

The protective effects of melatonin and Vitamin E on antioxidant enzyme activities and epididymal sperm characteristics of homocysteine treated male rats

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Abstract

The aims of this study were to investigate the effects of homocysteine (Hcy) on epididymal sperm characteristics, plasma testosterone level and biochemical changes related to oxidative stress and to examine the effects of melatonin (Mlt) or Vitamin E (VE) administration on these parameters in Hcy-treated male rats. In this study, 32 adult male albino rats of Wistar strain were used. The rats were randomly divided into four groups. The first group of rats received only Hcy (0.71 mg/kg/day) intraperitoneally (ip) for 6 weeks. The second group of rats was given Hcy along with simultaneous administration of Mlt (1 mg/kg/day) subcutaneously. The third group of rats received Hcy along with simultaneous administration of VE (125 mg/kg/day, ip). The fourth group of rats served as control during 6 weeks and was daily given 0.1 mL of physiological saline (NaCl, 0.9%) ip. While the plasma malondialdehyde level significantly ($p < 0.05$) increased, the plasma superoxide dismutase, glutathione peroxidase and catalase activities significantly ($p < 0.05$) decreased in Hcy-treated rats when compared to control rats. Furthermore, the epididymal sperm concentration, the percentage of progressive sperm motility and plasma testosterone level were significantly ($p < 0.05$) lower in Hcy-treated rats than those of the control rats. The simultaneous administration of Mlt or VE to Hcy-treated animals impeded the decrease in the plasma antioxidant enzyme activities, testosterone level, the epididymal sperm concentration and motility. In conclusion, this study indicates that chronic administration of Hcy has the harmful effect on the epididymal sperm characteristics of male rats. The administration of Mlt or VE can prevent adverse effects of Hcy on the plasma antioxidant enzyme activities, testosterone level, epididymal sperm count and motility in male rats.
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1. Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid that is generated from metabolism of methionine. Hcy is not present in the diet but it is an essential intermediate product in normal metabolism of methionine. Hcy metabolism consists of the intersection of two metabolic pathways; transsulfuration and remethylation. The cellular levels of Hcy are regulated by transsulfuration of Hcy to cysteine and remethylation of Hcy to methionine. While transsulfuration pathway contributes the maintenance of normal Hcy concentration, the

remethylation pathway maintains normal fasting concentrations [1,2].

The plasma Hcy concentration is higher in males than females. It is affected by genetic and dietary factors [3]. There is a relationship plasma Hcy level and cardiovascular disease. The increase in plasma Hcy level has several toxic effects and, in particular, it is considered as a risk factor for arteriosclerosis [4].

Although the effect of Hcy on male reproductive system is unknown, it is reported that there may be a positive correlation between the increase in plasma Hcy level and reduction of semen parameters [5]. The increase in Hcy concentration also alters regulatory proteins associated with cell membrane, which results inhibition of sperm motility [6]. However, there is no published record about the effect of Hcy administration on sperm function of male rats.

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There is a significant relationship between the plasma Hcy level and lipid peroxidation. The high plasma Hcy concentration leads to oxidative stress. The highly reactive thiol group of Hcy is readily oxidized in plasma and it causes the generation of reactive oxygen species (ROS) [7]. ROS are free radicals such as the hydroxyl radical ($\cdot\text{OH}$) and the superoxide anion (O_2^-) or molecules like hydrogen peroxide (H_2O_2). The production of ROS is a normal physiological event in various organs including the testis. However, the overproduction of ROS causes structural damage of sperm membranes, which results in the formation of cytotoxic secondary products such as malondialdehyde (MDA) [8,9]. Antioxidants have an important role in maintaining the motility and the genetic integrity of sperm cells against oxidative damage [10]. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) are natural antioxidant enzymes, which eliminate ROS. Besides, a number of non-enzymatic antioxidants exist in the intracellular and extracellular medium [11].

