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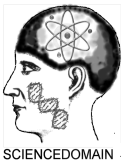

Effect of Arsenic (As) on the Spermatogenesis of Black Bengal Goat (*Capra hircus*) Reared at the Arsenic Prone Area of Mymensingh District in Bangladesh

M. A. Awal[1*], J. Alam[1], M. M. Hossain[2] and M. Matsumoto[3]

[1]Department of Anatomy and Histology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.
[2]Department of Anatomy, Histology and Physiology, Faculty of Animal Science and Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh.
[3]Department of Veterinary Anatomy, Joint Faculty of Veterinary Medicine, Kagoshima University, 21-24-1, Korimoto-1, Kagoshima-890-0055, Japan.

Authors' contributions

This work was carried out in collaboration between all authors. Author MAA planed and designed the study and wrote the protocol. Authors MAA and JA conducted the total research work with the financial assistance of Ministry of Science & Technology, the People's Republic of Bangladesh. *Author MMH wrote the first draft of the manuscript and performed the statistical analysis while author MM revised the final text.*

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ABSTRACT

Aim: Arsenic (As) alters the spermatogenic process as well as testicular histology has been reported in different species of domestic, wild, aquatic life, and laboratory animals. The present study was conducted to investigate the impact of toxicity of arsenic poison on the spermatogenesis of the Black Bengal goat at the most arsenic polluted area of Mymensingh district in Bangladesh by using histopathological techniques.
Methods: A total of 12 adult Black Bengal male goats (*Capra hircus*) were used in this study.

**Corresponding author: Email: professor_awal@yahoo.com;*

Among these, 6 were selected from the arsenic polluted area, and the rest were from the less contaminated area as control. Goats were sacrificed by using conventional animal killing method adopted in the laboratory. Both the right and left testes were collected aseptically. Testicular tissues were cut perpendicular to the long axis of the testis and preserved in Bouin's solution. Paraffin block was made and tissue sections were cut at 5-µm in thickness. Tissues were processed for routine hematoxylin and eosin and Periodic Acid-Schiff (PAS)-hematoxylin stains. Thickness of tunica albugenia, spermatigenic cell layer, diameters of the seminiferous tubules, number of spermatogenic, sertoli, and leydig cells were counted and tabulated. Apoptotic spermatogenic cells were detected by using Apoptosis Detection Kit. The data collected was statistically analyzed for any significant differences between the arsenic exposed and control goats.

Results: Our results revealed comparatively increased thickness of the tunica albugenia, wide inter-tubular spaces, low height of the spermatogenic cell layer, decreased diameter of the seminiferous tubules, decreased spermatogenic, sertoli, and leydig cell counts, and marked increased of apoptotic spermatogenic cells in the arsenic affected goats. The data differences between the arsenic affected and control goats were statistically significant (P<0.01).

Conclusion: Our histopathological study revealed alteration of testicular tissues in arsenic affected goats. This morphological changes of testes significantly affected on the spermatogenic processes. But it was not possible to determine the possible stage of the spermatogenesis was interrupted by the arsenic. Decreased number of spermatogenic, sertoli, and leydig cell counts, and distinctly increased number of apoptotic spermatogenic cell indicates high toxic effects of arsenic poisoning on the male gonad. The mechanism of action of toxicity of the arsenic could not be understood clearly. It is suggested here that the Black Bengal goats can be experimentally used as animal model in the laboratory for investigating the role of arsenic on the reproduction of the domestic animals.

Keywords: Arsenic; spermatogenesis; black Bengal goat.

