Arsenic-induced oxidative stress, apoptosis and alterations in testicular steroidogenesis and spermatogenesis in wistar rats: ameliorative effect of curcumin

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The present study was undertaken to explore the ameliorative potential of curcumin (CMN) against arsenic (As) induced reproductive effects in male rats. Administration of As (40 ppm in drinking water for 28 days) caused increase in lipid peroxidation and As content with concomitant reduction in glutathione and activities of catalase, superoxide dismutase and histopathological impairments in testes. As caused decrease in reproductive organs weight, sperm count, motility, testosterone level and increase in sperm abnormalities, sperm DNA damage with negative fertility index. As induce apoptosis by ROS generation, loss of mitochondrial transmembrane potential, upregulation of Bax, caspase-9 and -3 and repression of Bcl 2 mRNA level in mitochondrial pathways. As lowered 17β-hydroxysteroid dehydrogenase activity and caused down regulation of steroidogenic acute regulatory protein and Cytochrome P450 scc enzyme mRNA level. CMN co-treatment (100 mg/kg body weight orally for 28 days) was found to be effective in reversing arsenic-induced all these effects and could be able to antagonize reproductive toxicity.

Key words: Curcumin, Arsenic, Rats, Testes, Oxidative stress, Apoptosis, Steroidogenic enzymes.

INTRODUCTION

Arsenic is a wide spread environmental pollutant and is classified as a human carcinogen (NRC, 2001). It has become a major public health concern worldwide and as many as 70 million people in Indo–Bangladesh region alone are suffering from chronic arsenic poisoning through drinking water where its concentrations in groundwater range from 50 to 1200 μg/l, far above the current maximum permissible limit of 10 ppb has established by Environmental Protection Agency (USEPA, 2001). As is a major causative factor which impair male reproductive function like decreased spermatogenesis, testosterone release, inhibition of testicular enzyme function and reduction in the weight of the male sex organs in rodent models (Sarkar et al., 2003). The exact mechanism by which arsenic exerts its toxic effect is not clear. Indeed, some studies suggest that As III exert its toxicity by generating reactive oxygen species (ROS) and thereby oxidative stress (Chang et al., 2007). Oxidative stress occurs when the intracellular reactive oxygen species (ROS) levels are in excess of the cells scavenging capacity and caused sperm damage and apoptosis (Das et al., 2009). Excessive ROS production and depletion of reduced glutathione (GSH) caused dissipation of membrane permeability transport pore (PTP), resulted in loss of mitochondrial transmembrane potential (ΔΨm) and increased release of cytochrome c from mitochondria into the cytoplasm which is a potent stimulus for caspase-9 and caspase-3 activation in the intrinsic pathway (Green and Reed, 1998). Sperm DNA integrity is important for its fertilizing ability and resistant to aggressors because of its highly compacted structure after nuclear chromatin condensation but environmental toxicants caused sperm DNA damage which has been positively correlated with the reduced ability of spermatozoa to fertilize oocyte (Sakkas et al., 1998: Recio et al., 2001) . However, the exact mechanism(s) by which arsenic impairs male reproductive functions remains to be elucidated.
Plant products are known to exert their protective effects by scavenging free radicals and modulating antioxidant defense system. Curcumin (diferuloyl methane), an important constituent of turmeric (Curcuma longa L.) has been widely used for centuries as an indigenous medicine. Curcumin (CMN) has been shown to possess a broad spectrum of pharmacological activities including antineoplastic, antimutagenic, anti-inflammatory and antioxidant (Naik et al., 2004). CMN is a potent scavenger of a variety of reactive oxygen species including superoxide anion radicals, hydroxyl radicals and inhibit lipid peroxidation and effectively block thiol depletion (Chattopadhyay et al., 2005). Furthermore, CMN was also found to be effective in preventing methyglyoxal (MG)-induced oxidative DNA damage, cell injury, apoptosis, and generation of ROS in mononuclear cells (Chan and Wu, 2006). To determine whether CMN could attenuate As-induced apoptosis and oxidative stress in testicular tissue, this study was designed to examine the antagonistic actions of CMN on pathological and molecular biological abnormalities in rat testes, induced by As. The results of this study could clarify the role of the popular herbal drug CMN in prevention of As reproductive toxicity in India, Bangladesh and other parts of world where As contamination of drinking water supplies is a major public health problem.

MATERIALS AND METHODS

Chemicals

Sodium arsenite, curcumin, dehydroepiandrosterone, testosterone, bovine serum albumin, fetal calf serum (FCS), RPMI 1640, Dulbecco’s phosphate-buffered saline, 2', 7'-dichlorofluorescein diacetate (DCFH-DA) and 3, 3'-dihydroxyxacarbocyanine iodide (DiOC<sub>6</sub>) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Chloro-2, 4-dinitrobenzene, 5, 5'-dithiobis (2-nitrobenzoic acid), reduced glutathione, and thiobarbituric acid were purchased from Sisco Research Laboratory (Mumbai, India). The Annexin V-FITC apoptosis detection kit was purchased from Pharmingen (Becton Dickinson, San Diego, CA).

Animals and experimental design

Sixty male albino rats (Rattus norvegicus) of Wistar strain (12-14 weeks of age, 180-200 g) were procured from Laboratory Resource Section, Indian Veterinary Research Institute, Iznatnagar. Before the start of experiment, animals were kept in laboratory conditions for a period of 7 days for acclimatization. They were maintained under standard conditions of temperature at 24 ± 1°C and 55–60% relative humidity with “12-h dark-light cycle. They were provided access to standard rations (Feed Unit, IVRI) and water ad libitum. All animal experiments were performed according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (IAEC) of the Indian Veterinary Research Institute and Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment and Forests, Government of India (CPCSEA, 2003). The animals were randomly divided into five groups, comprising 12 rats in each but only 6 from each group cohabited with six female to assess mating and fertility indices in last five days of treatment remaining six used for measure biochemical parameters. Rats in Group I were received only distilled water daily for 28 days to serve as normal control. Group II were treated with 40 ppm As (69.6 mg of sodium arsenite dissolved in one liter of distilled water to obtain 40 ppm of As daily for 28 days). In Group III, rats were treated with CMN 99% pure (suspended in 1% gum acacia, 100 mg/kg body weight daily for 28 days).