Melatonin (Mlt), secretory product of the pineal gland, is an effective antioxidant. It is the most potent physiological scavenger of hydroxyl radical and blocks its devastating actions [12]. Vitamin E (VE), chain-breaking antioxidant, is also one of the primary components of the antioxidant system. It intercepts peroxy and alkoxy radicals, which are generated during the conversion of lipid hydroperoxides that have an important role in the peroxidative chain reaction [13,14]. There is relationship between plasma Hcy level and these antioxidants. The administration of both VE [15] and Mlt [16,17] decreases the plasma MDA level and, protects cells against oxidative damage. In addition, it was reported that plasma Hcy level significantly decreased both Mlt [18] and VE [19] treated rats.

The aims of this study were to investigate the effect of Hcy administration on epididymal sperm count and motility, plasma testosterone level and biochemical changes related to oxidative stress and to examine the effects of Mlt or VE administration on these parameters in Hcy-treated male rats.

2. Materials and methods

2.1. Chemicals

Mlt ($\text{C}_{13}\text{H}_{16}\text{N}_2\text{O}_2$) was obtained from MERCK (Darmstadt, GERMANY). VE (DL- α -tocopheryl acetate, Rovimix[®] E-50 Adsorbate) was purchased from ROCHE (Roche Inc., Istanbul, Turkey). DL-Hcy (2-amino-4-mercaptobutyric acid, $\text{C}_4\text{H}_9\text{NO}_2\text{S}$) and the other chemicals were purchased from Sigma–Aldrich Co.

2.2. Animals and treatment

All protocols for animal care and use in the present study were approved by the National Institutes of Health and Local Committee on Animal Research. Thirty-two adult male albino rats of Wistar strain, 6–7 months of age and weighting 350–400 g were used in this study. The animals were obtained from Experimental Research Centre, University of Firat, Elazığ, Turkey. The rats were individually housed in plastic cages and kept under standard laboratory conditions (12 h light:12 h dark and $24 \pm 3^\circ\text{C}$) during experimental period. The rats were fed standard commercial laboratory chow (pellet form, Elazığ Food Company). The composition of rat chow is given in Table 1. Feed and water were provided ad libitum.

Table 1
The composition of standard commercial rat chow

Contents	Percent
Wheat	10.0
Corn	24.0
Barley	16.0
Bran	9.0
Soybean	26.0
Fish flour	3.2
Meat-bone flour	2.6
Molasses	5.0
Salt	2.3
Calcium phosphate	1.7
Vitamin compound ^a	0.1
Inorganic substances ^b	0.1

^a Vitamin mixture (in kg^{-1}): Vit A (12,000,000 IU), Vit C (50 g), Vit D₃ (2,400,000 IU), Vit E (30 g), Vit K₃ (2.5 g), Vit B₁ (3.0 g), Vit B₂ (7.0 g), Vit B₆ (4.0 g), Vit B₁₂ (15 mg), nicotinamide (4 g), folic acid (1 g) and biotine (45 mg).

^b Inorganic mixture (in kg^{-1}): Manganese (80 g), iron (40 g), zinc (60 g), copper (5 g), iodine (0.5 g), cobalt (0.2 g) and selenium (0.15 g).

The rats were randomly divided into four groups of eight rats each. The first group of rats intraperitoneally (ip) received only Hcy (0.71 mg/kg/day) for 6 weeks. The dose of homocysteine was chosen based on the study of Chen et al. [20]. The second group of rats was given Hcy (0.71 mg/kg/day, ip) along with simultaneous administration of Mlt subcutaneously at a dose of 1 mg/kg/day for 6 weeks. The third group of rats received Hcy (0.71 mg/kg/day, ip) along with simultaneous administration of VE ip at a dose of 125 mg/kg/day for 6 weeks. The fourth group of rats served as control during 6 weeks and was daily given 0.1 mL of physiological saline (NaCl, 0.9%) ip.

2.3. Necropsy

The rats were sacrificed using ether anesthesia at the end of 6 weeks. Blood samples were collected into glass tubes containing EDTA and centrifuged at 3000 rpm for 10 min. Plasma was separated and then stored at -20°C until analysis. Testes, epididymides, seminal vesicles and prostate were removed. They were carefully cleaned from adhering connective tissue and weighed.

