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Studies on the Restoration of Testicular Function in Cadmium-exposed Rats by Vitamins C and E

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ABSTRACT: The study was carried out to evaluate the ability of vitamins C and E to restore testicular function in cadmium-exposed rats. Thirty male wistar rats (180-200g) randomly divided into 3 groups; group 1 served as control, group 2 received only cadmium while group 3 was treated with cadmium before a combination of vitamins C and E. The vitamins (100 mg kg^{-1} body wt vitamin C and 150 $mgkg^{-1}$ body wt vitamin E) were administered daily by gavage while $CdCl_2$ (5 $mgkg^{-1}$ body wt.) or its vehicle (normal saline) was administered subcutaneously on day 1 of study. The testis, epididymis, prostate and blood from 6 rats in each group were used for biochemical analysis. The remaining 4 males were exposed to females in the ratio of 1male: 2 females for 17 days, at the end of which the implantation sites in the females were examined for reproductive success. Cadmium significantly ($p<0.05$) increased catalase activity, malondialdehyde and cholesterol levels in the testes and significantly ($p<0.05$) reduced catalase activity and testosterone/luteinizing hormone ratio relative to the control. It also reduced the quantity and quality of sperm cells compared with the control. Vitamin treatment significantly ($p < 0.05$) reduced malondialdehyde and total cholesterol levels in testes and prostate relative to the cadmium only group. No successful fertilization was observed in all the cadmium treated rats compared with the control. This study shows that at the dose of cadmium used, vitamins C and E could not effectively restore testicular function in rats.

Keywords: Vitamins C and E, cadmium, testicular function, rats.

Introduction

The adverse effect of certain occupational exposures on reproduction has led to the exclusion of women of childbearing age from such environments. There has been an upsurge in studies into the contribution of occupational and environmental exposures to toxic metals, in declining sperm count and human male fertility (1).

Among the highly toxic agents in the environment is cadmium. Free radicals and lipid peroxidation are potentially important mediators in testicular physiology and pathology. Exposure to cadmium has been reported to induce the formation of reactive oxygen species (ROS) and consequently, oxidative stress (2).It is suggested that the mechanism involves the inhibition of SOD activity accompanied by an elevation in malondialdehyde (MDA) levels.The spermatozoa, being rich in polyunsaturated fatty acids (PUFA) and low in scavenging enzymes are particularly susceptible to ROS-induced damage (3).

Vitamin E, the most potent natural antioxidant readily donates its ring hydrogen from the hydroxyl group to free radicals, which then become unreactive. On donating the hydrogen atom, vitamin E itself, becomes a relatively unreactive free radical because the unpaired electron on the oxygen atom is usually delocalized into the aromatic ring structure thereby increasing its stability (4). After its reaction with free radicals, the reactive form of vitamin E is rapidly regenerated by vitamin C. Therefore, the consumption of foods rich in antioxidants, which are potentially able to quench or neutralize free radicals, may play an important role in the prevention of toxic effects due to cadmium. The aim of this study is to determine whether the dose of vitamins C and E used in this study, can restore testicular function in rats exposed to cadmium

Materials and Methods

Experiment (i)

A total of 30 male rats, randomly divided into 3 groups of 10 rats each were allowed a 2-week acclimatization period prior to the commencement of the experiment. All animals were allowed free access to commercial rat chow and water throughout the period of the study. Animals in group 1 served as control and were neither treated with cadmium nor vitamin. Group 2 received cadmium alone while group 3 was pretreated with cadmium before treatment with a combination of vitamins C and E. The vitamins (100 mg kg^{-1} body wt vitamin C and 150 $mgkg^{-1}$ body wt vitamin E) were administered daily by gavage (16 gauge 4″ curved. FN 16) while cadmium in the form of $CdCl_2$ (5 $mgkg^{-1}$ body wt.) or its vehicle (normal saline) was administered subcutaneously. The animals were weighed once every week and accordingly, the dose of $CdCl_2$ and vitamins were adjusted on weekly basis.

At the end of the 28 days of vitamin treatment, 6 rats from each group were sacrificed by chloroform anaesthesia and the blood, testes, prostate and epididymis removed and kept aside for biochemical and histological studies. Epididymal sperm cells were harvested and used for the study of the sperm population.

Experiment (ii)

The 4 male rats remaining from each group after the animal sacrifice in experiment (i) were housed in separate cages and exposed to females in the ratio 1 male: 2 females. Female partners were necropsied approximately 17 days later to determine the number and condition of implantation sites.

