

## Effects of dietary magnesium on testicular histology, steroidogenesis, spermatogenesis and oxidative stress markers in adult rats

Amar K Chandra<sup>a\*</sup>, Pallav Sengupta<sup>a</sup>, Haimanti Goswami<sup>a</sup> & Mahitosh Sarkar<sup>b</sup>

<sup>a</sup>Endocrinology & Reproductive Physiology Laboratory, Department of Physiology, University of Calcutta, Kolkata 700 009, India

<sup>b</sup>Department of Physiology, Gurunanak Institute of Dental Science & Research, Kolkata, India

*Received 12 April 2012; revised 19 October 2012*

The available information on the effect of excess dietary magnesium on male reproduction is inadequate, though consumption of hard water rich in magnesium salt is not uncommon in many geographical areas. The present study has thus been undertaken to evaluate the morphological as well as cytological and functional changes in testis of magnesium administered sexually mature male Wistar rats. Significant increase in the activities of androgenic enzymes viz.  $\Delta^5$  $\beta$ -hydroxysteroid dehydrogenase and  $17\beta$ -hydroxysteroid dehydrogenase with concomitant increase in serum testosterone level, followed by progressive development in cytoarchitecture of genital organs, without any significant alteration in quantitative spermatogenesis were observed. The results were more marked in the groups treated for longer duration. The results further suggests that the changes that occurred after excessive magnesium in testis were not for the enhanced adrenocortical activities or for the generation of oxidative stress in reproductive organs, but for the direct action of excess magnesium on male gonads. Magnesium supplementation thus has an apparent beneficial effect on male gonadal system.

**Keywords:** Antioxidant enzymes, Lipid peroxidation,  $MgSO_4$ , Oxidative stress, Sperm count, Steroidogenic enzymes, Testis

Role of various micronutrients in male reproductive health has been reported<sup>1</sup>. Magnesium (Mg) is one of such essential micronutrients, found in different foods (whole grain cereals, green vegetables, soy milk etc). Human body contains approximately 20-28 g of Mg, 60% of which is found in the bones and teeth, while the remaining 40% is found in muscle. It is the second most plentiful cation found within the cells of the body, signifying its importance in the multitudes of cellular functions. One of the most important metabolic processes, the synthesis and utilization of ATP is directly linked to Mg. Mg-linked ATP processes activate approximately 300 different enzymes which are involved in diverse functions such as DNA and RNA synthesis, glycolysis, intracellular mineral transport, nerve impulse generation, cell membrane electrical potential, muscle contraction, blood vessel tone, and regeneration of ATP<sup>2</sup>. Factors that increase the need for Mg due to limited intake or increased losses include excess consumption of phosphate (soft drinks) and alcoholic

beverages, diuretics, digitalis, strenuous exercise (high-performance athletes lose a considerable amount of Mg in sweat), pregnancy and lactation, diabetes, severe diarrhea, or kidney disease<sup>3</sup>. Excess Mg occurs mainly due to increased consumption of foods containing Mg and also drinking of hard water rich in magnesium salts<sup>4</sup>. Magnesium plays important role in carbohydrate metabolism and thus in the management of diabetes mellitus<sup>5</sup>, lung physiology and pathophysiology including asthma<sup>6</sup>. Magnesium deficiency impairs reproductive functions without any concomitant generation of oxidative stress<sup>7</sup> though its importance in maintaining balance between oxidants and antioxidants is well recognized<sup>8</sup>. The effect of excess magnesium on adult male gonadal physiology is almost not available. Therefore, the present study has been undertaken to investigate the action of excessive dietary magnesium for different durations on adult male reproductive histology, steroidogenesis, spermatogenesis in relation to the generation of oxidative stress and adrenocortical activities in rats.

### Materials and Methods

*Reagents*—Magnesium sulphate ( $MgSO_4$ ), thiobarbituric acid (TBA), NAD, NADPH, chloramines-T and -estradiol (98%) were procured

\*Correspondent author  
Telephone: +91 033 2498 2083;  
Fax: +91 033 235109755.  
E-mail: physiology.ac@gmail.com

from Sigma Chemical Company, St. Louis, M.O., USA. Potassium dichromate ( $K_2Cr_2O_7$ ), Triton X, triethanol amine, diethanolamine, EDTA and  $MnCl_2$  were from E-Merck, Mumbai, India. ELISA kit was obtained from Equipar Diagnostic, SRL, Italy (code no. 74010). All other reagents were procured from Sisco Research Laboratories (SRL), Mumbai, India and were of analytical grade.

**Animal treatment**—Sixty four healthy adult ( $90 \pm 10$  days) male albino rats (*Rattus norvegicus*) of Wistar strain weighing  $140 \pm 10$  g used in the present study were collected and maintained as per guidelines and protocols of Indian National Science Academy (INSA) and the proposal was approved by the Institutional Animal Ethics Committee (PHY/CU/IAEC/16/09 dated 15.05.2008). The animals were housed in clean polypropylene cages and maintained in an air-conditioned animal house at  $22 \pm 2$  °C and 40-60% RH, with constant 12:12 light:dark schedule. The animals were fed on standardized normal diet (20% protein) prepared in laboratory. The diet contained 70% wheat, 20% Bengal gram, 5% fish meal powder, 4% dry yeast powder, 0.75% refined til oil, 0.25% shark liver oil and 4% salt. Water was available *ad libitum*<sup>9</sup>. In the 13 and 26 days treatment respectively, the experimental animals were divided into following 8 groups (four groups for 13 days and four groups for 26 days), of 8 animals each: The first group (Gr. 1) was kept as control and fed with normal diet, Gr. 2 animals received 0.5 g  $MgSO_4/100$  g diet/day, Gr. 3 animals were treated with 1.0 g  $MgSO_4/100$  g diet/day. Animals in Gr. 4 were subjected to 1.5 g  $MgSO_4/100$  g diet/day treatment<sup>9</sup>. All animals were caged individually. Feed consumption, corrected for feed waste was measured. Treatment schedule was selected to determine the effect of Mg on one and two seminiferous cycles as duration of one seminiferous cycle is 13.2 days in albino rats<sup>10</sup>. All animals were sacrificed 24 h after the last treatment following protocols and ethical procedures by cervical dislocation. Blood samples for hormone assay were collected from the hepatic vein. Plasma samples were separated by centrifugation, frozen and stored at  $-50$  °C until assayed. The testis and accessory sex organs viz. ventral prostate and cauda epididymis, were dissected out, trimmed off the attached tissues and weighed. The left testis of each rat was fixed immediately for histological study and the right one for other biochemical estimations.