2.4. Epididymal sperm concentration and motility

The epididymal sperm concentration was determined with a hemocytometer (Improved Neubauer, Weber, UK) using a modification of the method described by Yokoi et al. [21]. Briefly, the right epididymis was finely minced by anatomical scissors in 1 mL of physiological saline (NaCl, 0.9%) in Petri dish. It was completely squashed by a tweezers for 2 min. Then, it was incubated at room temperature for 5 min to provide the migration of all spermatozoa from epididymal tissue to fluid. After incubation, the epididymal tissue-fluid mixture was filtered via strainer to separate the supernatant from tissue particles. The supernatant fluid was drawn into the capillary tube up to 0.5 lines of the pipette which designed for counting red blood cell. The solution containing 5 g sodium bicarbonate, 1 mL formalin (35%, v/v) and 25 mg eosin per 100 mL distilled water was pulled up to 101 lines of the pipette. Approximately 10 μL of the diluted sperm suspension was transferred to counting chambers of hemocytometer and allowed to stand for 5 min. The sperm cells in both chambers were counted with the help of light microscope at the magnification of $200\times$.

The percentage of progressive sperm motility was evaluated using a light microscope with heater table as described in our previous study [22]. For this process, a slide was placed on microscope stage and, allowed to warm a temperature of 35°C by heater table. Several droplets of Tris buffer solution [Tris (hydroxymethyl) aminomethane 3.63 g, glucose 0.50 g, citric acid 1.99 g and distilled water 100 mL] were dropped on the slide, and a very small droplet of fluid obtained from left cauda epididymis with a pipette was added on the this solution and mixed by a cover-slip. The percentage of progressive sperm motility was visually evaluated using a score ranging from 0 to 100% at a magnification

of 400× [23]. Motility estimations were performed from three different fields in each sample. The mean value of three successive estimations was used as the final motility score.

2.5. Biochemical studies

2.5.1. Testosterone

The plasma testosterone level was assayed using Coat-a-Count Radioimmunoassay kit (Active Testosterone RIA DSL-4000, Diagnostic System Laboratories Inc., Texas, USA) and expressed as ng/mL.

2.5.2. Lipid peroxidation

The plasma lipid peroxidation level was measured according to the concentration of thiobarbituric acid reactive species [24]. The amount of produced MDA was used as an index of lipid peroxidation. Briefly, one volume of the test sample and two volume of stock reagent (15%, w/v trichloroacetic acid in 0.25N HCl and 0.375%, w/v thiobarbituric acid in 0.25N HCl) were mixed in a centrifuge tube. The solution was heated for 15 min in boiling water. After cooling, the precipitate was removed by centrifugation at 2500 rpm 10 min. Then, absorbance of the supernatant was measured at 532 nm against a blank containing all reagents except test sample on a spectrophotometer (Shimadzu 2R/UV-visible, Tokyo, Japan). The plasma MDA level was expressed as nmol/mL.

2.5.3. Superoxide dismutase

The plasma SOD activity was measured using xanthine and xanthine oxidase to generate superoxide radicals, which react with nitroblue tetrazolium (NBT) [25]. Briefly, each sample was diluted 1:10 with phosphate buffer (50 mM, pH 7.5). The assay solution containing sodium-carbonate buffer (50 mM, pH 10), 0.1 mM xanthine, 0.025 mM NBT, 0.1 mM EDTA, xanthine oxidase (0.1 U/mL in ammonium sulfate 2 M) and sample were mixed in a cuvette. One unit of SOD activity was defined as the amount of enzyme, which required to inhibition of NBT. The plasma SOD activity was measured at 560 nm by the degree of inhibition of this reaction on a spectrophotometer and expressed as U/mL.

2.5.4. Catalase

The plasma CAT activity was measured as previously described by Goth [26]. Briefly, 0.2 mL of plasma samples was incubated in 1.0 mL substrate (65 μmol/mL hydrogen peroxide in 50 mM phosphate buffer, pH 7.0) at 37 °C for 60 s. The enzymatic reaction was terminated with 1.0 mL of 32.4 mM ammonium molybdate. Hydrogen peroxide was measured at 405 nm against blank

containing all the components except the enzyme on a spectrophotometer. The plasma catalase activity was expressed as KU/L.