1. INTRODUCTION

Arsenic, a non essential trace element, a potent toxin, mutagen and xenobiotic metalloid, has recently appeared as a major pollutant of drinking water in several districts of West Bengal [1] Bangladesh [2], Northern Chile, Thailand, Taiwan, China, Mongolia, Mexico, Argentina, Finland and Hungary [3]. Arsenic contamination in water has for a long time been a global concern. Survey revealed that 59 out of 64 districts and about 29% of the total tube wells in Bangladesh are contaminated with arsenic [4,5,6] and about 85 million people are at risk of drinking arsenic contaminated water and foodstuffs [7,8]. Convincing evidence exists that a variety of environmental pollutants, some of which can disrupt endocrine development in wildlife and laboratory animals, is found in rain water, well water, lakes, oceans, freshwater, soil and food chains. Exposure to such chemicals in earlier life may cause severe health risks among humans and animals including decreased fertility [9,10,11,12]. In Asia, ground water and sediments in Vietnam and in West Bengal have been reported contaminated by inorganic arsenic [13,14]. Arsenic can be used as herbicide, fungicide and rodenticides. It causes air, soil and water pollution. Polluted drinking water resulted the arsenic poisoning in wide range of domestic, aquatic life including human being [15,16,17]. Exposure to arsenic has been associated with various metabolic disorders, hypertrophy of adrenal gland, skin lesions, cardiovascular disorder, endocrine disruption as well as reproductive dysfunctions [18,19,20]. Arsenic exerts its effects on mitochondrial enzymes and impairs tissue respiration which seems to be related to the cellular toxicity [21]. Gonadal effects of arsenic were fist evaluated in mice and later in fishes [22,23,24]. Most of the available data on arsenic toxicity indicates that the main concern is with the developmental toxicity on the fetus [25]. Until recently, there is little study available on the effect of arsenic on the microscopic anatomy of testis using domestic animals [26,27]. So far known most of the study using arsenic was restricted in the human health concern. Gosh [28] in his study reported that female goat reproduction dramatically impaired when the animal exposed to the high concentration of arsenic contaminated grazing land. In his experiment he uses poultry, cattle and female goats. Arsenic effects on the female reproductive organs of Black Bengal goats have been reported in recent time [29,30,31]. The present study will clarify the effects of arsenic on the goat spermatogenesis. Many reports in the

literature have indicated decreasing sperm counts and increasing incidences of testicular cancer, hypospadias and cryptorchidism in human [32,33]. *In vivo* and *in vitro* study revealed that male fertility can be impaired markedly by various environmental toxicants when the animals exposed to their earlier life. Most of the environmentally found toxicants those are known to impair male fertility, mainly targeted the sertoli cells of the seminiferous tubules of the testes [34]. Chemotherapeutics such as cisplatin, drugs like gossypol, and plasticizers such as phthalate esters, alkyl phenols, and heavy metals targeted the sertoli cells and modulated its normal functions [35,36,37]. Sertoli cell plays a crucial role in the spermatogenesis [38]. In most species, including man sertoli cell replicate only during fetal, neonatal and prepubertal life. This proliferation was mainly controlled by the follicle stimulating hormone (FSH) and stops with maturations of sertoli cell at the onset of the puberty [39]. Exposure to such environmental toxins and exogenous estrogens of animal at the earlier stages of life can lead to suppression of FSH secretion by pituitary gland resulting reduced rate of sertoli cell replication [40]. Sertoli cell provides all nutrients and hormones needed by the germ cells. As a consequence alteration in sertoli cell function may lead to impaired spermatogenesis, germ cell loss and resulted atrophy of the testes as well as male infertility. The present study was undertaken to evaluate the effect of arsenic on the testes of Black Bengal goats in the most arsenic affected areas of Mymensingh district in Bangladesh.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Ishwarganj upazilla was considered as most of the arsenic affected area of Mymensingh district, and Nirlakha Char area of the Guripur Upazilla under Mymensingh district was considered as less arsenic contaminated area for the control goats (Fig. 1A). A total of 12 adult male Black Bengal goats (C*apra hircus*) were used in this investigation (Fig. 1B). Among these, 6 goats were collected from the Ishwarganj upazilla of the Mymensingh district as a targeted affected group, and the rest 6 were from the Nirlakha Char area as a control group. Both the right and left testes (Fig. 1C) were removed from the goats immediately after killing the animals using conventional animal killing method followed in the laboratory of the Department of Anatomy and Histology, Bangladesh Agricultural University, Mymensingh. Gross morphology of the testis was carefully observed. Weight of the both right & left testis were taken by using electric balance. Testes were cut into small pieces and preserved in the Bouin's solution for further routine histopathological processes. The specimens were kept at 40ºC for overnight. After several washes with phosphate buffer solution (0.1 M, pH 7.4), the specimens were dehydrated in a series of ascending grades of alcohol, cleared in several changes of xyline, and infiltrated with graded of melted paraffin in the heated oven at 56ºC. The tissues were then embedded in paraffin and finally the sections were cut at 5-µm in thickness using rotatory microtome. Tissue sections were allowed to dry 40ºC for overnight and stained with Hematoxylin and Eosin (H & E) and Periodic Acid Schiff (PAS) stains for histopathological studies. Apoptosis in spermatogenic cell was performed by using the In Situ Apoptosis Detection Kit (Takara Chemical Co., Ltd., Japan). The procedure and protocols used in this study was followed by Awal et al. [9,10] in his previous study in guinea pig testes. One hundred (n=100) rounded seminiferous tubule was selected for counting spermatogenic, sertoli, and leydig, cells. The results were recorded and analyzed for drawing the effects of arsenic on the spermatogenesis in the Black Bengal goat.