**Body and sex organ weights**—The body weights were recorded on the first day before treatment (initial) and on the day of sacrifice (final). The relative weight of organs was expressed per 100g body weight.

**Food consumption pattern**—Food consumption of per rat per day was recorded daily during the period of treatment. Food consumption (g/rat/day) was calculated, for each rat as,

$$\text{Food Consumption (g)} = \text{Food given (g)} - \text{Food wasted (g)}$$

**Histopathological study, sperm and germ cell count**—Immediately after removal, the testis, accessory sex organs and adrenal glands were fixed in formol fixative and testis was fixed in Bouin's fluid and embedded in paraffin. Sections (5  $\mu$ m thick) were taken from the mid portion of each organ and stained with hematoxylin-eosin. Each slide was examined under a light microscope. Seminiferous tubular diameter was measured by an ocular micrometer under light microscope. Quantitative analysis of spermatogenesis was carried out by counting the relative number of each variety of germ-cells at stage VII of the seminiferous epithelium cycle, i.e. type-A spermatogonia (ASg), preleptotene spermatocytes (pLSc), mid pachytene spermatocytes (mPSc) and step 7 spermatids (7Sd), following the method of Leblond and Clermont<sup>11</sup>. Theoretically the mPSc to 7Sd ratio should be 1:4<sup>12</sup>. The percentage of 7Sd degeneration was calculated from this ratio. Subtraction of the percentage of 7Sd degeneration in vehicle-treated rats showed the effective percentage of spermatid degeneration. Sperm samples were collected from the cauda epididymis and counted by a haemocytometer chamber under light microscope<sup>13</sup>. To minimize error, count was repeated at least five times for each rat.

**Determination of  $\Delta^5 3\beta$ -hydroxysteroid dehydrogenase activity**—To study testicular and adrenal  $\Delta^5 3\beta$ -hydroxysteroid dehydrogenase ( $\Delta^5 3\beta$ -HSD) enzyme activity<sup>14</sup>, tissues were homogenized, maintaining chilling conditions (4 °C) in 20% spectroscopic- grade glycerol containing 5mM of potassium phosphate and 1mM of EDTA at a tissue concentration of 100 mg/mL homogenizing mixture in a Potter-Elvehjem glass homogenizer. This mixture was centrifuged at 10,000 g for 30 min at 4 °C in a cold centrifuge (REMI, C40). Supernatant (200  $\mu$ L) was mixed with 1mL of 100  $\mu$ M sodium pyrophosphate buffer (pH 8.9) and 20  $\mu$ L of 30  $\mu$ g

17  $\beta$ -estradiol.  $\Delta^5$  3 $\beta$ -HSD activity was measured after the addition of 1 mL of 0.5  $\mu$ M nicotinamide adenine dinucleotide phosphate (NAD) to the cuvette in a UV spectrophotometer (UV-1240 Shimadzu, Japan) at 340 nm against a blank without NAD. One unit of enzyme activity is equivalent to a change in absorbance of 0.001/min at 340 nm.

*Determination of 17 $\beta$ -hydroxysteroid dehydrogenase activity*—For testicular 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$  HSD) enzyme assay<sup>15</sup>, the same supernatant fluid (200  $\mu$ L) (prepared as described above) was added with 1.5 mL of 440  $\mu$ M sodium pyrophosphate buffer (pH 8.9), 0.5 mL of bovine serum albumin (25 mg crystalline BSA) and 40  $\mu$ L of 0.3  $\mu$ M 17 $\beta$ -estradiol. 17 $\beta$ -HSD activities were measured after the addition of 1 mL of 1.35  $\mu$ M NAD to the cuvette in a UV spectrophotometer (UV-1240 Shimadzu, Japan) at 340 nm against a blank without NAD. One unit of enzyme activity is equivalent to a change in absorbance of 0.001/min at 340 nm.

*ELISA of serum testosterone*—Serum testosterone was assayed using ELISA kit obtained from Equipar Diagnostici, srl, Italy (code no. 74010). In this method serum sample (25  $\mu$ L) was taken in a micro-plate well and enzyme testosterone conjugate was added, then the reactant was mixed. After the completion of the required incubation period (60 min at 37 °C) the antibody bound enzyme testosterone conjugate was separated from the unbound enzyme testosterone conjugate by decantation. The activity of the enzyme present on the surface of the well is quantitated by the reaction with tetramethylbenzidine (TMB) substrate solution with 15 min incubation and finally by adding 0.3 M H<sub>2</sub>SO<sub>4</sub> as stop solution. The absorbance was read against blanking well at 450 nm within 30 min in ELISA Reader (Merck). The sensitivity of the testosterone assay was 5 pg/mL and inter and intra-run precision had a coefficient of variation of 3.9 and 6.2% respectively.

*Radioimmunoassay (RIA) of follicle stimulating hormone (FSH) and luteinizing hormone (LH)*—Serum levels of FSH and LH were assayed by RIA<sup>16</sup> using reagents supplied by Rat Pituitary Distribution and NIDDK (Bethesda, MD, USA). Carrier free <sup>125</sup>I for hormone iodination was obtained from Bhabha Atomic Research Center (BARC), Mumbai, India. Pure rat FSH (NIDDK-r FSH-I-11) and LH (NIDDK-r LH-I-11) were iodinated using chloramine-T as the oxidizing agent following the standard procedure<sup>17</sup>.

NIDDK anti-rat FSH-S-11 and anti-rat LH-S-11 in rabbit were used as antiserum at a final dilution of 1:35000 and 1: 70000, respectively. The second antibody was goat anti-rabbit  $\gamma$ -globulin purchased from Indo-Medicine (Friendswood, TX, USA). The intra-assay variation for FSH and LH was 5.0 and 4.5% respectively. All samples were run in one assay to avoid inter-assay variation.

*Spectrofluorometric determination of serum corticosterone*—Serum corticosterone level was determined by spectrofluorometry, according to the method of Glick *et al.*<sup>18</sup> modified by Silber<sup>19</sup>, and Biswas *et al.*<sup>20,21</sup>. The fluorescence was measured at 463 nm (excitation) and 518 nm (emission) by setting the instrument at a spectrofluorometric reading 80 with standard corticosterone (Sigma Chemical Company, St. Louis, MO, USA) solution having concentration 1.6  $\mu$ g/mL. A minimum 1.6  $\mu$ g of corticosterone per 100 mL serum can be measured by this method.

*Determination of lipid peroxidation*—Lipid Peroxidation (LPO) was measured following the method of Ohkawa *et al.*<sup>22</sup> using TBA-TCA-HCl reagent. Tissue (200 mg) was taken with 2 mL of phosphate buffer (50 mM, pH 7.4) solution and was homogenized at 4 °C. Following homogenization, 2 mL of homogenate was mixed thoroughly with 2 mL of TBA-TCA-HCl mixture and heated in a boiling water bath for about 15 to 20 min. After cooling the precipitate was removed by centrifugation at 10000 rpm for 10 min. Finally reading of the sample was measured against the blank at 532 nm.