Rats in Group IV were simultaneously treated with As and CMN in combination identically as in Groups I and II. In Group V, rats were treated with 1% gum acacia dissolved in distilled water, 1 ml/kg body weight for the duration of the treatment to serve as vehicle control. Rats of all the groups were injected orally over a period of 28 days. Dosage of As and CMN were selected as per available literature and previous study from our laboratory which is unpublished data (Shankaramurthy, 2007). 24 h after the last treatment, the animals were sacrificed by decapitation and testes, epididymis, seminal vesicles and prostate were collected. The tissues were blotted with tissue paper, weighed and kept at -20 °C for further analyses.

Preparation of testicular homogenate

500 mg of testes was homogenized in 5 ml of ice-cold 1.15% KCl-0.01 M sodium, potassium phosphate buffer (pH 7.4). Another 200 mg of testes was taken in 2 ml of 0.02 M EDTA for GSH estimation. The homogenates (10%) were prepared by using Polytron PT 1600E homogenizer (Switzerland) under cold conditions. The resultant homogenates were centrifuged at 12,000g for 30 min at 4°C. The supernatant was collected and used for the experiments within 24 hours.

Estimation of lipid peroxidation and protein content

The extent of lipid peroxidation in terms of thiobarbituric acid-reactive substances (TBARS) formation was measured (Esterbauer and Cheeseman, 1990). Tissue supernatant was mixed with 1 ml TCA (20%), 2 ml TBA (0.67%) and heated for 1 h at 100 °C. After cooling, the precipitate was removed by centrifugation. The supernatant was transferred to a quartz cuvette and the
absorbance was measured at 535 nm with a spectrophotometer (UV 5704SS, ECIL, India). As 99% TBARS are malondialdehyde (MDA), so TBARS concentrations of the samples were calculated using the extinction co-efficient of MDA, which is 1.56×10³ M⁻¹ cm⁻¹. For the expression of results of certain parameters, the protein content in testes homogenate was estimated using bovine serum albumin as the standard protein (Lowry et al., 1951).

**Epididymal arsenic estimation**

As content in epididymal samples was measured following wet digestion of the all experimental samples as per the method (Das et al., 1995). All epididymal samples were digested under a condenser in a mixture of 12.78 ml concentrated nitric acid and 2.25 ml 75% perchloric acid. When the solutions were cleared they were filtered, diluted to volume with Milli-Q purified water and filtered again. The concentration of arsenic in digested sample was measured at 193.7 nm wave length and 10 mA current using Atomoc Absorption Spectrophotometer (Electronic Corporation of India Limited-4141) equipped with arsenic lamp. Vapour generation accessory (VGA) was used to produce hydride vapour using 0.6% sodium borohydride and 10M HCl.

**Assessment of testicular antioxidative defense status**

Antioxidant status was assessed by evaluating both the nonenzymatic and enzymatic antioxidants in testes homogenate. Reduced glutathione (GSH) level was determined by estimating free-SH groups, using 5, 5'-dithiobis-2-nitrobenzoic acid (Prins and Loos, 1969). The enzymatic antioxidants evaluated were catalase superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione S-transferase (GST). Catalase activity was assayed by the method as described by (Aebi, 1984). The activity of SOD was estimated by the method (Madesh and Balasubramanian, 1998). GR activity was determined by the method (Goldberg and Spooner, 1983). The activities of GPx and GST were measured by the method described by (Paglia and Valentine, 1967) and (Habig et al., 1974), respectively.

**Testicular enzymes and testosterone assay**

The enzymatic activities of 3β-hydroxysteroid dehydrogenase (HSD) and 17β-HSD were analyzed by following the methods (Talalay, 1962) and (Jarabak and Adams, 1962). Testosterone is a key hormone that regulates spermatogenesis. The secretion of testosterone by the Leydig cells in testes is dependent upon the secretion of Luteinizing hormone by the pituitary gland. Since the testosterone level in experiment measured in testicular homogenate. Testosterone level in all experimental rats was determined in auto-gamma counter (Packard Bio Science Company Model CobraII, USA) using a commercial Radioimmunoassay (RIA) kits (Immunotech, France) following the instructions of the manufacturer.

In brief, testes tissues were homogenized in 250 μl potassium phosphate buffer (pH 8.9). After adding 1 ml of diethyl ether to the homogenate and vortexing for 2 min, the mixture was allowed to stand at room temperature (RT) for 10 min. After centrifugation at 5000 rpm for 15 min at 4 °C, the upper diethyl ether phase was carefully removed. This process was repeated another two times, after which the ether phases from the three extractions were combined, allowed to evaporate at room temperature in a fume hood, and resuspended in dextran-coated charcoal-treated calf serum. For testosterone, the intra-assay and inter-assay coefficients of variation were 7.5% and 11.5% respectively. Because the anti-testosterone antibody used in our assay has a high cross-reactivity with dihydrotestosterone, assay assays are referred to as testosterone + dihydrotestosterone levels (T + DHT).