2.5.5. Glutathione peroxidase

The plasma GSH-Px activity was determined according to the method of Lawrence and Burk [27]. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide (NaN₃), 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 EU/mL oxidized glutathione (GSSG)-reductase, 1 mM GSH, and 0.25 mM H₂O₂. Enzyme source (0.1 mL) was added to 0.8 mL of the above mixture and incubated for 5 min at 25 °C before initiation of the reaction with the addition of 0.1 mL of peroxide solution. The absorbance at 340 nm was recorded for 5 min on a spectrophotometer. The activity was calculated from the slope of the lines as micromoles of NADPH oxidized per minute. The blank value (the enzyme was replaced with distilled water) was subtracted from each value. The protein concentration was also measured by the method of Lowry et al. [28]. The results were expressed as IU/g protein.

2.6. Statistical analyses

All values are presented as mean ± standard error of means (S.E.M.). The differences were considered to be significant at $p < 0.05$. Statistical analyses were performed using analysis of variance (One-way ANOVA) and post hoc Tukey test using the SPSS/PC (Version 10.0) software program.

3. Results

3.1. Organ weights

The values of organ weights are shown in Table 2. There was no significant difference among groups relative to average weights of the testes, epididymides, seminal vesicles and prostate.

3.2. Testosterone, lipid peroxidation and antioxidant enzyme activities

The plasma SOD, GSH-Px, CAT activities, MDA and testosterone level are shown in Table 3. While the plasma MDA

Table 2
The weights of testes, epididymides, seminal vesicles and prostate in all groups

	Control	Hcy	Hcy + Mlt	Hcy + VE
Body weight (g)	377.0 ± 4.3	368.8 ± 4.3	365.8 ± 3.2	372.1 ± 4.9
Testis weight (mg)	2364.1 ± 72.6	2200.8 ± 45.6	2389.4 ± 90.8	2176.9 ± 59.8
Epididymis (mg)	632.9 ± 31.5	593.3 ± 27.7	585.3 ± 31.8	621.5 ± 29.7
Seminal Vesicles (mg)	383.8 ± 12.8	346.3 ± 16.3	353.3 ± 14.1	374.5 ± 16.4
Prostate (mg)	178.6 ± 3.2	185.1 ± 2.6	184.6 ± 2.9	182.8 ± 4.7

The data are expressed in mean ± S.E.M. for eight animals per group. No significant changes were observed among any of groups.

Table 3
The plasma MDA, SOD, GSH-Px, CAT activities and testosterone level in all groups

Plasma	Control	Hcy	Hcy + Mlt	Hcy + VE
MDA (nmol/mL)	1.42 ± 0.06 ^a	1.72 ± 0.08 ^b	1.34 ± 0.11 ^a	1.39 ± 0.06 ^a
SOD (U/mL)	2.94 ± 0.08 ^a	2.67 ± 0.07 ^b	3.15 ± 0.04 ^c	3.01 ± 0.07 ^a
GSH-Px (IU/g protein)	2.55 ± 0.09 ^a	2.22 ± 0.06 ^b	3.17 ± 0.02 ^c	2.76 ± 0.04 ^d
CAT (KU/L)	18.92 ± 0.60 ^a	14.81 ± 0.48 ^b	22.25 ± 0.63 ^c	20.82 ± 0.73 ^d
Testosterone (ng/mL)	2.78 ± 0.15 ^a	1.47 ± 0.17 ^b	2.46 ± 0.16 ^a	2.58 ± 0.19 ^a

The data are expressed in mean ± S.E.M. for eight animals per group. Different letters (a–d) within same line show significant ($p < 0.05$) differences among the groups.

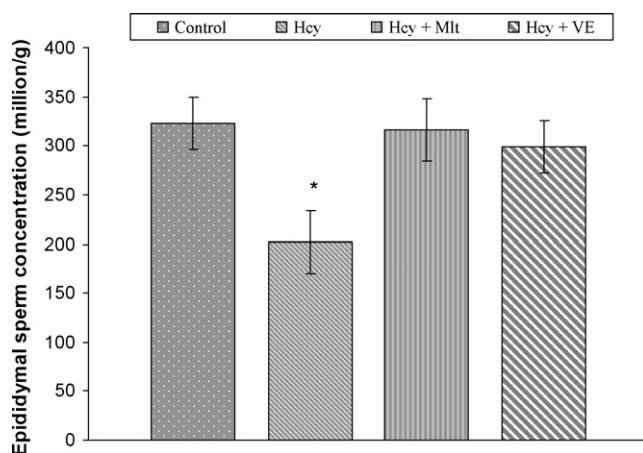


Fig. 1. Epididymal sperm concentrations in all groups (* $p < 0.05$; vs. control).