*Protein estimation*—Proteins were estimated by the method of Lowry *et al.*<sup>23</sup> using bovine serum albumin (BSA) as the standard protein.

*Estimation of serum magnesium*—Serum magnesium was estimated as per Connerty *et al.*<sup>24</sup> and values were expressed in mM/L.

*Statistical analysis*—Results were expressed as mean $\pm$ SD. One-way analysis of variance (ANOVA) test was first carried out to test for any difference between the mean values of all groups. If differences between groups were established, the values of the treated groups were compared with those of the control group by a modified *t*-test. A value of  $P < 0.05$  was interpreted as statistically significant<sup>25</sup>.

## Results

Excessive dietary magnesium resulted in a substantial reduction of net body weight gain (Table 1) and no significant alterations were found in

reproductive organ weights, except prostate, over the control values in a dose- and time-dependent fashion though all the control and treated animals took food and water properly. No significant alteration was found in food consumption pattern (g/rat/day) in any of the cases of magnesium treatment (Table 1).

On magnesium treatment, there was a significant elevation in the activities of testicular  $\Delta^5_3$ -hydroxysteroid dehydrogenase (HSD) and 17 $\beta$ -hydroxysteroid dehydrogenase (HSD) over control values (Fig. 1A and B). Thus, in rats, magnesium treatment increased serum testosterone levels over the control values (Fig. 1C).

Histological section of testis from control rats revealed the presence of normal seminiferous tubules undergoing spermatogenic cycle composed of spermatogonia, spermatocytes, round spermatids and elongated spermatids consistent with general spermatogenesis. Similarly, testicular sections from treated rats also showed almost equal number of elongated spermatids as well post-spermatogonial germ cells. Testicular sections from rats treated with magnesium showed not significant, but increase in the number of spermatogonia A (ASg), preleptotene spermatocytes (pLSc), mid pachytene spermatocytes (mPSc) and step-7 spermatid (7Sd) over the control values. The percentage of spermatid degeneration is even less in magnesium treated rats than the respective control (Table 2).

In rats treated with high doses of magnesium, LH level was significantly increased than the corresponding value for the respective control rats, but serum FSH was showed no such alteration following treatment (Table 3).

No change in the activities of testicular lipid peroxidation (LPO) was observed. Both adrenal  $\Delta^5_3$ -hydroxysteroid dehydrogenase (HSD) activities as well serum corticosterone level were also found to be unaltered in treated rats than respective controls in dose- and time-dependant manner (Table 3).

Magnesium treated rats showed almost equal number of mature spermatozoa with normal features of boundary tissues. Histoarchitecture showed progressive improvement in sections (Fig. 2).

Cauda epididymis of control animals showed no pathologic changes (Fig. 3). Epididymal sperm count in magnesium treated rats also showed no such difference than control.

Histopathological analysis of ventral prostate showed progressive improvement in structure of secretory columnar epithelial cells lining ducts, with respect to controls (Fig. 4).

## Discussion

Supplementation of dietary magnesium found to decrease the net body weight gain at the highest dose as used in this study (1.5 g %) as compared to relevant control in time-dependent manner despite almost equal food intake as controls (Table 1).

Table 1—Effect of Mg on body weight (g), food consumption, testicular weight, ventral prostate weight, and weight of cauda epididymis and adrenal [Values are mean  $\pm$  SD, from 8 observations each. Figures in parenthesis are % increase over initial body weight]

Parameters	Control	13 days treatment			Control	26 days treatment		
		Mg (%)				Mg (%)		
		0.5	1.0	1.5		0.5	1.0	1.5
Body weight (Initial)	148 $\pm$ 5.5	147.1 $\pm$ 6.0	148.3 $\pm$ 4.5	145.0 $\pm$ 7.5	148.2 $\pm$ 4.94	142.1 $\pm$ 3.3	143.1 $\pm$ 5.4	147.6 $\pm$ 6.0
Body weight (Final)	173.5 $\pm$ 5.7 (+17.2%)	174.9 $\pm$ 6.0 (+18.9%)	170.9 $\pm$ 8.1 (+15.5%)	164.8 $\pm$ 10.8 (+13.7%) <sup>a</sup>	191.7 $\pm$ 6.88 (+29.3%)	183.3 $\pm$ 5.1 (+28.9%)	180.1 $\pm$ 9.3 (+25.8%) <sup>a</sup>	177.8 $\pm$ 8.4 (+20.4%) <sup>ab</sup>
Food consumption (g/rat/day)	24.5 $\pm$ 0.92	24.3 $\pm$ 2.04	25.2 $\pm$ 2.07	25.5 $\pm$ 2.52	25.2 $\pm$ 1.48	25.2 $\pm$ 1.50	25.8 $\pm$ 1.71	26.1 $\pm$ 1.41
Testicular weight (g/100g body weight)	1.14 $\pm$ 0.11	1.14 $\pm$ 0.06	1.14 $\pm$ 0.03	1.13 $\pm$ 0.09	1.19 $\pm$ 0.12	1.17 $\pm$ 0.03	1.17 $\pm$ 0.03	1.18 $\pm$ 0.02
Ventral prostate weight (mg/100g body weight)	228.23 $\pm$ 12.22	229.1 $\pm$ 5.22	230.1 $\pm$ 5.13	232.2 $\pm$ 6.42	230.15 $\pm$ 7.41	220.2 $\pm$ 6.51	238.5 $\pm$ 8.82 <sup>a</sup>	241.1 $\pm$ 5.91 <sup>ab</sup>
Cauda epididymis weight (mg/100g body weight)	190.81 $\pm$ 15.15	190.2 $\pm$ 4.71	189.93 $\pm$ 4.72	189.6 $\pm$ 3.63	190.95 $\pm$ 10.1	192.0 $\pm$ 3.81	192.3 $\pm$ 6.24	195.63 $\pm$ 7.05
Adrenal weight (mg/100g body weight)	13.05 $\pm$ 1.19	13.4 $\pm$ 1.20	13.8 $\pm$ 1.23	14.1 $\pm$ 0.45	14.02 $\pm$ 1.14	14.1 $\pm$ 0.66	14.3 $\pm$ 0.75	14.5 $\pm$ 1.32

*P* values <0.05. when compared with <sup>a</sup>control; <sup>b</sup>magnesium.