2.2 Data Collection

The data, calculated as the mean ± SEM. Significance between groups (P<0.01) was determined by single factor analysis of variance (ANOVA) with a Fisher's least significant differences test comparison using Stat View software (Abacus Concepts Inc., Berkeley, USA).

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Light microscopy

The effects of arsenic on the spermatogenesis were studied and evaluated in this investigation. Light microscopic examination revealed arsenic altered the general histological features of the testes which subsequently affects on the normal process of spermatogenesis. Histological feature of the spermatogenic cells were found to be different between arsenic affected and control goats. The minimum alteration of testicular histology due to arsenic poisoning negatively

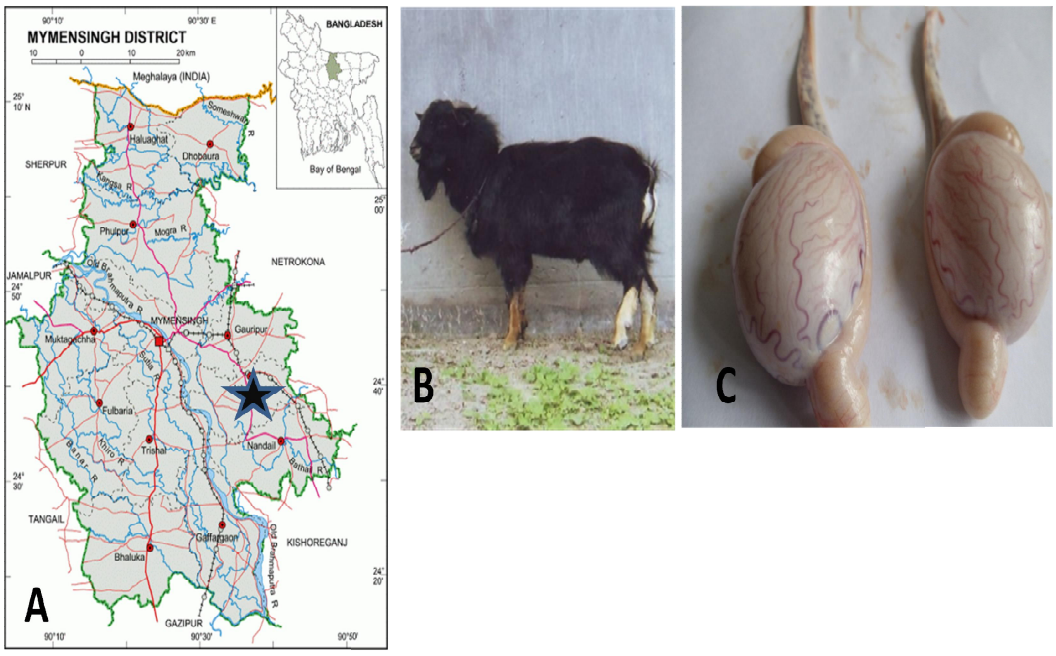

Fig. 1A. Map of the Mymensingh district of Bangladesh. *(asterisk) indicates experimental area of Ishwarganj Upazilla of Mymensingh district. B. Experimental Black Bengal goat (*Capra hircus*). C. Testis of Black Bengal goat.