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Biochemical Analysis
Serum testosterone and Luteinizing hormone were estimated by Enzyme Immunoassay method. Superoxide dismutase (SOD) activity was estimated by the method of (5) while the method of (6) was adopted for the assay of Catalase (CAT). The concentration of malondialdehyde was assayed as thiobarbituric acid reactive substances (TBARS) in the testes. Malondialdehyde (MDA) was determined according to the method of Buege and Aust (7). Total lipid in the testes was extracted by the method of Jensen (8), while total cholesterol in the sample and serum were determined by the method of Richmond (9).

Sperm Count
The epididymis were dissected immediately from the testes after sacrifice and immersed in 1ml of phosphate buffered saline (pH 7.2) at 37^0C. The distal end of the epididymis was punctured in 20-30 locations with a 19-G needle, and the sperm were flushed out with a gentle stream of buffer. The suspension was filtered through an 80 μm nylon mesh. An aliquot from the suspension was taken in leukocyte hemocytometer and diluted with phosphate buffered saline up to the mark 11. The count was assisted with 0.2% formolsaline to immobilize the sperm cells. The suspension was well mixed and charged into Neubauer´s counting chamber. The total sperm in 5 squares of $1mm^2$ each was determined and multiplied by 10^6 to express the number of spermatozoa / ml.

Sperm Morphology test
For the evaluation of sperm morphology, the filtrate obtained was mixed with 1% papanicolaou (10:1). One drop of the stained sperm suspension was used to prepare the slide smear. Smears were air dried and analyzed under high power objective (magnification x 100). Two hundred sperms per animal were observed and classified into normal (long tail) and abnormal (short tail) types. Total abnormalities were expressed in percentage incidence / rat.

Analysis of sperm motility
Sperm were isolated from dissected epididymis at time of sacrifice according to the method of Huang *et a*l., (10), as described above. Following incubation at 37^0C for 10 minutes, a drop of the sperm suspension was placed on a prewarmed haemocytometer. Sperm motility was determined in triplet counts of 100 sperms each, noting the percentage of sperm moving. A sperm was considered motile if it did not remain at the same location for 5-10 second.

Determination of the cadmium content of Testis
The amount of cadmium in the testes and animal diet was determined by atomic absorption spectrophotometery (AAS) with a Varian AA 1475 series atomic absorption spectrophotometer.
One gramme of tissue (testes) or diet was digested with 20ml HNO_3 - $HClO_4$ mixture (4:1) in a Pyrex tube. The sample was heated at 100^oC in a sand bath until it was completely dissolved. Each digest was then made up to 100ml with distilled deionized water and the cadmium content read against a blank at 228.8nm.

Statistical Analysis
Statistical analysis was performed using One Way Analysis of Variance (ANOVA). The Turkey-Kramer Multiple Comparison Test was used to test the differences between means. Values were considered significant at $p < 0.05$

Results

A significant difference in the activities of antioxidant enzymes and malondialdehyde level were observed in the testes of all the cadmium exposedrats when compared with the control (Table 1). While catalase activity was significantly ($p<0.05$) elevated, SOD activity was significantly ($p<0.05$) reduced in the cadmium-treated rats when compared with the control. Treatment with the vitamins significantly ($p<0.05$) reduced SOD and increased catalase activity when compared with the control and the group that was treated with only cadmium.
In all the cadmium treated rats, testicular MDA was significantly ($p<0.05$) increased when compared with the control group (Table 1). Treatment with the vitamins after cadmium exposure attenuated the cadmium–induced increase in MDA level when compared with their level in the group that was treated with only cadmium.
The effect of cadmium and cadmium plus vitamin treatments on testes cholesterol level are presented in Table 1. Cadmium significantly ($p<0.05$) increased testicular total cholesterol level when compared with the control but daily administration of vitamins C and E after exposure to cadmium reduced total cholesterol to levels that were not statistically different from control. However, cadmium treatment alone, did not have any significant ($p>0.05$) effect on plasma and prostate total cholesterol levels. Treatment of cadmium-exposed rats with vitamins C and E significantly ($p<0.05$) reduced the total cholesterol to levels that were lower than the control.