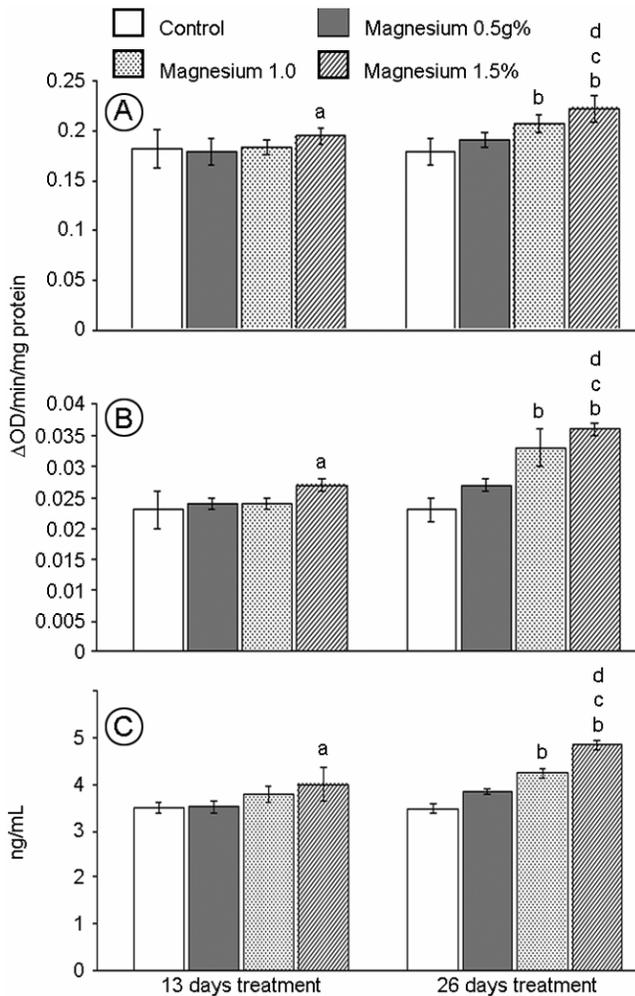


Fig. 1—Effect of magnesium of different doses (0.5, 1.0 and 1.5g%) for different durations (13 and 26 days treatment) on testicular  $\Delta^5$ -3 $\beta$ -HSD (A) and 17 $\beta$ -HSD (B) activities and serum testosterone level (C) of adult rats. [Values are mean  $\pm$  SD of 8 animals per group.  $P$  values  $< 0.05$ ; <sup>a</sup>control versus other groups in 13 days treatment; <sup>b</sup>control versus other groups in 26 days treated groups; <sup>c</sup>0.5g% 26 days group versus 1.5g% 26 days group; <sup>d</sup>1.5g% 13 days group versus 1.5g% 26 days group.]

The exact mechanism by which excess magnesium decreases body weight is not known; as it plays major role in glucose homeostasis<sup>5</sup>, therefore, its imbalance may cause alteration in body weight. There are reports that oral magnesium supplementations reduce blood glucose level and raise high density lipoprotein (HDL) level<sup>26</sup>, while other studies showed magnesium inhibits sodium-glucose co-transporter<sup>27</sup> and thus reduces cellular glucose uptake that may be the plausible basis of less body weight gain of rats supplemented with excess dietary magnesium.

However, no significant alteration in testicular weight was found after regular ingestion of

magnesium for 2-4 weeks. In histological examination of testicular sections no such changes were observed after magnesium treatment, even after its supplementation at higher doses. There is no as such evidence showing the effect of excess magnesium on testicular weight, but its deficiency was found to increase testicular weight<sup>28,29</sup>. Testicular weight is solely dependent on the number of germ cells present in it, if the number is altered, as in oxidative stress induced damage; there is concomitant decrease in testicular weight<sup>30</sup>; there was no significant alteration in the histological sections of testis and oxidative stress was not generated in this organ (Table 3). Furthermore, the serum testosterone level was increased in the excess  $Mg^{2+}$  treated groups. Therefore, excess  $Mg^{2+}$  enhances the testicular weight not only by preventing the generation of oxidative stress, but also enhancing the release of testosterone through LH, because, growth and maturation of testicular germ cells, number of spermatogonia and spermatocytes also corresponds with the serum level of testosterone<sup>31</sup>.

Testosterone is responsible for the growth, structural integrity and functional activities of accessory sex organs<sup>32,33</sup>. Increase in the mean ventral prostate weight as observed in the present study may correspond with the increase in serum testosterone concentration that influences the maturation of secretory columnar epithelial cells lining of the prostatic vacuoles. Drastic reduction of magnesium concentration may be associated with prostatitis and other disorders leading to male fertility<sup>34</sup>. The weight of the accessory sex organs were increased except cauda epididymis but its histological sections showed progressive improvement in cell lining as well as in the number of mature spermatozoa present within it.

The activity of two testicular key steroidogenic P-450 enzymes (viz.  $\Delta^5$  3 $\beta$  HSD and 17 $\beta$  HSD) were found to be significantly increased at moderate and high doses of  $Mg^{2+}$  that results in increased serum testosterone level in dose- and time-dependent manner.

FSH, LH and testosterone levels are the useful markers in the diagnosis and management of male infertility. Serum LH level was increased after supplementation of excessive dietary magnesium that is for the decreased corticosterone level that may regulate the HPG axis through the HPA axis. However, serum FSH level showed no alteration after magnesium treatment. FSH is required for the initial phase of spermatogenic restoration in adult

Table 2—Effect of magnesium on the relative number of germ cells per tubular cross section at stage VII of the seminiferous epithelial cycle [ASg = spermatogonia A; pLSc = preleptotene spermatocytes; mPSc = mid pachytene spermatocytes; 7Sd = step 7 spermatid] [Values are mean  $\pm$  SD, from 8 observations each]