tense the spermatocytogenesis, spermatogenesis and spermiogeneis. Our study in arsenic affected goats revealed thickened tunica albugenia, the covering of the testis with sparse and collapsed blood vessels while the control goats revealed the normal histological structures (Figs. 2A and C). Most of seminiferous tubules were found shrunken and had a wavy outline. The basement membrane of the tubules was thickened and discontinued in some places (Fig. 2B). The mean tubular diameter was reduced; the myoepithelial cells surrounding the seminiferous tubules moved closer to each other and were projected more prominently. Most of the lumen of the seminiferous tubules was occupied with the debris of shredded cells (Figs. 2B, D and F). Seminiferous tubular lumen frequently contained spermatogonia and spermatocytes, which were large in size and with darkly stained nuclei (Fig. 2F, big arrow). In some cells, the nuclear membrane had been ruptured and was accompanied by fragmentation of nucleus (karyorrhexis). The blood vessels were found to be both parse and collapsed. Leydig cells were reduced in number and their characteristic tendency of forming groups and/or cluster was also disappeared. Nuclei of these cells were decreased in size and lost the characteristic nucleoli (Figs. 2B, F). Decreased spermatogenic cell layer height was observed in arsenic affected goats (Fig. 2D). A low count of spermatozoa was found to attach with the apical surface of the sertoli cells of the affected seminiferous tubules of the testis in goat (Fig. 2F), but a normal count of spermatozoa were recorded in the control group (Fig. E).

A reduced number of small and medium size blood vessels were observed in the interstitial tissues (Figs. 2B and D). Number of leydig cell in the affected goats was decreased significantly (Fig. 5; $P< 0.01$).). Inter-tubular space was observed wide in the arsenic affected goats (Fig. 2B).

3.1.2 Comparative study between the arsenic affected and control goats

Mean thickness of the tunica albugenia and the testicular trabeculae of the testes of affected goats were 647.170±2.56, and 33.690±0.87 µm respectively, where as it was 551.010±2.43 and 20.630±0.93 µm in control group. Inter-tabular space in affected goat was 60±7.74 µm, but in control group, it was 37.03±5.33 µm. The diameter of the seminiferous tubule was 213.10±18.48 and 163.100±18.48 µm in control and affected goats respectively. The mean height of the spermatogenic cell layer in seminiferous tubule in control goats was 54.380±8.7 and in

affected group it was 27.65±5.52 µm. Number of spermatogenic, and sertoli cell in control goats was 177.99±22.17, and 14.500±1.51, and but this number was decreased to 137.610±13.15, and 11.20±1.41, in affected goats respectively. A decreased number of leydig cell was observed in the arsenic affected goats 450±0.31, but this number was 590±0.85 in control group (n=30 focus in each slide). Increased number of apoptotic spermatogenic cells was observed in the arsenic affected goats (14.68±3.4), but this number was 11.57±2.15 in control goats. The mean value between the arsenic affected goat and control goat was significantly different (P<0.01).

3.1.3 Significant test

Data for the tunica albugenia, testicular trabeculae, inter-tubular spaces, diameters of the seminiferous tubules, spermatogenic, sertoli, and leydig cell, and apoptotic spermatogenic cells counts were analyzed using student *t* test. All the data analyzed sowed significantly different between arsenic affected goats & control group (P<0.01; Figs. 3, 4, 5 and 6).