Table 1: The Effect of Supplementation with Vitamins C and E on Cadmium-induced Changes in testicular superoxide dismutase (SOD), catalase (CAT) activities and malondialdehyde (MDA) and cholesterol levels in the testes, prostate and plasma of rats

Groups	Parameters					
	SOD (units / g tissue)	CAT (K / min) x 10^{-2}	MDA (units / g tissue)	Total Cholesterol (Testes) (mg/ g tissue)	Total Cholesterol (Prostate) (mg/ g tissue)	Total Cholesterol (Plasma) (mg / dl)
Control	108.11± 2.47	2.61 ± 0.37	4.61± 1.43	2.06 ± 0.19	4.52 ± 0.34	82.48 ± 9.75
Cadmium Only	86.10 ± 3.87*	26.37 ± 5.26*	24.13 ± 5.13*	10.52 ± 1.71*	4.08 ± 0.99	84.60 ± 10.98
Cd1V	36.13 ± 6.67***	31.60 ± 3.48*	10.85 ± 1.31***	4.47 ± 0.77**	1.77 ± 0.36***	87.97 ± 8.88

Values are mean ±SD (n = 5)
**Values on the same column differ significantly from control (p< 0.05).*
*** Values on the same column are significantly (p<0.05) different from the cadmium only group*
**** Values on the same column are significantly (p<0.05) different from both control and cadmium only groups*

Table 2 shows the effect of cadmium on serum levels of LH and testosterone. Results show that though cadmium decreased serum testosterone levels, this decrease was not significant when compared with the control. Treatment of cadmium exposed rats with vitamins C and E did not change this pattern. However, a significant ($p<0.05$) increase in luteinizing hormone level was observed, in rats that were treated with cadmium or cadmium plus vitamins C and E, when compared with the control. Results also show a significant ($p<0.05$) reduction in T/LH ratio of the cadmium treated groups of rats relative to the control.

The effect of cadmium and cadmium plus vitamins C and E treatments on the cadmium content of the testes, sperm count, morphology and motility in rats are presented in Table 2. In this study, long-tailed sperm cells were considered normal while short-tailed sperm cells represented abnormal or deformed sperm cells. Results show that treatment with cadmium alone or with vitamins C and E, significantly ($p<0.05$) reduced sperm count (Table 2), increased cellular debris and the number of deformed cell when compared with the control. Results also show a significant ($p<0.05$) elevation in the testicular cadmium level of all the cadmium-treated rats when compared with the untreated control. Daily treatment of rats with vitamins C and E did not reduce the accumulation of cadmium in the testes.

Table 2: The Effect of Supplementation with Vitamins C and E on Cadmium-induced Changes in Selected Androgenic Hormones and their Ratios as well as Sperm Morphology and Motility in Rats

Groups	Parameters								
				Sperm Morphology					
	Testosterone [(T) ng/ml]	Luteinizing Hormone [(LH) mIU/ml]	T/LH Ratio	Long Tailed (%)	Short Tailed (%)	Motility (%)	Cellular Debris (%)	Cadmium Content (µg/g tissue)	Sperm Count ($x10^6$ Spermatozoa/ml)
Control	0.88 ± 0.23	4.10 ± 0.78	0.24 ± 0.06	85	15	70	10	0.121 ±0.045	919.83 ± 10.02
Cadmium Only	0.83 ± 0.06	9.48 ± 0.81*	0.09 ± 0.03	02	98	0	60	0.349 ± 0.029*	123.50 ± 6.85*
Cd1V	0.83 ± 0.12	11.70 ± 0.85***	0.07 ± 0.01	08	92.3	0	70	0.369 ± 0.061*	122.99 ± 4.30 *

Values are mean ± SD (n = 5)
**Values on the same column differ significantly from control (p< 0.05).*
*** Values on the same column are significantly (p<0.05) different from the cadmium only group.*
**** Values on the same column are significantly (p<0.05) different from both control and cadmium only groups*

In experiment (ii), the cadmium-treated and untreated rats were tested for reproductive success. No successful implantation was observed in all the cadmium-treated groups relative to the control.

Table 3: The Effect of Cadmium- and Cadmium plus Vitamin Treatments on Fertility of Male Rats

Male Rats	Female rats	
Treatment Groups	Weight (g) Mean ± SD (6)	Implantation Sites
Control (No Cd; No Vit)	147.88 ± 16.81	10
Cadmium only	154.00 ± 21.27	0
Cd1 V	149.75 ± 15.52	0

Discussion

The restoration of testicular function in cadmium-exposed rats by vitamins C and E was investigated in this study.

Some of the toxic effects of cadmium are known to be mediated through the generation of Reactive Oxygen Species (ROS). The mechanism by which cadmium is able to do this is not well understood but it is generally believed that since the xenobiotic is not a redox active metal, it cannot by itself initiate the Fenton reactions (11). Waisberg *et al.*, (12) reported that cadmium induces oxidative stress through indirect processes. One of these mechanisms involves the inhibition of complex III of mitochondrial electron Transport Chain, thereby increasing the production of ROS intracellularly (13). Another mechanism by which cadmium increases reactive oxygen species involves the inhibition of antioxidant enzymes like superoxide dismutase (SOD). Cadmium can displace Zn from Cu-Zn SOD. This can lead to dysfunctional conformational changes in the enzyme, with a consequent loss of activity (14), as observed in the present study.