Parameters		13 days treatment					26 days treatment			
		Control	Mg (%)			Control	Mg (%)			
			0.5	1.0	1.5		0.5	1.0	1.5	
Spermatogenesis pattern at stage VII	ASg	0.67 $\pm$ 0.04	0.67 $\pm$ 0.06	0.69 $\pm$ 0.05	0.69 $\pm$ 0.04	0.66 $\pm$ 0.04	0.66 $\pm$ 0.06	0.68 $\pm$ 0.06	0.69 $\pm$ 0.06	
	pLSc	18.42 $\pm$ 1.17	18.51 $\pm$ 1.08	18.6 $\pm$ 0.7	18.9 $\pm$ 1.17	18.3 $\pm$ 0.81	18.33 $\pm$ 1.41	18.69 $\pm$ 0.75	18.96 $\pm$ 1.17	
	mPSc	19.2 $\pm$ 1.44	19.4 $\pm$ 1.05	19.8 $\pm$ 1.14	19.9 $\pm$ 1.11	19.1 $\pm$ 0.9	19.24 $\pm$ 1.11	19.8 $\pm$ 1.2	19.9 $\pm$ 0.84	
	7Sd	64.62 $\pm$ 1.2	64.9 $\pm$ 1.1	67.1 $\pm$ 1.2	68.1 $\pm$ 1.38	64.0 $\pm$ 1.14	65.1 $\pm$ 1.17	67.6 $\pm$ 1.47	68.4 $\pm$ 1.14	
	mPSc:7Sd	1:3.36	1:3.34	1:3.38	1:3.40	1:3.35	1:3.37	1:3.40	1:3.42	
7Sd degeneration (%)	16.0	16.20	15.25	14.0	16.25	15.5	14.5	14.0		
Effective 7Sd degeneration	-	+0.20	-0.75	-2.00	-	-0.75	-1.75	-2.25		
Epididymal Sperm Count (million cells/cauda epididymis)	116.94 $\pm$ 5.80	116.55 $\pm$ 5.34	117.51 $\pm$ 5.72	117.84 $\pm$ 6.44	117.05 $\pm$ 5.94	118.24 $\pm$ 6.15	120.77 $\pm$ 4.79	122.79 $\pm$ 7.53		

*P* values <0.05. when compared with <sup>a</sup>control; <sup>b</sup>magnesium.

Table 3—Effect of Mg on serum LH, FSH, corticosterone, magnesium levels, testicular LPO and adrenal  $\Delta^5$  3 $\beta$  HSD activity [Values are mean  $\pm$  SD, from 8 observations each]

Parameters		13 days treatment					26 days treatment		
		Control	Mg (%)			Control	Mg (%)		
			0.5	1.0	1.5		0.5	1.0	1.5
Serum LH level (ng/mL)	4.65 $\pm$ 0.41	4.7 $\pm$ 0.38	4.7 $\pm$ 0.70	4.78 $\pm$ 0.42	4.60 $\pm$ 0.41	4.80 $\pm$ 0.48	4.80 $\pm$ 0.51	5.1 $\pm$ 0.55 <sup>a</sup>	
Serum FSH level (ng/mL)	18.69 $\pm$ 0.33	18.72 $\pm$ 1.07	18.81 $\pm$ 0.85	18.87 $\pm$ 2.03	18.70 $\pm$ 0.32	18.75 $\pm$ 1.99	18.63 $\pm$ 1.89	18.60 $\pm$ 1.96	
Serum magnesium level (mmol/L)	0.78 $\pm$ 0.02	0.78 $\pm$ 0.01	0.84 $\pm$ 0.02 <sup>a</sup>	0.96 $\pm$ 0.01 <sup>a</sup>	0.79 $\pm$ 0.02	0.81 $\pm$ 0.02	0.93 $\pm$ 0.01 <sup>ab</sup>	1.08 $\pm$ 0.01 <sup>ab</sup>	
Testicular LPO (nmol TBARS/g tissue)	3.96 $\pm$ 0.51	4.00 $\pm$ 0.31	4.71 $\pm$ 0.36	4.88 $\pm$ 0.38	4.12 $\pm$ 0.49	4.32 $\pm$ 0.25	5.01 $\pm$ 0.82	5.40 $\pm$ 1.36	
Adrenal $\Delta^5$ 3 $\beta$ HSD Activity ( $\Delta$ OD/mg protein/min)	0.189 $\pm$ 0.024	0.191 $\pm$ 0.023	0.193 $\pm$ 0.025	0.0193 $\pm$ 0.019	0.191 $\pm$ 0.027	0.189 $\pm$ 0.025	0.189 $\pm$ 0.019	0.196 $\pm$ 0.013	
Serum corticosterone level ( $\mu$ g/dl)	31.24 $\pm$ 1.08	30.43 $\pm$ 2.05	30.25 $\pm$ 2.12	29.10 $\pm$ 1.65	30.66 $\pm$ 2.07	30.36 $\pm$ 2.36	29.13 $\pm$ 2.13	28.26 $\pm$ 1.72	

*P* values <0.05. when compared with <sup>a</sup>control; <sup>b</sup>magnesium.

rats<sup>35</sup> and once FSH stimulation is completed and spermatogenesis is restored, this gonadotrophic hormone is no longer required; testosterone then maintains active spermatogenesis. In infertile men, chronic higher concentration of FSH is considered to be a reliable indicator of germinal cell damage, and

associated with azoospermia and severe oligospermia. Therefore, the initial level of FSH is a prime determinant for the activity of testicular key steroidogenic P-450 enzymes<sup>30</sup>, which is found to be slightly increased, followed by decrease after prolonged exposure.

The number of spermatogonia A (ASg), preleptotene spermatocytes (pLSc), mid pachytene spermatocytes (mPSc) and step 7 spermatid (7Sd) were found normal in excess magnesium treated animals. Theoretically, the mPSc: 7Sd is 1:4<sup>12</sup>. This ratio was 1:3.40 and 1:3.42 after Mg exposure at

1.5 g% for 13 and 26 days treatment respectively, as compared with control (1:3.36). The percentage of spermatid degeneration as calculated from above ratio was less, but insignificant, after Mg treatment. There have been reports of Mg deficiency induced morphological changes up to 40% of the spermatids<sup>29</sup>,