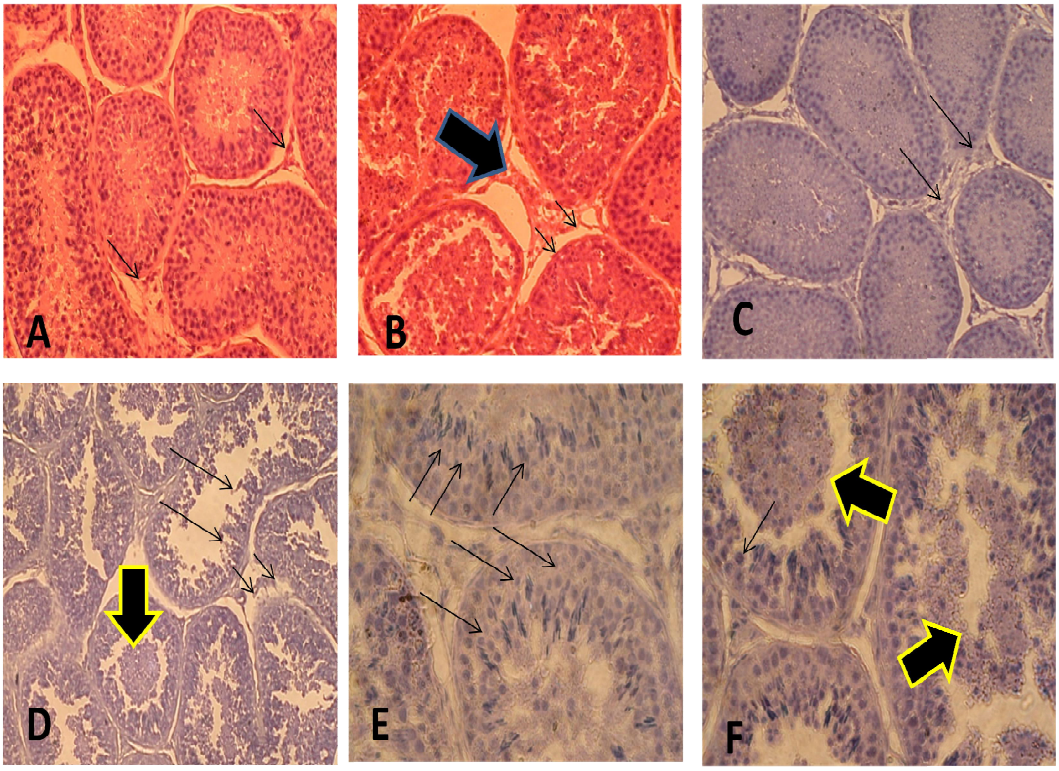

Fig. 2A. Seminiferous tubules appear normal. Inter-tubular space is narrow (small arrows-control). B. Widen inter-tabular space (Big arrow). Basement membrane of the seminiferous tubules discontinued in some places (small arrows-arsenic affected group). H & E stain. X 20. C. Normal structure of the testis of Black Bengal goat (*Capra hircus).* Comparatively narrow inter-tubular space (Thin long arrows-control). D. Sloughed off spermatogenic cell appears in the lumen of the seminiferous tubules (Big arrow). Low Spermatogenic cells height due to desquamation of the spermatogenic cells from the tubular basement membrane (Thin long arrows-arsenic affected group). PAS stain. X 20. E. A huge number of spermatozoa are seen attached with the apical surface of the sertoli cells (Thin long arrows-control). F. A reduced number of spermatozoa are seen attached with the apical surface of the sertoli cells. Sloughed of spermatogenic cells accumulated within the lumen of the seminiferous tubules (Big arrows). Spermatogenic cell layer height appears thin (long thin arrow). PAS stain X 40