Gupta *et al.*, (15) reported an inverse relationship between tissue SOD activity and the level of Thiobarbituric acid reactive substances. In this study, cadmium administration caused a decrease in the activities of the antioxidant enzyme, SOD and an increase in malondialdehyde (MDA) level, which is indicative of lipid peroxidation. This result is in consonance with our earlier findings (2).There is a direct relationship between the degree of tissue damage and the level of MDA (16). Therefore the concentration of MDA can be used as an index of peroxidative injury and susceptibility of tissues to oxidative stress. Thus the increased MDA in the testes of cadmium treated rats in this study is an indication of increased membrane lipid peroxidation. MDA has been used in biochemical assays to monitor the degree of peroxidative damage sustained by spermatozoa (3). The results of such an assay exhibit an excellent correlation with the degree to which sperm function is impaired in terms of motility and the capacity for sperm-oocyte fusion (17).

Reactive oxygen species (ROS) have been associated with impaired fluidity of sperm plasma membrane, motility (18) and increased number of morphologically deformed sperm cells (19) and DNA damage (4) leading to the decline in sperm count as observed in this study. In addition, ROS-induced changes in oxidative metabolism at mitochondrial level carry an energy deficit, which affects the essential functions of the sperm cells (20). The result of most studies on laboratory animals and humans suggest that abnormally shaped spermatozoa may not reach the oviduct and / or participate in fertilization (21). This claim is supported by the report that

infertility correlates positively with increased tail abnormalities in sperm cells (22). Increase in ROS may therefore be one of the mechanisms by which cadmium reduces fertility in male rats.

This study also shows that testicular cholesterol level is significantly elevated in the testes of rats exposed to only cadmium. This is probably due to the secretion of cholesterol from the prostate into the seminal plasma (23) to protect the spermatozoa against environmental shock and /or decreased androgen production by the testes. Leydig cells in the presence of LH use cholesterol to produce testosterone. Since cholesterol is an intermediate in the synthesis of testosterone, its elevation in the testes will be indicative of decreased androgenesis. However, the present study shows that testosterone is not significantly reduced in the cadmium only group when compared with the control. Based on the available data, one is inclined to think that the excess cholesterol is not endogenous to the testes but must have been secreted into it by the surrounding glands, probably the prostate.

Treatment of rats with vitamins C and E after cadmium exposure effectively reduced testicular cholesterol to levels that were not statistically different from the cadmium-free control rats. This reduction in testicular cholesterol as a result of the vitamin treatments might not be unconnected with the α-tocopherol-induced inhibition of hydroxymethylglutaryl-CoA (HMG-CoA) reductase activity (24). The inhibition of HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis, has been reported by several workers (25; 26). This is supported by the report that cholesterol reduces cellular lathosterol concentration, an index of cholesterol synthesis (26). The results presented here also show that prostate cholesterol levels of the vitamin treated rats were significantly lower than all the other treatment groups, thus suggesting an inhibition of cholesterol synthesis in this gland. This is supported by the report that the vitamin E status of rats and rabbits can reduce their plasma cholesterol and total fat concentration (26).

Shimamoto and Sofikitis, (27) reported secretory dysfunction of stimulated leydig cells in a hypercholesterolemic environment.It is therefore possible, that the elevation of testicular cholesterol level observed in the testes of cadmium-exposed rats in the present study, will negatively affect leydig cell function.This issue of Leydig cell functionin infertility was addressed by comparing hormonal serum markers of Leydig cell function in cadmium-exposed male rats with those of non-exposed control rats. The hormonal serum markers measured include T, LH and the T/LH ratio. Using the method of Anderson *et al.,* (28) for the classification of infertile males, the hormonal data of the present study show reduced testosterone level, T/LH ratio and significantly elevated LH level. These, according to Anderson *et al.,* (28) are characteristic of a state of compensated leydig cell failure.

Leydig cells are the predominant interstitial cells involved in the production of testosterone for spermatogenesis through cytochrome P450 dependent reactions (29). Optimal leydig cell function and testosterone secretion are therefore, prerequisites for the normal activation of spermatogenesis (30).

Treatment with vitamins C and E after cadmium exposure did not change this effect of cadmium on leydig cell function. This result was supported by the fact that no successful fertilization was observed in the female partners of all the cadmium exposed male rats.

This study shows that cadmium causes infertility in male rats by increasing reactive oxygen species (ROS) and inducing a hypercholesterolemic environment in the testes, leading to the impairment of leydig cell function. It also shows that the damage caused by cadmium on the germinal components of the testes cannot be repaired by treatment with vitamins C and E post-cadmium exposure.

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