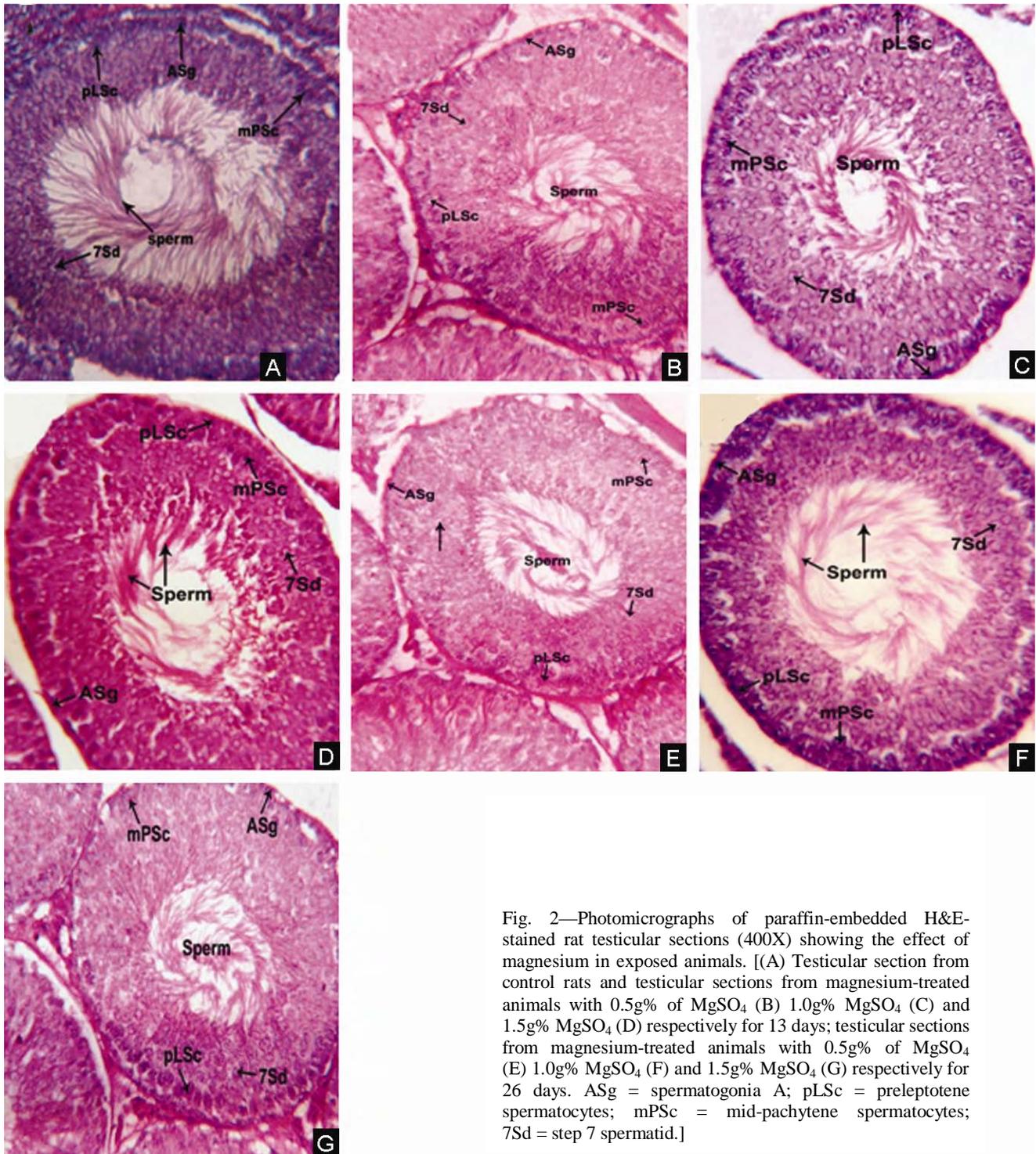


Fig. 2—Photomicrographs of paraffin-embedded H&E-stained rat testicular sections (400X) showing the effect of magnesium in exposed animals. [(A) Testicular section from control rats and testicular sections from magnesium-treated animals with 0.5g% of MgSO<sub>4</sub> (B) 1.0g% MgSO<sub>4</sub> (C) and 1.5g% MgSO<sub>4</sub> (D) respectively for 13 days; testicular sections from magnesium-treated animals with 0.5g% of MgSO<sub>4</sub> (E) 1.0g% MgSO<sub>4</sub> (F) and 1.5g% MgSO<sub>4</sub> (G) respectively for 26 days. ASg = spermatogonia A; pLSc = preleptotene spermatocytes; mPSc = mid-pachytene spermatocytes; 7Sd = step 7 spermatid.]

but its excess supplementation and possible impact on germ cell count is not evident. The epididymal sperm count also showed an increasing trend that is consistent with the increase in testicular weight; however, it is well known that testicular size or

weight does not determine the level of sperm production<sup>30</sup>. No alterations were also found in germ cells count in seminiferous epithelial cycle even in presence of profuse testosterone, as observed in the study.



Fig. 3—Photomicrographs of paraffin-embedded H&E-stained rat cauda epididymis sections (400X) showing the effect of magnesium in exposed animals. [(A) Epididymal section from control rats and epididymal sections from magnesium-treated animals with 0.5g% of MgSO<sub>4</sub> (B) 1.0g% MgSO<sub>4</sub> (C) and 1.5g% MgSO<sub>4</sub> (D) respectively for 13 days; epididymal sections from magnesium-treated animals with 0.5g% of MgSO<sub>4</sub> (E) 1.0g% MgSO<sub>4</sub> (F) and 1.5g% MgSO<sub>4</sub> (G) respectively for 26 days.]

While evaluating the role of magnesium in the induction of oxidative stress in testicular tissues, no such oxidation of lipid was observed as evidenced by testicular LPO level. Merker *et al.*<sup>29</sup> reported magnesium deficiency causes increased MDA as

an indicator of LPO whereas Suzer *et al.*<sup>36</sup> reported excess magnesium could be used as a neuroprotective agent on LPO.

Generated ROS act via HPA-axis and in chronic stress hypothalamus releases CRH from medial

