-homocysteine methyltransferase (BHMT, EC 2.1.1.5) was assessed based on reductions in sulfhydryl groups in the incubation medium [3].

The results were given as mean±SEM of 8-10 tests. Statistical analysis was performed with standard statistical software and *Student's t*-test. The level of $P<0.05$ was regarded as statistical significance.

RESULTS

Dosing experimental animals with L-thyroxin for 21 days has led to significant increases in S-AMS activity levels in the liver (a 35% increase) and in the kidneys (a 63% increase) compared to control animals (tab.1). Hyperthyroidism also caused an increase in activity of S-AHH (another enzyme of methylation cycle, which cleaves S-adenosylhomocysteine to adenosine and HCy): hepatic and renal S-AHH increased by 33% and 59%, respectively. The activity of BHMT, another enzyme in the methylation cycle (which converts HCy to methionine), increased in the liver and in the kidneys by 45% ($P<0.05$) and 47% ($P<0.05$), respectively (vs controls). The therapeutic agents that we have selected (used either as a single agent or in combination) have failed to correct the L-thyroxine-induced abnormalities of S-AMS- and S-AHH-catalyzed reactions in the liver and in the kidneys.

In hyperthyroidism, the activity of BHMT significantly increased vs. controls in the liver and in the kidneys: by 45% ($P<0.05$) and 47% ($P<0.05$), respectively. Betaine was found to increase BHMT activity when co-administered with L-thyroxine. The activity of BHMT increased by 38% in the liver and by 69% in the kidneys compared to the group of animals receiving L-thyroxine only. The activity of BHMT increased to an even greater degree in the animals receiving a combination of vitamin B_6 +vitamin B_9 + vitamin B_{12} + betaine (by 43% and 70% in hepatic tissue and in the kidneys, respectively, compared to animals with hyperthyroidism).

Table 1. Activity (nmol/min*mg of protein) of methylation cycle enzymes in the liver and in the kidneys of rats with a model of hyperthyroidism when using pyridoxine, betaine, folic acid and cyanocobalamin (M±m; n=8-10)
Tabela 1. Aktywność (nmol / min * mg białka) enzymów cyklu metylacji w wątrobie szczura i nerce z modelem nadczynności tarczycy podczas stosowania pirydoksyny, betainy, kwasu foliowego tacianokobalaminy (M ± m; n = 8-10)

The object of study	Group of animals						
	Enzyme	Intact	Hyperthyroidism (21 days)	Correction of metabolism of sulfur-containing amino acids in hyperthyroidism (21 days)			
			L-thyroxine	L-thyroxine + B_6	L-thyroxine + betaine	L-thyroxine + B_9 +B_{12}	L-thyroxine +B_6+ B_9 + B_{12} + Betaine
Liver	S-AMS, nmol/min*mg of protein	5.83±0.45	7.88±0.52*	7.51±0.62	8.20±0.58*	7.30±0.71	8.53±0.82*
	S-AHH, nmol/min*mg of protein	5.65±0.32	7.53±0.50*	7.43±0.60*	7.91±0.59*	7.73±0.63*	7.96±0.65*
	BHMT, nmol/min*mg of protein	8.65±0.50	12.50±0.95*	12.25±1.02*	17.25±1.21*#	13.50±1.23*	17.85±1.41*#
Kidneys	S-AMS, nmol/min*mg of protein	3.04±0.29	4.96±0.30*	5.02±0.51*	4.98±0.55*	5.09±0.61*	5.21±0.58*
	S-AHH, nmol/min*mg of protein	3.83±0.38	6.08±0.53*	6.57±0.59*	6.32±0.55*	6.20±0.52*	6.17±0.65*
	BHMT nmol/min*mg of protein	3.33±0.17	4.90±0.35*	5.10±0.45*	8.28±0.56*#	5.15±0.49*	8.33±0.53*#

Note: * – the changes were significant vs. the group of intact animals; # – the changes were significant vs. uncorrected animal model of hyperthyroidism

Table 2. Activity of methylation cycle enzymes (nmol/min*mg of protein) in the liver and in the kidneys of rats with a model of hypothyroidism when using pyridoxine, betaine, folic acid and cyanocobalamin (M±m; n=8-10)
Tabela 2. Aktywność (nmol / min *mg białka) enzymów cyklu metylacji w wątrobie szczura i nerce z modelem niedoczynności tarczycy przy zastosowaniu kwasu foliowego pirydoksyny, cyjanokobalaminy i betainy (M ± m; n = 8-10)