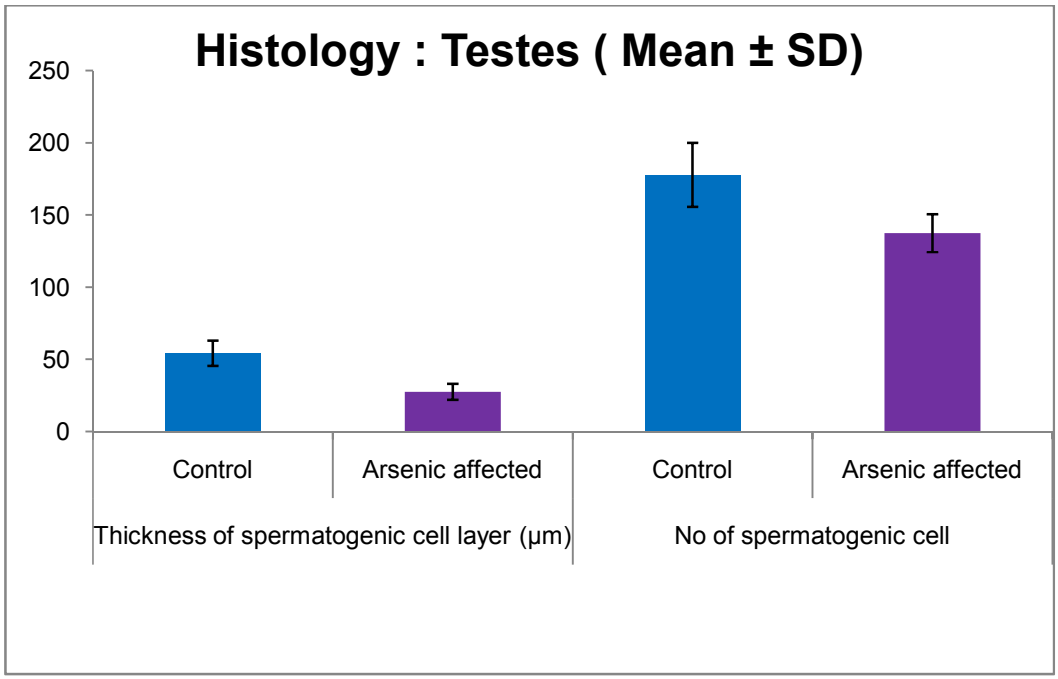

Fig. 3. Significantly decreased spermatogenic cell layer height along with spermatogenic cell count were noticed in the arsenic affected goats (*P*<0.01)

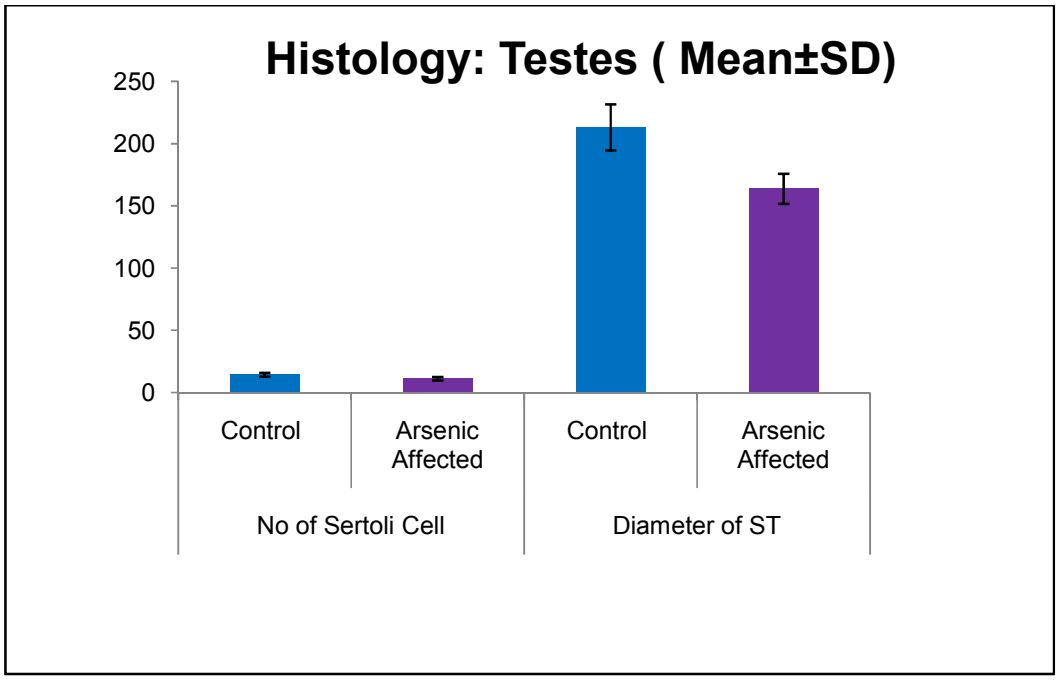

Fig. 4. Significantly decreased diameters of seminiferous tubules together with the decreased number of sertoli cells were detected in the arsenic affected goats (*P*<0.01)