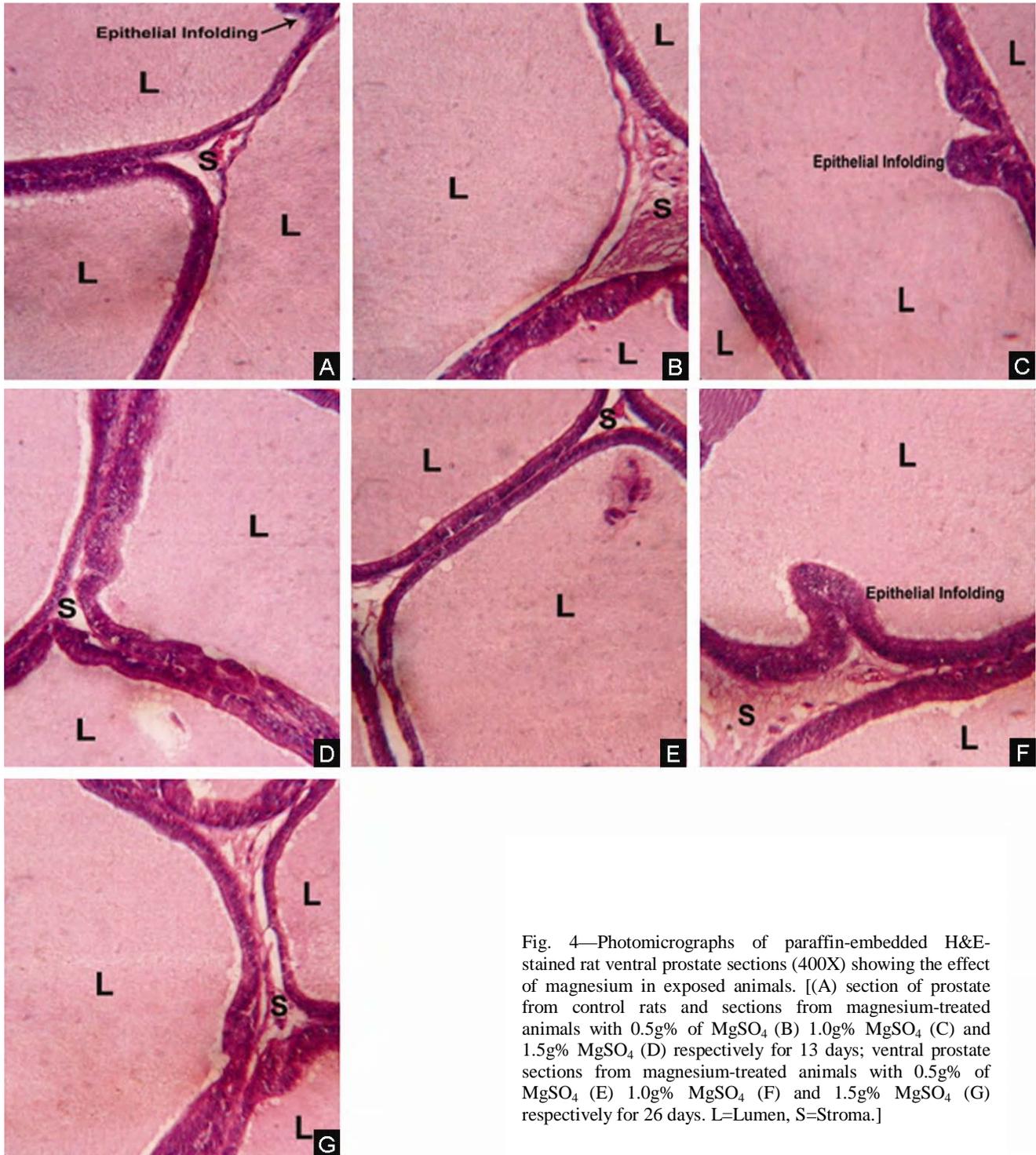


Fig. 4—Photomicrographs of paraffin-embedded H&E-stained rat ventral prostate sections (400X) showing the effect of magnesium in exposed animals. [(A) section of prostate from control rats and sections from magnesium-treated animals with 0.5g% of MgSO<sub>4</sub> (B) 1.0g% MgSO<sub>4</sub> (C) and 1.5g% MgSO<sub>4</sub> (D) respectively for 13 days; ventral prostate sections from magnesium-treated animals with 0.5g% of MgSO<sub>4</sub> (E) 1.0g% MgSO<sub>4</sub> (F) and 1.5g% MgSO<sub>4</sub> (G) respectively for 26 days. L=Lumen, S=Stroma.]

paraventricular nucleus causing increased secretion of ACTH from pituitary that in turn stimulates increased release of corticosterone which acts as a defense against stressful situations<sup>37</sup>. Caddell *et al.*<sup>38</sup> reported that in magnesium deficient rats plasma corticosterone level was increased. In the present study serum corticosterone level and adrenal  $\Delta^5 3\beta$  HSD activity were unaltered (Table 3) that has also been reflected in adrenal gland weight (Table 1), because, stress is always associated with the increase in adrenal weight<sup>39</sup>. All these findings are in line with the observations of Richer *et al.*<sup>40</sup> and Chandra *et al.*<sup>41</sup> who reported that magnesium at lower supplemented doses cause decreased plasma corticosterone level whereas larger magnesium supplement showed corticosterone levels are almost similar to those of the control rats. Based on their *in vitro* studies, Matthews and Saffran<sup>42</sup> observed that an increase in extracellular magnesium concentration markedly inhibits both basal and ACTH-stimulated corticosterone production.

In view of the findings of present investigation it is evident that excessive dietary magnesium shows an apparent beneficial effect on male reproductive system.

### Conclusion

The results demonstrate that excessive magnesium at the concentrations as used does not induce oxidative stress in the testis as evident in magnesium deficient condition. Conversely, it promotes overall testicular morphology and function (sex organ weight, enhanced activity of the androgenic P-450 enzymes, serum testosterone, and relative sperm count).

### Disclosure Statement

The authors declare that there are no conflicts of interest.

### Acknowledgement

Thanks are due to the Project of University Potential for Excellence (UPE), University of Calcutta under University Grants Commission (UGC), New Delhi, India for providing scholarship to Pallav Sengupta and to Dr. Syed N. Kabir, Scientist, Cell Biology & Physiology Division, CSIR-Indian Institute of Chemical Biology (CSIR - IICB), Kolkata, India for providing necessary facilities to assay serum LH and FSH in experimental animals.

### References

- Mathur PP & D'Cruz C, The effect of environmental contaminants on testicular functions, *Asian J Androl*, 13 (2011) 585.
- Durlach J, *New perspectives in magnesium research: Nutrition and health*, edited by Y Nishizawa, H Morii, J Durlach (Springer) 2007, 69.
- Al-Ghamdi SMG, Cameron EC & Sutton RAL, Magnesium deficiency: Pathophysiological and clinical overview, *Am J Kid Dis*, 24 (1994) 737.
- Chandra AK, Goswami H & Sengupta P, Dietary calcium induced cytological and biochemical changes in thyroid, *Environ Toxicol Pharmacol*, 34 (2012) 454.
- Paolisso G, Sgambato S, Gambardella A, Pizza G, Tesauro P, Varricchio M & D'onofrio F, Daily magnesium supplements improve glucose handling in elderly subjects, *Am J Clin Nutr*, 55 (1992) 1161.
- Bichara MD & Goldman RD, Magnesium for treatment of asthma in children, *Can Fam Physic*, 55 (2009) 887.
- Vernet P, Britan A, Gueux, Mazur A & Drevet JR, Dietary magnesium depletion does not promote oxidative stress but targets apical cells within the mouse caput epididymis, *Biochim Et Biophys Acta*, 1675 (2004) 32.
- Keenoy BM, Moorkens G, Vertommen J, Noe M, Neve J & Leeuw ID, Magnesium status and parameters of the oxidant-antioxidant balance in patients with chronic fatigue: effects of supplementation with magnesium, *J Am Coll Nutr*, 3 (2000) 374.
- Chandra AK, Tripathy S, Debnath A & Ghosh D, Bioavailability of iodine and hardness (Magnesium and Calcium Salt) in drinking water in the etiology of endemic goitre in Sundarban delta of West Bengal (India), *J Env Sc Engg*, 49 (2007) 139.
- Sarkar M, Roy Chaudhury G, Chattopadhyay A & Biswas NM, Effect of sodium arsenate on spermatogenesis, plasma gonadotrophins and testosterone in rats, *Asian J Androl*, 5 (2003) 27.
- Leblond PC & Clermont Y, Definition of the stages of the seminiferous epithelium in the rat, *Ann NY Acad Sci*, 55 (1952) 548.
- Clermont Y & Morgentaler H, Quantitative study of spermatogenesis in hypophysectomized rats, *Endocrinology*, 57 (1955) 369.
- Majumder GC & Biswas R, Evidence for the occurrence of ecto (adenosine triphosphatase) in rat epididymal spermatozoa, *Biochem J*, 183 (1979) 737.
- Talalay P, Hydroxysteroid dehydrogenases. *Methods Enzymol*, 5 (1962) 513.
- Jarabak J, Adams JA, Williams-Ashman HG & Talalay P, *J Biol Chem*, 237 (1962) 345.
- Moudgal NR & Madhawa Raj HG, Pituitary gonadotropin, in *Methods of hormone radioimmunoassay*, edited by BM Jaffe and, HR Berham (Academic Press, New York) 1974, 57.
- Greenwood FC, Hunter WM & Glover JS, The preparation of <sup>131</sup>I-labelled human growth hormone of high specific activity, *Biochem J*, 89 (1963) 114.
- Glick D, Recllich DV & Seyniour L, Fluorometric determination of corticosterone and cortisol in 0.02-0.05ml of plasma or submilligram samples of adrenal tissue, *Endocrinology*, 74 (1964) 653.
- Silber RH, in *Methods in biochemical analysis*, edited by D Glick, (Interscience Publishers, New York) 1966, 63.