**Sperm quantity and quality**

After sacrificing the animals, the cauda epididymis was removed and placed in a Petri-plate containing 2 ml of Hank’s balanced salt solution (HBSS) medium at room temperature. The epididymis was cut into small portions to allow the sperms to swim out. Ten μl of sperm suspension was taken and placed on hemocytometer. Sperm motility was assayed by the method (Freund and Carol, 1993). The motility of epididymal sperm was evaluated microscopically within 2–4 min of their isolation from the cauda epididymis and data were expressed as percentages of progressively motile spermatozoa. The spermatozoa were counted by haemocytometer using the improved Neubauer (Deep 1/10 mm, LABART,Germany) chamber. The total epididymal sperm per ml was calculated as follows. Total epididymal sperm /ml = (Average number of sperm per chamber) x 10³ x (Dilution Factor). For sperm abnormality and viability determination, a portion of the sperm suspension placed on a slide glass was smeared out with another slide and stained with Wells and Awa’s stain (0.2 g of eosin and 0.6 g of fast green dissolved in distilled water and ethanol in ratio of 2:1) for morphological examination and 1% eosin and 5% nigrosine in 3% sodium citrate dehydrate solution for determination of live/dead sperm were employed.

**Sperm chromatin structure assay (SCSA)**

SCSA was performed for assessment of sperm DNA integrity according to method described to detect the
susceptibility of sperm to acid denaturation of DNA in situ (Evenson and Melamed, 1983). Briefly, a 250 μL sperm suspension was treated with 750 μL an acid detergent solution (0.1% Triton X-100, 0.15 mol/l NaCl, and 0.08 mol/l HCl; pH 1.2) for 30 seconds and stained the DNA-specific fluorescent dye acridine orange 6 μg/l (Sigma, St. Louis, USA) in a phosphate-citrate buffer (pH 6.0) and subjected to flow cytometric analysis. Cells were analyzed in a Becton Dickinson-FACS Calibur Systmem (BD, San Jose, CA), equipped with an argon ion laser (488 nm), and calibrated for each session so that the mean green and red fluorescence of the reference sample corresponded to 450 and 125 channels, respectively.

Ten thousand cells were analyzed per sample at a cell flow rate of less than 200 cells/s. Data were acquired and analyzed using the SCSA Soft software (SCSA Diagnostics Inc., Brookings, SD). The extent of DNA denaturation/damage for each sperm was expressed as the percentage of cells with the DNA fragmentation index (%DFI), which is the metachromatic shift from green (native, double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence and displayed as red versus green fluorescence intensity cytogram patterns. The percentage of sperm showing more than 50% red fluorescence was considered as “cut-off” value to characterize an abnormal chromatin status, which can negatively affect the fertilization rate.

Mating and fertility indexes

After exposure period, treated and control rats were housed together with untreated proven fertile, with regular estrus cycle, females (6) for 5 days. Mating was confirmed by the presence of vaginal plugs or deposition of spermatozoan at the vaginal orifice upon vaginal examination. The day that a vaginal plug was found was considered day 0 of gestation and females that copulated successfully was necropsied to check for pregnancy on around Day 15 of gestation. Then fertility indexes was estimated and recorded.

Flow cytometry analysis

Apoptosis-associated changes were investigated by Flow cytometry. After exposure period testes were decapsulated under aseptic conditions and Leydig cells were isolated by collagenase (type I, Invitrogen, Grand Island, NY, USA) digestion and purified on discontinuous Percoll (Sigma, St. Louis, USA) gradient by the method previously described (Mondillo et al., 2009). The viability of Leydig cells was determined by Trypan blue dye exclusion method and that was 95%. The yield of Leydig cells per isolation was more than 5× 10⁶ cells plated in 24 well plates containing RPMI 1640 medium for Flow cytometry analysis. In all studies, cell debris (characterized by a low FSC/SSC) was excluded from analysis. All data were analyzed using Cell Quest software (provided by BD) and mean fluorescence intensity was obtained by histogram statistics.

Measurement of ROS generation

Oxidative stress induced by As was examined via measures of cell ROS generation using the method (Wang et al., 1996). Non-fluorescent 2, 7-dichlorodihydrofluorescein diace-tate (DCFH-DA) passively diffuses into the cells where it is cleaved by cellular esterases and the resulting diol is retained. With ROS generation, the diol is oxidized to fluorescent 2', 7'-dichloro-fluorescein (DCF) that is qualitatively detected by flow cytometry. Leydig cells in each well were collected into dedicated microcentrifuge tubes and pelleted at 200 x g for 10 min at 20°C. Cells were then washed with PBS and re-suspended in 950 μl PBS containing 25 mM 3-amino 2,3,4-triazole. DCFH-DA (50 μl, final concentration = 100 μM) was added to each tube and the cells kept in the dark for 15 min at 25°C. Flow cytometric analyses for DCF were performed and results reported as Counts vs. FL-1 (530 nm). The percentage (%) right shift in fluorescence peak was recorded. A minimum of 10,000 events/sample was acquired.

Mitochondrial transmembrane potential (MTP) assay

Loss in MTP was verified by flow cytometry at the single cell level. Loss in 3,3'-dihyxyloxacarbocyanine iodide (DiOC₆) staining indicated disruption of the mitochondrial inner transmembrane potential (Castedo et al., 2002). Cells were collected in microcentrifuge tube and pelleted at 200 x g for 10 min at 20°C. After washing with PBS, cells were incubated in 475 μl of PBS and 25 μl of 40 nM DiOC₆ for 15 min at 37°C in darkness. Loss in MTP (ΔΨm) was measured by changes in counts vs. FL-1 (fluorescence intensity at 530 nm) and the percentage (%) left shift in the florescence peak was recorded. A minimum of 10,000 events/sample was acquired.