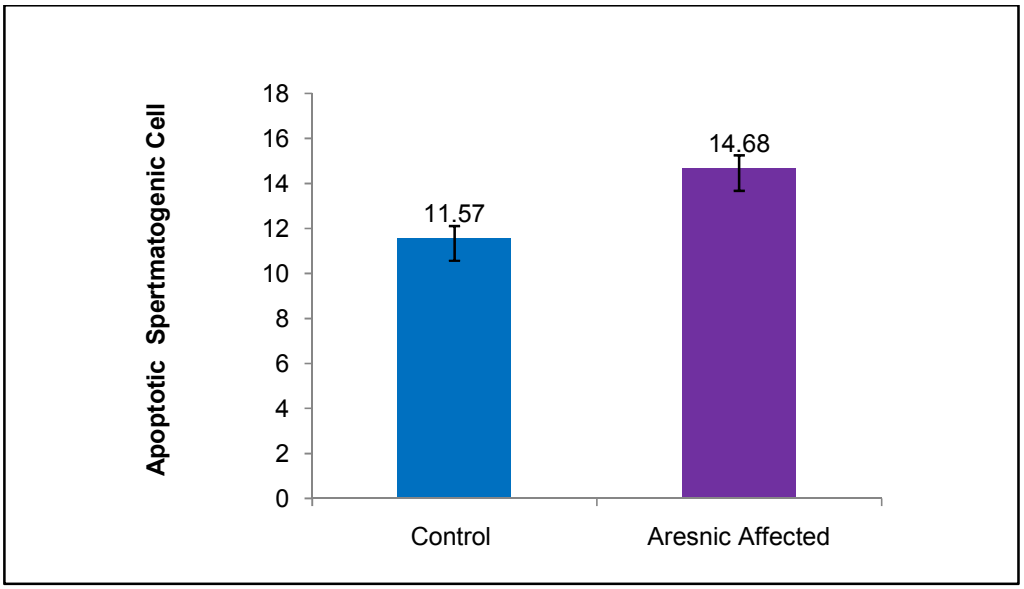

Fig. 5. Significantly increased number of apoptotic spermatogenic cells were observed in the arsenic affected goats (*P*<0.01)

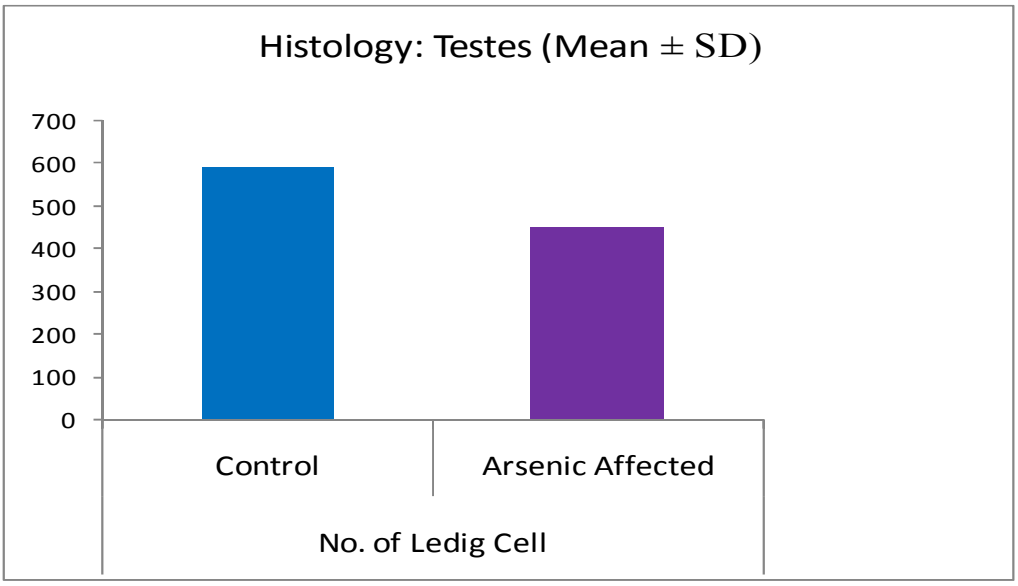

Fig. 6. A significantly decreased number of leydig cell was counted in the arsenic affected goats (*P*<0.01)