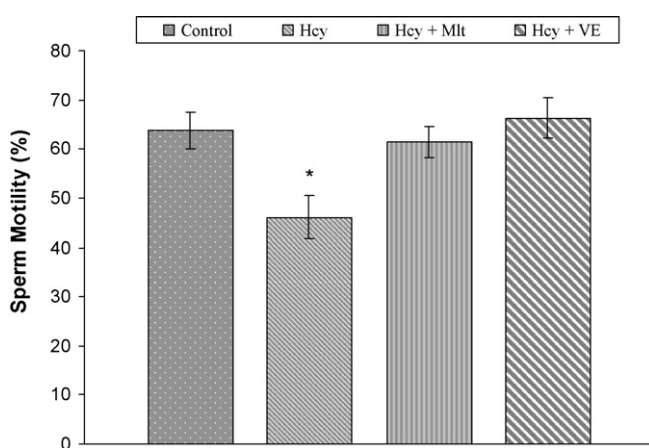


Fig. 2. Sperm motility in all groups (* $p < 0.05$; vs. control).

level significantly ($p < 0.05$) increased, the plasma SOD, GSH-Px, CAT activities and testosterone level significantly ($p < 0.05$) decreased in Hcy-treated rats when compared to control rats. On the other hand, the simultaneous administration of Mlt or VE to Hcy-treated rats significantly ($p < 0.05$) reduced the plasma MDA level and prevented the decrease in the plasma testosterone level and activities of antioxidant enzymes.

3.3. The epididymal sperm count and motility

The epididymal sperm count and the percentage of progressive sperm motility for all groups are presented in Figs. 1 and 2, respectively. The epididymal sperm count and motility were significantly ($p < 0.05$) lower in Hcy-treated animals than those of the control animals. However, the simultaneous administration of Mlt or VE to Hcy-treated animals significantly ($p < 0.05$) impeded the decrease in the epididymal sperm count and the percentage of progressive sperm motility.

4. Discussion

Hcy is an essential intermediate product in normal metabolism of methionine. The increase in plasma Hcy level

has several toxic effects although its exact mechanism of action is not fully understood [4]. Chen et al. [20] reported that the intraperitoneal infusion of Hcy is an effective method to increase plasma Hcy level in rats. The fasting homocysteine metabolism causes generation of ROS because the reactive thiol group of Hcy is readily oxidized in plasma [7]. In the present study, while the plasma MDA level significantly ($p < 0.05$) increased, the plasma SOD, GSH-Px, and CAT activities significantly ($p < 0.05$) decreased in Hcy-treated group when compared to control group. This is in agreement with the findings of Venture et al. [29] who demonstrated that the increase in plasma Hcy level caused the decrease in antioxidant enzyme activities. In addition, it was reported that Hcy promoted the formation of ROS [30,31] and, it caused inhibition of antioxidant enzymes such as SOD [32] and GSH-Px [33].

Mlt acts as a regulatory agent on Hcy metabolism. The administration of melatonin prevented the increase in plasma Hcy concentration which caused oxidative damage [34]. It was observed that while the simultaneous administration of Mlt to Hcy-treated rats significantly ($p < 0.05$) reduced the plasma MDA level, it increased in the plasma enzyme activities in the present study. This is confirmed by results of Baydas et al. [18] who reported that plasma MDA level significantly decreased in Mlt-treated animals, and administration of Mlt had a beneficial effect on decrease in plasma Hcy levels. On the other hand, Mlt has a very efficient neutralizer of the hydroxyl radical ($\cdot\text{OH}$), which arises from auto-oxidation of Hcy [35]. Reiter et al. [36] stated that Mlt stimulates GSH-Px activity which metabolizes reduced glutathione to its oxidized form and, this enzyme has an important role that it converts H_2O_2 to H_2O .