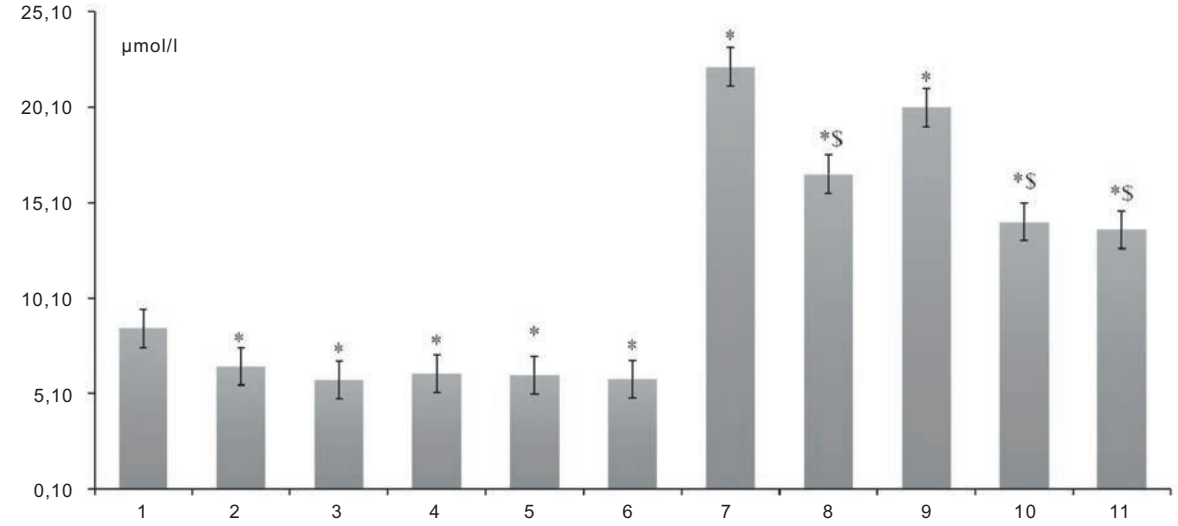
The object of study	Group of animals						
	Enzyme	Intact	Hypothyroidism (21 days)	Correction of metabolism of sulfur-containing amino acids in hypothyroidism (21 days)			
			Thiamazole	Thiamazole + B_6	Thiamazole + betaine	Thiamazole + B_9 +B_{12}	Thiamazole + B_6+ B_9 +B_{12} + betaine
Liver	S-AMSnmol/min*mg of protein	5.83±0.45	4.02±0.38*	4.62±0.45	4.14±0.41*	4.13±0.32*	4.24±0.38*
	S-AHH,nmol/min*mg of protein	5.65±0.32	4.26±0.35*	4.29±0.32*	4.36±0.39*	4.60±0.41	4.52±0.38
	BHMT,nmol/min*mg of protein	8.65±0.50	4.94±0.29*	5.43±0.41*	6.82±0.65*$	5.53±0.49*	6.92±0.61*$
kidneys	S-AMSnmol/min*mg of protein	3.04±0.29	1.82±0.28*	1.98±0.32*	1.87±0.30*	2.06±0.33	2.15±0.35
	S-AHH,nmol/min*mg of protein	3.83±0.38	2.08±0.19*	2.27±0.22*	2.18±0.20*	2.33±0.24*	2.37±0.28*
	BHMT,nmol/min*mg of protein	3.33±0.17	1.76±0.15*	1.85±0.16*	2.72±0.18*$	1.82±0.15*	2.70±0.14*$

Note: * – the changes were significant vs. the group of intact animals; $ – the changes were significant vs. uncorrected animal model of hypothyroidism

Experimentally induced hypothyroidism reduced activities of S-AMS (by 31% and 40%) and S-AHH (by 25% and 46%) in the liver and in the kidneys, respectively (vs. controls). As with the model of hyperthyroidism, none of the investigational therapies or combination thereof has produced any significant effects on S-AMS and S-AHH activity levels in either organ.

Hypothyroid status inhibited BHMT activity in rats by 43% and 47% in the liver and in the kidneys, respectively, compared to healthy animals. Correction with betaine caused the activity of the latter enzyme to increase both in the liver and in the kidneys. In particular, BHMT activity increased 38% in hepatic tissue and 55% in the kidneys compared to the group of animal model of hypothyroidism. The use of a "B_6 + B_9 + B_{12} + betaine" combination as an intervention to improve enzymatic status increased BHMT activity by 40% in the liver and by 53% in the kidneys compared to the group of animals that were dosed only with thiamazole.