Assessment of apoptotic and necrotic cells by Annexin V assay

An early indicator of apoptosis is a rapid translocation/accumulation of membrane phosphatidylserine from the cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected using Annexin V. Apoptotic and necrotic cell distribution here was analyzed by Annexin V binding and PI uptake. Dot-plot positioning of quadrants was performed and living cells (Annexin V/PI-), early
apoptotic/primary apoptotic cells (Annexin V+/PI), late apoptotic/secondary apoptotic cells (Annexin V+/PI*), and necrotic cells (Annexin V+/PI*) were distinguished (Vermes et al., 1995). From this, the total apoptotic proportion included the percentage of cells with fluorescence (i.e., Annexin V+/PI and Annexin V+/PI*).

Cells were harvested, placed in microcentrifuge tubes, pelleted, and then re-suspended in 100 μl binding buffer (1X). Cells then received 5 μl Annexin V-FITC and 5 μl PI and were incubated for 15 min at room temperature in the dark before 400 μl binding buffer (1X) was added. FITC and PI fluorescence were measured through FL-1 filter (530 nm) and FL-2 filter (585 nm) respectively, and 10,000 events were acquired.

mRNA extraction, cDNA synthesis and Real-time PCR

Total cellular RNA was isolated from the Leydig cells using TRI reagent (Sigma, St. Louis, MO, USA) according to the manufacturer’s instructions and quantified using ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and RNA samples with an A260/A280 of 1.9 or higher were employed for reverse transcription. Total RNA was dissolved in diethylpyrocarbonate (DEPC)-treated distilled water and stored at -20°C until complementary DNA (cDNA) synthesis.

The RT reaction was conducted in 20μl of reaction mixture at 37°C for 15 min, and terminated by heating at 85°C for 5 s followed by cooling at 4°C with 5 μg of RNA, in 5-reaction buffer (Fermentas, USA) containing 100 pmol random hexamer primer, 10 mM dNTP mixture (Fermentas, USA), 20 units of RNase inhibitor (Bioline, USA) and 200 units of moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Fermentas, USA). The published specific primer sequences for rat were used (Table 1). The singleplex real-time were performed in 96-well optical reaction plates using Stratagene Mx3000P real-time thermo cycler (Stratagene, La Jolla, CA, USA). Reactions were set up in a total volume of 20 μl using 5 μl of cDNA (diluted 1:4), 10 μl of 2x Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and 20 pmol of each gene-specific primer following manufacturer’s instructions for amplification.

The cycling program consists of 95°C for 10 s followed by 40 cycles of denaturation at 94°C for 30s, appropriate annealing temperature for 1min and extension at 72 °C for 2 min, final extension at 72 °C for 5min. Finally, the dissociation curves of PCR products were obtained by a following cycle of 60°C for 30 s and 95°C for 15 s, and reaction specificity was determined when there was only one specific peak in the dissociation curve. The real time PCR yields a value (Ct) having the threshold cycle of specific target gene amplification at which the PCR products were first detected via fluorescence. The Ct values were used to quantify the PCR product, i.e., the relative expression level of the target gene was expressed as Fold change, the 2^ΔΔCT method (Livak KJ, Schmittgen, 2001). The fold change in gene expression was calculated by, "Fold change = 2^ΔΔCT where ΔΔCT = (CT, target gene-CT, housekeeping gene: GAPDH) control - (CT, target gene-CT, GAPDH) treatment and mRNA amounts are expressed relative to those in control group arbitrarily set to the value of 1.

Histopathological studies

Small pieces (5 mm thickness) of testes and epididymis were fixed with Bouin’s solution for at least 24h. The fixed specimens were processed through the conventional paraffin embedding technique to obtain 5 μm thick sections (Culling, 1983). The sections were then stained with haematoxylin and eosin to evaluate the histopathological changes with microscopy.

Statistical analysis

The data were expressed as Mean ±SEM. Significant differences between the groups were analyzed by one-way analysis of variance supplemented with the Tukey’s multiple comparison post hoc test. All statistical analyses were performed by using SPSS 11.0 software (SPSS Inc, Chicago, IL, USA). P<0.05 was considered to be statistically significant.

RESULTS

Reproductive organ weights

The weights of testes, epididymis, prostate gland and seminal vesicle of in As-treated rats were significantly lower (p<0.05) as compared to control. However, there was no significant change in the weights of testes, epididymis, prostate gland and seminal vesicle of rats administered with AS and CMN combination as compared to control (Table 2).

LPO extent, GSH level in testes and arsenic deposition in epididymis

As exposure caused significant higher (p<0.05) in TBRs extents, As contents and decreased GSH levels than the control rat’s testes. In contrast, co-treatment with CMN significantly reduced (p<0.05) TBRS and As contents and significantly higher GSH levels to control levels in rats (Tables 3).

Activities of antioxidant enzymes in testes

The activities of SOD, CAT, GR, GPx and GST enzymes
in testes were decreased (p<0.05) significantly in As treated group as compared to control group. There was a significant increase (p<0.05) in activities of these enzymes in As plus CMN treated group as compared to As alone exposed animals. In CMN alone and in vehicle treated groups, there was no significant difference in the activities of antioxidant enzymes as compared to control animals presented in (Tables 4).

**Sperm indices**

Sperm count and motility in the cauda epididymis were significantly reduced (p<0.05) after treatment with As compared with control. However, co-administration of CMN with As reversed the decrease in sperm count and motility when compared with the As treated rats, and both parameters reached near to the values of control animals.

There was a significant increase (p<0.05) in the sperm abnormal morphology (head and tail) and decrease in viability of As treated rats, however, co-administration with CMN reduced the percentage abnormal morphology and enhanced viability in comparison with As treated rats, though the values remained higher to that of control shown in (Table 5).

**Sperm DNA integrity**

Arsenic caused significant increase (p<0.05) in cells with DNA damage with respect to the control. There was significant decrease (p<0.05) in DNA damage in As plus CMN treated group as compared to As alone exposed rats.

In CMN alone and in vehicle treated groups there was no significant difference in cells with DNA damage as compared to control animals (Figure 1).