VE is the primary components of the antioxidant system. It is particularly important in protecting cells against oxidative damage by induced ROS [15]. The simultaneous administration of VE to Hcy-treated rats significantly ($p < 0.05$) reduced the plasma MDA level, and increased in the plasma enzyme activities in the present study. These results are supported by the findings of Can et al. [19] who reported that the administration of VE significantly decreased the serum Hcy levels by preventing the folate depletion. The folate is essential for the remethylation of Hcy to methionine and a low folate status is associated with high plasma level of Hcy [37]. The chronic administration of Hcy develops progressive folate deficiency and it causes an impairment of Hcy metabolism [38].

The plasma testosterone level was significantly lower in the Hcy-treated animals than those of the control animals in this study. This is agreement with finding of Papadopoulos et al. [39] who reported that the Hcy diminished directly testosterone production. Similarly, Llanos et al. [40] demonstrated that the high level of Hcy has a role inhibition of testosterone synthesis in rat Leydig cells. On the other hand, the hydrogen peroxide is generated during oxidation of the sulfhydryl groups of Hcy [41]. Hydrogen peroxide can directly act to reduce testosterone production in rat Leydig cells [42].

VE plays an ameliorative role against adverse effects of oxidative stress on Leydig cell steroidogenesis, but this increase in VE level does not change basal testosterone level in normal rats [43]. Similarly, Mlt has no direct effect on testosterone

production in Leydig cells [44]. In this study, the plasma testosterone level was increased to control levels by simultaneous administration of Mlt or VE to Hcy-treated animals. This result may explain that both Mlt and VE prevent the increase in plasma Hcy concentration, which causes inhibition of testosterone synthesis in Leydig cells. In addition, they protect normal Leydig cell function against cellular damage, which induced by ROS [45].

The auto-oxidation of Hcy leads to the formation of homocystine, homocysteine thiolactone and sulfhydryl group. Homocysteine thiolactone is a highly reactive Hcy derivative that can react easily with proteins. The increase in plasma level of homocysteine thiolactone blocks intracellular protein-carboxyl methylation reaction, which results in the inhibition of sperm motility [6,46]. Furthermore, ROS are generated during oxidation of sulfhydryl group of Hcy [7]. The cellular damage in the semen is the result of an improper balance between ROS generation and antioxidant enzyme activities. The reduction in the activities of antioxidant enzyme such as SOD, GSH-Px and CAT and the increase in the level of hydrogen peroxide cause failure of sperm function [47]. The sperm plasma membrane contains a high amount of unsaturated fatty acids, and so it is particularly susceptible to peroxidative damage. The lipid peroxidation destroys the structure of lipid matrix in the membranes of spermatozoa, and it is associated with loss of motility and impairment of spermatogenesis [9]. The percentage of progressive sperm motility significantly ($p < 0.05$) decreased in the Hcy-treated animals when compared to the control animals in the present study. This decrease may be explained with either inhibition of enzymatic methylation or peroxidative damage by ROS.

There is a significant positive correlation between total seminal plasma folate and sperm concentration [5]. Sauls et al. [38] found that the chronic administration of Hcy caused progressive folate deficiency. The epididymal sperm concentration significantly ($p < 0.05$) decreased in the Hcy-treated animals compared to the control animals in the present study. This reduction may be depending on chronic administration of Hcy induced low seminal plasma folate concentration and/or adverse effect of ROS on spermatogenesis.

Previous studies reported that the administration of Mlt [48] or VE [49] prevented ROS induced negative changes in sperm count and motility although they did not affect these sperm functions in normal rats. VE is the most effective antioxidant, which presents in the cell membranes; it is likely considered that it plays a major role in maintaining cell membrane integrity [14]. Mlt is also an effective antioxidant and, it protects mitochondrial function of sperm against oxidative damage [12,50]. Besides, both Mlt [18] and VE [19] administration decreases directly plasma Hcy level in rats. It was observed that the simultaneous administration of Mlt or VE to Hcy-treated rats impeded the reduction in the epididymal sperm concentration and motility in this study.

In conclusion, this study indicates that chronic administration of Hcy has the harmful effect on the epididymal sperm characteristics of male rats. The administration of Mlt or VE can prevent adverse effects of Hcy on the plasma antioxidant

enzyme activities, testosterone level, sperm count and motility in male rats.

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