- 20 Biswas NM, Chattopadhyay A, Sengupta R, Roychowdhury G & Sarkar M, Protection of adrenocortical activity by dietary casein in rats treated with estrogen, *Med Sc Res*, 27 (1999) 415.
- 21 Biswas NM, Roychowdhury G, Sarkar M & Sengupta R, Influence of adrenal cortex in testicular activity in the toad during the breeding season, *Life Sc*, 66 (2000) 1253.
- 22 Ohkawa H, Ohishi N & Yagi K, Assay for lipid peroxidases in animal tissues by thiobarbituric acid reaction, *Anal Biochem*, 95 (1989) 351.
- 23 Lowry OH, Rosebrough NJ, Farr AL & Randall RJ, Protein measurement from phenol reagent, *J Biol Chem*, 193 (1951) 265.
- 24 Connerty HV, Lau HSC & Briggs AR, Spectrophotometric determination of magnesium by use of methyl thymol blue, *Clin Chem*, 17 (1971) 629.
- 25 Fisher RA & Yates R, *Statistical tables for biological, agricultural and medical research*, (Longman Group, London) 1974.
- 26 Song Y, He K, Levitan EB, Manson JE & Liu S, Effects of oral magnesium supplementation on glycaemic control in Type 2 diabetes: A meta-analysis of randomized double-blind controlled trials, *Diab Med*, 23 (2005) 1050.
- 27 List JF, Woo V, Tang W & Fiedorek FT, Sodium-glucose co-transport inhibition with dapagliflozin in type 2 diabetes, *Diabetes Care*, 32 (2009) 650.
- 28 Zavaczki Z, Szollosi J, Kiss S, Koloszar S, Fejes I, Kovacs L & Pal A, Magnesium-orate supplementation for idiopathic infertile male patients: A randomized, placebo-controlled clinical pilot study, *Mag Res*, 16 (2003) 131.
- 29 Merker HJ, Günther T, Höllriegel V, Vormann J & Schümann K, Lipid peroxidation and morphology of rat testis in magnesium deficiency, *Andrologia*, 28 (1996) 43.
- 30 Chandra AK, Sengupta P, Goswami H & Sarkar M, Excessive dietary calcium in the disruption of structural and functional status of adult male reproductive system in rat with possible mechanism, *Mol Cell Biochem*, 364 (2012) 181.
- 31 Sharpe RM, Maddocks S, Millar M, Saunders PTK, Kerr JB & McKinnel C, Testosterone and spermatogenesis: identification of stage dependant, androgen regulated proteins secreted by adult rat seminiferous tubules, *J Androl*, 13 (1992) 172.
- 32 Chandra AK, Roy Choudhury S, De N & Sarkar M. Effect of green tea (*Camellia sinensis L.*) extract on morphological and functional changes in adult male gonads of albino rats, *Indian J Exp Biol*, 49 (2011) 689.
- 33 Steinberger E & Steinberger A, Hormonal control of testicular function in mammals, in *Handbook of physiology, Endocrinology*, Vol. IV, Part 2, edited by DW Hamiltol, and RO Greep., (Williams and Wilkins, Baltimore, MD) 1975, 325.
- 34 Edorh AP, Tachev K, Hadou T, Gbeassor M, Sanni A, Creppy EE, Le Faou A & Rihn BH, Magnesium content in seminal fluid as an indicator of chronic prostatitis, *Cell Mol Biol*, 49 (2003) 419.
- 35 Meachem SJ, Wreford NG, Stanton PG, Robertson DM & McLachlan RI, Follicle-stimulating hormone is required for the initial phase of spermatogenic restoration in adult rats following gonadotropin suppression, *J Androl*, 19 (1998) 725.
- 36 Suzer T, Coskun E, Islekel H & Tahta K. Neuroprotective effect of magnesium on lipid peroxidation and axonal function after experimental spinal cord injury, *Spinal Cord*, 37 (1999) 480.
- 37 Viau V, Functional cross talk between hypothalamic-pituitary-gonadal and adrenal axis, *J Neuroendocrinol*, 14 (2002) 506.
- 38 Caddell JL & Dunn JD, Plasma corticosterone in stressed magnesium-deficient weanling rats, *Mag Trace Elem*, 10 (1992) 40.
- 39 Pellegrini A, Grieco M, Materazzi G, Gesi M & Ricciardi MP, Stress-induced morphohistochemical and functional changes in rat adrenal cortex, testis and major salivary glands, *The Histochem J*, 30 (1998) 695.
- 40 Richer CL, Veilleux R & Bois P, Effect of magnesium deficiency on corticosterone in rats. *Endocrinology*, 82 (1968) 954.
- 41 Chandra AK, Sengupta P & Goswami H, *Excessive magnesium intake reduces testicular oxidative stress and promotes male reproductive function in rats*, paper presented in the Symposium on Man and Modern Day Stress, Physiological Society of India, Guntur, 21-23 December, 2011.
- 42 Matthews EK & Saffran M, Ionic dependence of adrenal steroidogenesis and ACTH induced changes in the membrane potential of adrenocortical cells, *J Physiol*, 234 (1973) 43.