Therefore, prolonged hyper- and hypothyroidism causes oppositely directional changes of HCy metabolism in the liver and in the kidneys of experimental animals. Therefore, it was



Group 1 = intact rats; Group 2 = animals with hyperthyroidism; Group 3 = hyperthyroid rats receiving daily pyridoxine; Group 4 = hyperthyroid rats receiving daily betaine; Group 5 = hyperthyroid rats receiving daily folic acid and cyanocobalamin; Group 6 = hyperthyroid rats receiving daily folic acid, betaine, cyanocobalamin and pyridoxine; Group 7 = animals with hypothyroidism; Group 8 = hypothyroid rats receiving daily pyridoxine; Group 9 = hypothyroid rats receiving daily betaine; Group 10 = hypothyroid rats receiving daily folic acid and cyanocobalamin; Group 11 = hypothyroid rats receiving daily dosing with a combination of folic acid, betaine, cyanocobalamin and pyridoxine.
Note: * = the changes were significant vs. the group of intact animals; # = the changes were significant vs. uncorrected animal model of hyperthyroidism; $= the changes were significant vs. uncorrected animal model of hypothyroidism.
1-a – nienaruszone szczury; 2-a – zwierzęta z nadczynnością tarczycy; 3-a – szczury z nadczynnością tarczycy, którym codziennie podawano pirydoksynę; 4-a – szczury z nadczynnością tarczycy, które codziennie podawano betainę; 5-a – szczury z nadczynnością tarczycy, którym codziennie wstrzykiwano kwas foliowy i cyjanokobalaminę; 6-a – szczurom z nadczynnością tarczycy, którym codziennie wstrzykiwano kwas foliowy, betainę, cyjanokobalaminę i pirydoksynę; 7-a – zwierzęta z niedoczynnością tarczycy; 8-a – szczury z niedoczynnością tarczycy, którym codziennie podawano pirydoksynę; 9-a – szczury z niedoczynnością tarczycy, którym codziennie podawano betainę; 10-a – szczury z niedoczynnością tarczycy, którym codziennie wstrzykiwano kwas foliowy i cyjanokobalaminę; 11-a – szczury z niedoczynnością tarczycy, którym codziennie podawano kombinację kwasu foliowego, betainy, cyjanokobalaminy i pirydoksyny.
Uwaga: * – zmiany są znaczące w odniesieniu do nietkniętych zwierząt; # – zmiany są znaczące w stosunku do wskaźników zwierząt z modelem nadczynności tarczycy, które nie zostały skorygowane; $ – zmiany są znaczące w stosunku do wskaźników zwierząt z modelem niedoczynności tarczycy, które nie zostały skorygowane.

Figure 1. Plasma HCy levels (µmol/L) in model rats with hyper- and hypothyroidism when using pyridoxine, betaine, folic acid and cyanocobalamin; n=8-10
Rycina 1. Zawartość HCy (µmol/l) w osoczu krwi szczurów z modelem nadczynności i niedoczynności tarczycy przy użyciu pirydoksyny, betainy, kwasu foliowego i cyjanokobalaminy; n=8-10

interesting to perform a direct assessment of level of this amino acid in the blood of rats used as models of hyper- and hypothyroidism.

We have found administration of L-thyroxine to reduce HCy levels in the animals by 23% vs controls (Fig. 1). Unlike L-thyroxine, prolonged exposure to thiamazole was accompanied by a 160% increase in HCy levels compared to control animals.

Administration of pyridoxine to animals with hypothyroidism has led to a 25% reduction in HCy levels compared to the group of animals with hypothyroidism. The use of folic acid and cyanocobalamin in animals with inhibited thyroid function has caused a 37% HCy reduction compared to animals with no correction.

It is possible to predict that the use of combination of vitamins B_6, B_9 and B_{12} and betaine may activate HCy utilization via both remethylation to methionine and trans-sulfonation to cysteine. Combined use of the above-mentioned agents in animals with hyperthyroidism caused a 38% reduction in HCy levels compared to the group of hyperthyroid animals where no correction was undertaken.