**Mating and fertility indices**

Mating and fertility indexes were significantly decreased (p<0.05) in As intoxication as compared to control. Rats given CMN had fertility index of 100% while it was 66% in male rats given combination of CMN and As in (Table 6).

**Steroidogenic enzyme activities**

Treatment with As significantly reduced (p<0.05) the testicular 3β-HSD and 17β-HSD activities compared to the control rats. In contrast, 3β-HSD and 17β-HSD activities of the animals given CMN plus As were significantly increased (p<0.05) compared to As alone treated rats and were almost similar to that of control group (Figure 2).

**Testicular testosterone levels**

The levels of testosterone were significantly decreased (p<0.05) in the As-treated rats when compared with the control group. Co-treatment with CMN significantly elevated (p<0.05) the testicular testosterone levels compared to As alone treated rats but the levels were slightly lower than the values measured in control animals (Figure 3).

**ROS generation in Leydig cells**

There was a significant increase (p<0.05) in cells with ROS generation following exposure to As as compared to control. A significant decrease (p<0.05) in cells with ROS generation was seen in As plus CMN treated group as compared to arsenic alone exposed animals. In CMN alone and in vehicle treated groups there was no significant difference in cells with ROS generation as compared to control animals (Figure 4).

**Mitochondrial trans-membrane potential**

Flow cytometry results (Figure 5) revealed that there was a significant decrease (p<0.05) in the fluorescence in As-treated cells. In As-treated cells, the percentage of cells with loss of MTP was significantly increased (p<0.05) relative to control. Co-treatment with CMN resulted in significant decreases in cells that lost MTP. There was no significant change in MTP vs. control values in CMN alone and vehicle-treated cells.

**Phosphatidylserine externalization in apoptotic cells**

The results shown in (Figure 6) illustrate that the proportion of apoptotic cells was significantly increased (p<0.05) after the As treatment as compared to the control. CMN co-treatments caused significant decreases (p<0.05) in the number of apoptotic cells. Among CMN alone and vehicle-treated cells, no significant differences in apoptosis were noted as compared to the controls.

**Expression of genes responsible for cell cycle, apoptosis and steroid genesis**

We used real-time RT-PCR to quantify the fold change in expression of the testes specific mRNA transcripts levels of gene involved in apoptosis and steroid genesis namely Bax, Bcl-2, caspases-9, -3, StAR and CYP450scc were determined and compared with the control. The result indicated that As treatment caused upregulation of Bax, caspases-9, -3, and downregulation of StAR protein, CYP450scc enzyme and Bcl2 transcripts levels (Figure 7,
Table 1. Description of primers employed in analysis of real-time RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Annealing</th>
<th>Annealing</th>
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<tr>
<td>Bcl-2</td>
<td>sense: 5'-TGCACCTGACGCCCTTCAC-3'</td>
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<td></td>
<td>antisense: 5'-AGACAGCAGAGGAATCAAACAG-3'</td>
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<tr>
<td>Bax</td>
<td>sense: 5'-ACCAAGAAGCTGACAGTGTC-3'</td>
<td>55ºC</td>
<td>55ºC</td>
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<tr>
<td></td>
<td>antisense: 5'-ACAAAGATGGTCACGGTGCAC-3'</td>
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<td></td>
</tr>
<tr>
<td>Caspase 9</td>
<td>sense: 5'-AGCCAGATGCTGCATCCACAT-3'</td>
<td>65ºC</td>
<td>65ºC</td>
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<tr>
<td></td>
<td>antisense: 5'-CAGGAGACAAACCTGAGGAA-3'</td>
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<tr>
<td>Caspase 3</td>
<td>sense: 5'-AGTACGAGCTGACTCCGACAT-3'</td>
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<td>57ºC</td>
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<td></td>
<td>antisense: 5'-TGATTTCCCTTGACTTTGAGTCC-3'</td>
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<tr>
<td>StAR</td>
<td>sense: 5'-CTGCTAGACCGGCCATGACAG-3'</td>
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<td></td>
<td>antisense: 5'-TGATTTCCCTTGACTTTGAGTCC-3'</td>
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<tr>
<td>P450scc</td>
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<td></td>
<td>antisense: 5'-TGGAAGGAGCTGGTGACATG-3'</td>
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<td>GAPDH</td>
<td>sense: 5'-TTCAATTGACCTGAACTACAT-3'</td>
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<td></td>
<td>antisense: 5'-GAGGGGCCATCCAGTCTT-3'</td>
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Table 2. Mean ± SEM values of Absolute reproductive organ weights (As=Arsenic, CMN= Curcumin).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>Testis weight (g)</th>
<th>Epididymis weight (g)</th>
<th>Seminal vesicles weight (g)</th>
<th>Prostate weight (g)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>2.96 ± 5.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.522 ± 0.019&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.87 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.47 ± 0.071&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>2.81 ± 3.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.494 ± 0.007&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.91 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.45 ± 0.053&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>As</td>
<td>1.55 ± 1.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.226 ± 0.003&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.43 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27 ± 0.035&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>CMN</td>
<td>2.89 ± 5.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.463 ± 0.012&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.97 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.47 ± 0.064&lt;sup&gt;c&lt;/sup&gt;</td>
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</tr>
<tr>
<td>As + CMN</td>
<td>2.42 ± 4.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.359 ± 0.009&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.68 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.35 ± 0.042&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Values bearing different superscript in the same column vary significantly from each other p<0.05 (ANOVA) with the Tukey’s multiple comparison post hoc test.

Table 3. Mean ± SEM values of GSH, TBRS level testicular tissue homogenate and As content in epididymides (As=Arsenic, CMN= Curcumin)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>GSH (µmol/g tissue)</th>
<th>TBRS (mol/g tissue)</th>
<th>As content (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.21 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86.37 ± 5.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.91 ± 2.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>13.67 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90.58 ± 6.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.26 ± 2.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>As</td>
<td>4.67 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>155.33 ± 8.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78.84 ± 3.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>CMN</td>
<td>15.08 ± 0.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75.52 ± 4.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.86 ± 2.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>As + CMN</td>
<td>9.67 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>110.39 ± 7.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.53 ± 2.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Values bearing different superscript in the same row vary significantly from each other p<0.05(ANOVA) with the Tukey’s multiple comparison post hoc test.

8 and 9). Co-administration of CMN with As and CMN alone normalized their expression levels to controls.

Histopathological changes in testes

In control healthy rats, testicular sections had normal histoarchitecture that consisted of uniform, well-organized seminiferous tubules with complete spermatogenesis and normal interstitial connective tissue (Figure 10 A). In As alone exposed rats, the majority of the seminiferous tubules showed moderate to severe degenerative and necrotic changes with depletion of spermatogonia, primary spermatocyte and secondary spermatocyte cell layers. These changes were characterized by shrunken, disorganized seminiferous tubules with irregular, buckled basement membrane and devoid of spermatids and spermatozoa. Degenerated germinal epithelial cells were sloughed in the lumina of most seminiferous tubules. As intoxication caused decrease in the number of Leydig cells, edema in interstitial tissue and proliferation of connective tissue around the seminiferous tubules.
Table 4. Mean ± SEM values of activities of antioxidant enzymes in testes (As= Arsenic, CMN=Curcumin).

<table>
<thead>
<tr>
<th>Groups</th>
<th>GST</th>
<th>SOD</th>
<th>CAT</th>
<th>GR</th>
<th>GPx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>76.23± 2.67</td>
<td>162 ± 5.66a</td>
<td>212.67 ± 5.41a</td>
<td>266.33 ± 6.18a</td>
<td>87.85 ± 2.42a</td>
</tr>
<tr>
<td>Vehicle</td>
<td>71.20 ± 2.19a</td>
<td>159 ± 4.67a</td>
<td>205.34 ± 4.67a</td>
<td>251.76 ± 4.76a</td>
<td>82.29 ± 2.11a</td>
</tr>
<tr>
<td>As</td>
<td>18.67 ± 0.57c</td>
<td>46.14 ± 1.33c</td>
<td>62.33 ± 2.45c</td>
<td>88.34±2.08c</td>
<td>32.63 ± 1.31c</td>
</tr>
<tr>
<td>CMN</td>
<td>84.00 ± 3.15a</td>
<td>167.00 ± 6.89a</td>
<td>215.67 ± 5.29a</td>
<td>278.56 ± 5.49a</td>
<td>97.28 ± 2.84a</td>
</tr>
<tr>
<td>As + CMN</td>
<td>52.00 ± 1.75b</td>
<td>113.00 ± 3.89b</td>
<td>184.67 ± 4.40b</td>
<td>197.58 ± 4.41b</td>
<td>68.39 ± 2.01b</td>
</tr>
</tbody>
</table>

SOD= Superoxide dismutase (Units/mg protein)
GPx = Glutathione peroxidase (μmol NADH oxidized to NAD/min/mg protein)
GR = Glutathione reductase (μmol NADPH oxidized to NADP/min/mg protein)
CAT= Catalase (mmol H2O2 utilized/min/mg protein)
GST= Glutathione S-transferase (μM CDNB-GSH conjugate formed/min/mg protein)

Means values within a column not sharing a common superscript letter (a, b, and c) were significantly different, p < 0.05(ANOVA) with the Tukey’s multiple comparison post hoc test.

Table 5. Effect of As and/or CMN exposure following 28 days on different sperm related parameters of rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Vehicle</th>
<th>As</th>
<th>CMN</th>
<th>As + CMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>TESC</td>
<td>58.50 ± 0.76a</td>
<td>58.00 ± 0.86a</td>
<td>28.33 ± 0.71c</td>
<td>58.67 ± 0.95a</td>
<td>57.67 ± 3.29a</td>
</tr>
<tr>
<td>Live SC</td>
<td>94.50 ± 0.76a</td>
<td>94.17 ± 0.48a</td>
<td>58.50 ± 2.23c</td>
<td>95.33 ± 0.67a</td>
<td>81.83 ± 1.45a</td>
</tr>
<tr>
<td>Dead SC</td>
<td>5.50 ± 0.76a</td>
<td>5.83 ± 0.48a</td>
<td>41.50 ± 2.23c</td>
<td>4.67 ± 0.67a</td>
<td>18.17 ± 1.45a</td>
</tr>
<tr>
<td>Motility</td>
<td>94.33 ± 1.12a</td>
<td>93.33 ± 0.99a</td>
<td>41.00 ± 2.18a</td>
<td>96.17 ± 0.60a</td>
<td>79.67 ± 1.50a</td>
</tr>
<tr>
<td>HA</td>
<td>2.83 ± 0.31c</td>
<td>3.50 ± 0.43c</td>
<td>13.33 ± 1.80a</td>
<td>2.33 ± 0.42c</td>
<td>6.83 ± 0.65c</td>
</tr>
<tr>
<td>TA</td>
<td>2.67 ± 0.49c</td>
<td>4.50 ± 0.43c</td>
<td>12.67 ± 1.02c</td>
<td>1.67 ± 0.33c</td>
<td>6.33 ± 0.67c</td>
</tr>
</tbody>
</table>

TESC= Total Epididymal Sperm Count (10⁶), Live SC= Live Sperm Count (%), Dead SC= Dead Sperm Count (%), Motility= Sperm Motility (%), HA= Head Abnormality (%) and TA= Tail Abnormality (%).

Means values within a column not sharing a common superscript letter (a, b and c) were significantly different, p < 0.05(ANOVA) with the Tukey’s multiple comparison post hoc test.