DISCUSSION

The results of our study have demonstrated that experimentally induced hyper- and hypothyroidism causes imbalances of HCy and methionine metabolism. In rats, L-thyroxine was causing an increase in hepatic and renal activities of S-AMS, S-AHH and BHMT. Such a directionality of changes in activity of enzymes in the remethylation cycle has apparently affected HCy levels in the blood of animals, the latter being significantly lower than in the control rats. At the same time, hypothyroidism reduced activities of S-AMS, S-AHH and BHMT in hepatic and renal tissue, which was accompanied by a significant increase in HCy levels in the blood of animals. Similar results were obtained in another work [2]. The authors have assessed plasma HCy levels in hypo- and hyperthyroid patients before and after treatment and have found plasma HCy levels to increase in hypothyroidism with a concomitant reduction in plasma folate and creatinine clearance; hyperthyroidism is conversely associated with increased creatinine clearance.

During analysis of the influence of investigational products on the course of remethylation cycle reactions in hypothyroidism in the liver and in the kidneys we have found betaine and the use of combination of all drugs to be the most effective. Such results of betaine effects may be considered expected, since betaine is a donor of methyl groups and an essential component of the reaction of conversion of HCy to methionine, the latter catalyzed by BHMT. In addition to that, HCy may be converted into methionine with homocysteine methyltransferase, a B_{12}-dependent enzyme; of note, N-5-methyltetrahydrofolate, which is formed in the active folate cycle, is a donor of methyl group for homocysteine methyltransferase. Obviously, that is the reason why the use of folic acid and cyanocobalamin as part of our experimentation in animals with hypothyroidism produced a significant reduction in plasma HCy levels. *Maier H. et al.* [11] have shown low levels of vitamin B_{12} and folic acid to be associated with increased HCy levels. Similar results have been established in a research study by *Gołyński M. et al*. [4]. The authors explored the relationships between total serum HCy levels, folic acid concentrations and the levels of thyroid hormones. Increased HCy concentrations and decreased levels of folic acid were demonstrated in patients with hypothyroidism, which may influence the development of multiple secondary alterations, including cardiovascular disease. The majority of studies that assess the relationship between hyperhomocysteinemia and cardiovascular risks in hypothyroidism are specifically focused on folic acid and cyanocobalamin deficiencies as the causes of impaired remethylation of HCy to methionine. A study of clinical chemistry findings in the blood of 17 patients who had a total thyroidectomy for thyroid cancer has shown progressive postoperative increases in serum HCy (by 27%), creatinine (by 37%) and cholesterol (by 100%) and these values returned to baseline during 4-6 weeks of treatment with L-thyroxine [10]. Folate levels in serum and red blood cells of such patients had demonstrated small but statistically significant downward trends. The authors came to a conclusion that elevated HCy levels could increase cardiovascular risk in patients with hypothyroidism.

A study of biochemical changes in a setting of post-puberty hypothyroidism has shown the use of folic acid to improve spermatogenesis, to increase concentrations of spermatozoids and to increase antioxidant levels [6]. The authors have found the use of folic acid together with replacement thyroxine therapy for hypothyroidism to reduce oxidative stress due to high HCy levels. *Ibrahim W. et al*. [7] studied biochemical changes in hypothalami of hypothyroid post-pubertal rats and the potential improving effects of folic acid. Compared to control group, the group of hypothyroid rats was observed to have substantially increased serum levels of HCy, malondialdehyde (MDA) and GSSG/GSH and reduced concentrations of NO, serotonin and dopamine in hypothalamus. This data suggests the development of hyperhomocysteinemia and oxidative stress in a setting of hypothyroidism. Treatment of hypothyroid rats with folic acid inhibited oxidative stress and normalized the levels of hypothalamus monoamines.

CONCLUSIONS

Our data have shown prolonged hyper- and hypothyroidism to caused an imbalance of metabolism of sulfur-containing amino acids. The activity levels of S-adenosyl-methionine synthase, S-adenosylhomocysteine hydrolase and betaine-homocysteine methyltransferase were increased in the liver and in the kidneys of animals with hyperthyroidism; in the meantime, HCy levels in the blood were decreased. Hypothyroidism reduces the activities of S-adenosyl-methionine synthase, S-adenosylhomocysteine hydrolase and betaine-homocysteine methyltransferase in the liver and in the kidneys and increases HCy levels in the blood. Betaine partially prevents impairments of betaine-homocysteine methyltransferase activity in hyper- and hypothyroidism. The use of folic acid, cyanocobalamin and pyridoxine significantly reduced HCy levels in the blood of animals with hypothyroidism. Such effects of the above mentioned agents suggest the possibility of their use to prevent endothelial dysfunction, a potential occurrence in hypothyroidism.

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