Figure 1. Effect of CMN on As-induced % DNA fragmentation index. After exposure period rat sperms were isolated and stained with AO. Values are expressed as Mean ± SEM (n=6). Values bearing different superscript on the bar diagram vary significantly (p<0.05).
Table 6. Functional fertility parameters of male rats after oral administration of As and/or CMN for 28 days. (As= Arsenic, CMN=Curcumin)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Number of males that used for mating</td>
<td>6</td>
</tr>
<tr>
<td>Mating index (%)</td>
<td>6/6&lt;sup&gt;a&lt;/sup&gt; (100)</td>
</tr>
<tr>
<td>Fertility index (%)</td>
<td>6/6&lt;sup&gt;a&lt;/sup&gt; (100)</td>
</tr>
</tbody>
</table>

Mating index (%) = Number of males inseminated females/total number of males cohabited with females x100.
Fertility index (%) = Number of cohabited females becoming pregnant/number of non pregnant with evidence of vaginal plug x100. Means values within a column not sharing a common superscript letter (a, b and c) were significantly different, p < 0.05(ANOVA) with the Tukey's multiple comparison post hoc test.

Figure 2. 3β- and 17β-hydroxy steroid dehydrogenase activities in testicular tissue of rat. Values are expressed as Mean ± SEM (n=6). Values bearing different superscript on the bar diagram vary significantly (p<0.05).

(Figure10 C). There were almost normal architectural details of seminiferous tubules with apparently lining epithelium and spermatogonial cells in testes of As plus CMN treated group. A few numbers of seminiferous tubules had sloughed necrotic germinal epithelium in their lumina and mild interstitial edema and slight vacuolization of the germinal epithelial cells. There was marked improvement of spermatogenesis, evidenced by presence of elongated spermatids and spermatozoa in the majority of seminiferous tubules (Figure 10E).

DISCUSSION

Arsenic is a major causative factor to impair male reproductive function like decreased spermatogenesis, testosterone release, inhibition of testicular enzyme function and reduction in the weight of the male sex organs in rats and mice (Sarkar et al., 2003). In the present study, various aspects of male reproductive function, oxidative stress and apoptosis markers were examined and ultimately, the protective role of curcumin on arsenic-induced male reproductive toxicity was assessed.

In the present study, exposure to arsenic at 40 ppm concentration in drinking water for 28 days caused a reduction in the absolute weights of the testes, epididymis, prostate and seminal vesicles in rats. The weight of the testes is largely dependent on the mass of the differentiated spermatogenic cells, and the reduction in the testes weight may be due to the reduced tubule size, spermatogenic arrest and inhibition of steroid biosynthesis of Leydig cells (Chiou et al., 2008). The decrease in weight of epididymis, prostate and seminal
vesicles as observed in our study may be due to ROS induced cell damage resulting in impairment of steriodogenesis leading to low androgen production.

A decrease in sperm count and motility and increase in abnormal sperms following arsenic exposure as observed in the present study may be due to its adverse effect on spermatogenesis. Mammalian sperms contain large amount of thiol rich protamines in their nuclear chromatin and sulphhydryl groups in the flagellum which are thought to be involved in their stability and in the maintenance of motility (Working et al., 1985). As arsenic is known to be a thiol inhibiting substance, the decrease in motility could be ascribed to the presence of high arsenic concentration in the epididymis where sperms undergo the process of maturation and acquire motility.

Recent standard parameters for the assessment of sperm function, such as concentration, motility and morphology are sensitive biological markers of exposure to toxicants, but they cannot predict alterations in sperm genetic integrity. Sperm chromatin/DNA integrity is essential for fertilizing capacity of the sperm cell, and abnormalities in sperm chromatin structure may affect its fertilizing ability (Virant-Klun et al., 2002). In the later stages of spermatogenesis in mammals, chromatin structure is reorganised and nucleosomal histones are replaced by protamines. Protamine 1 is present in the sperm nuclei of all mammals, while protamine 2 (P2) has been found only in a few species, but these include humans and mice. P2 is a zinc (Zn) finger protein important in chromatin organization (Cho et al., 2003).

Sperm DNA is normally resistant to aggressors due to its highly compacted structure. During spermatozoa maturation in the epididymis, chromatin stabilisation increases through the formation of disulphide bonds between cystein thiol residues of protamine, leading to a more rigid sperm nucleus, and concomitant reduction in cell size. As may have high affinity for P2, as well as for other Zinc-binding proteins; thus, as a consequence of As binding to P2, a decrease in P2 interaction with DNA may occur, potentially interfering with chromatin condensation (Ahmadi et al., 1999). In this study sperm DNA damage in sperm suspension as evident by SCSA and deviation in mating and fertility indices of arsenic-treated rats were observed.

Because of high concentration of polyunsaturated fatty acids and low antioxidant capacity, male germ cells could be susceptible to oxidative stress (Vernet et al., 2004). In our study, As treatment significantly increased arsenic content in epididymis and lipid peroxidation in rat testes with concomitant decrease in intracellular antioxidants and antioxidant enzyme activities. As is also known to produce oxidative damage by disturbing the prooxidant–antioxidant balance as it has very high affinity for sulphydryl groups of GSH, which have role in the maintenance of thiol-disulphide balance (Yamanaka et al., 1991). Intracellular antioxidant enzymes (SOD, CAT, GR, GPx and GST) are considered to be the first line of cellular defense that prevent oxidative damage to biological macromolecules like DNA, proteins, etc. SOD has an antitoxic effect against the superoxide anion. SOD accelerates the dismutation of superoxide to $\text{H}_2\text{O}_2$ which is removed by catalase (Usoh et al., 2005). In the present
study, the reduction in SOD activity in arsenic-treated rats might lead to the accumulation of superoxide radicals in the testes. In addition, arsenic intoxication also reduced the testicular CAT activity. The significant decrease in SOD and CAT activities in testes of rats treated with sodium arsenite is in agreement with the previous findings (Lenartowicz, 1990). Our results also demonstrated that arsenic exposure caused reduction in the activities of glutathione-dependent enzymes GR, GPx, and GST with decrease in GSH level. To gain insight into understanding the molecular mechanism involved in arsenic-induced apoptosis, mRNA expression of the pro-apoptotic Bax, anti-apoptotic protein Bcl2, caspase-9 and caspase-3 were assessed in Leydig cells. During As-induced apoptosis, mitochondrial events, which include excessive generation of ROS, collapse of mitochondrial transmembrane potential (MTP) and upregulation of Bax and downregulation of Bcl2 mRNA expression, resulted in release of mitochondrial cytochrome c leads to activation of caspase-9 and caspase-3 of mitochondrial pathway, which again confirmed by the increased mRNA levels of caspase-9 and caspase-3. The activation of caspase -9 in arsenic exposed rat strongly suggests an involvement of a

**Figure 4.** Representative histograms of flow cytometry depicting rat Leydig cells (%) showing ROS generation after staining with DCFDA.
mitochondrial pathway. During apoptosis mitochondrial proteins (Bax, Bcl 2), located in the intermembrane space are released into the cytosol where they activate the caspase -9 of mitochondrial pathway as observed in this study which are consistent with the earlier findings in rats (Gupta et al., 2003).

ROS such as superoxide anions and H$_2$O$_2$ play important roles in endocrine disruption by environmental compounds and also known to inhibit testicular steroidogenesis (Omura and Morohashi, 1995). In the present study, As treatment resulted in increased levels of mitochondrial ROS and the inhibition of steroidogenesis in testes. As exposure caused decrease in the activities of 3β-HSD, 17β-HSD and reduced testosterone (T) levels in the rat testes, are in agreement with previous study (Chang et al., 2007). STAR is essential in T synthesis and responsible for the transport of cholesterol into mitochondria of Leydig cells. P450scc convertes cholesterol to pregnenolone in the inner mitochondrial membrane of Leydig cells, the first enzymatic step in T biosynthesis (Miller, 2007). Our results revealed that As exposure decreased mRNA
Figure 6. Representative histograms of flow cytometry depicting rat Leydig cells (%) showing (phosphatidylserine externalization) apoptosis after staining with Annexin-V FITC/PI. A : Control; B : Vehicle; C : As; D: CMN; E : As + CMN.

expression of StAR protein and P450scc enzymes in Leydig cells.

CMN co-treatment of rats resulted in amelioration of arsenic-induced alterations in oxidative stress-related parameters, sperm functions, testicular enzyme activities, mRNA expression of 17β-HSD and CYP450scc and testosterone levels in testes. Histopathological changes caused by arsenic in testes and epididymus were also reversed by curcumin co-treatment. CMN exhibits protective effects against oxidative damage by decreasing the levels of free radicals, through its free radical scavenging activity, particularly against oxygen radicals, which inhibit SH-group oxidation (Manikandana et al., 2004). In earlier studies, CMN has been demonstrated to exert protective effect against adverse effects induced by arsenic in different rat organs (El-Demerdash et al., 2009) and aflatoxin-induced decrease in 3β- HSD and 17β-HSD activities and histopathological changes in mouse testes (Verma and Mathuria, 2009).

In conclusion, our results strongly suggest that As-induced male reproductive toxicity can be mitigated by co-treatment with CMN. The beneficial effects of CMN
Figure 7. mRNA transcripts level of Bax and Bcl2 relative to control in rat Leydig cells. Values are expressed as Mean ± SEM (n=6). Values bearing different superscript on the bar diagram vary significantly (p<0.05).

Figure 8. mRNA transcripts level of Caspase-3 and Caspase-9 relative to control in rat Leydig cells. Values are expressed as Mean ± SEM (n=6). Values bearing different superscript on the bar diagram vary significantly (p<0.05).

Figure 1. mRNA transcripts level of StAR and CYP450scc relative to control in rat Leydig cells. Values are expressed as Mean ± SEM (n=6). Values bearing different superscript on the bar diagram vary significantly (p<0.05).
A: Testes showing normal structure of seminiferous tubules in “control” of male rats.
B: Normal movement of spermatozoa in seminiferous tubules with complete spermatogenesis and normal interstitial connective tissue in “vehicle” rat testes.
C: Testes of male rats intoxicated with “As” alone showing complete testicular necrosis and sloughing of all layers.
D: Swirling movement of spermatozoa in seminiferous tubules with complete spermatogenesis and normal interstitial connective tissue in “CMN” rat testes.
E: Marked improvement of spermatogenesis, evidenced by presence of spermatids and spermatozoa in the majority of seminiferous tubules of rats treated with “As + CMN”.

**Figure 10.**
could be attributed to its ROS scavenging and antioxidant activities.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

REFERENCES


Abbreviations
As, arsenic; CMN, Curcumin; ROS, reactive oxygen species, MTP, mitochondrial transmembrane potential potential; DiOC₃, 3,3'-dihexyloxacarbocyanine iodide; DCFH-DA, 2',7'-dichlorofluorescein; AO, Acridine Orange; LPO, lipid peroxidation; TBARS, thiobarbituric acid-reactive substances; GSH, Reduced glutathione; CAT, Catalase; SOD, Superoxide dismutase; GR, Glutathione reductase; GPx, Glutathione peroxidase; GST, Glutathione S-transferase; transpeptidase; 17β -HSD, 17β-hydroxysteroid dehydrogenase; StAR, steroidogenic acute regulatory protein; CYP450scc, Cytochrome P450 side chain cleavage.