3.2 Discussion

The effects can be summarized in two categories as gross effects and histological effects on which the process of spermatogenesis depended. Arsenic alters the normal histological features of the testicular tissues along with the hormonal homeostasis of the body which directly affects the spermatogenic process resulting reproduction failure in the Black Bengal goats. The histological organizations of the testicular tissues and the hormone titer of the body are the main factors those influences on the process of spermatogenesis. Arsenic effect on the female reproductive organs of the Black Bengal goat was reported recently [29,30,31]. It is now recognized that numerous endocrine-disrupting chemicals have been released into the

environment in large quantities since World War II. Due to increased civilization and industrialization and urbanization accelerate the input of inorganic contaminants like phalate esters, Bisphenol-A including heavy toxic metals like arsenic, cadmium, copper, lead etc in drinking water, soils and food chains [12,20]. These toxic compounds many of them known as endocrine-disrupter and produces their effects on the male spermatogenesis by disrupting and /or interrupting the normal endocrine functions of the male gonad [10,11]. Inorganic arsenic is now serous alarming and threat for human health as well as animal production in Bangladesh [28,29,30,31]. Histological characteristics of the female reproductive organs altered significantly when the animals exposed to the arsenic affected area. Arsenic affected goat reveals considerably weak and debilitated [30]. This finding is in consistent with the observations of the Imran Ahmed et al. [41]. The mean weight and size of the testes were reduced in arsenic affected goats, but the differences between the arsenic affected and control goats were not significant. Testicular atrophy and decreased fertility has been described in mammals, birds, and aquatic life's when these animals exposed to the areas where the presence of multiple man-made chemicals, such as byproducts of industrial chemicals waste, pesticides and heavy metals [42]. Our present investigation in the arsenic affected goats supported their results. Interstitial cells or cells of leydig were present among the angles formed by the seminiferous tubules. Leydig cell populations were reduced in number significantly, and they lost their characteristics nucleoli. They were usually found dispersed in the interstitial connective tissues of the inter-tubular spaces and lost their grouping and/or cluster formation. Similar histological character was observed in shiba goats and guinea pig testes [9,10,11] when the animals exposed to the environmental toxicants at their earlier life. The average tissue ratio, which is a better way to assess the damage to the testes in relation to the body, was significantly reduced. All these effects were indicative of atrophic changes that had taken place in the testes. Our result indicates if the animals left for a longer period of time with out proper treatment atrophy of the testes, gradual body weight loss, and ultimate threat of life will be the final goal. Increased thickness of the trabeculae and wider inter-tubular spaces in our observation is due to increased connective tissue proliferation caused by the continuous irritation produced by arsenic poisoning. Decreased diameter of the seminiferous tubules is suggested to be increased pressure originated by the highly proliferated inter-tubular connective tissue on the seminiferous tubules of the testis. Decreased spermatogenic cell layer height due to the sloughing off and/or shedding off the spermatogenic cells from the basement membrane of the seminiferous tubules and was in consistent with our previous findings in guinea pig testes. The decreased number of the spermatogenic cell, sertoli, and leydig cells is probably due to arsenic toxicity to the targeted cells. Our previous study using Transmission Electron Microscope (TEM) revealed frequent appearance of numerous small and large size vacuoles in the sertoli cells of the guinea pig testis and shiba goats. Appearance of such vacuoles in the sertoli cell cytoplasm indicated the sertoli cell toxicity. Number of spermatozoa production for a species depends on the number of sertoli cells. Therefore, a reduced number of sertoli cells is the direct indicator of the reduced number of spermatozoa which in turn resulted the reduced testicular tissue mass i.e., reduced testicular size and weight. However, in our present investigation decreased spermatogenic, sertoli and leydig cell counts in arsenic affected goats is well agreement with the reports of Bibin et al. [11] in shiba goats and Awal et al. [9,10], in guinea pig testes. Testicular atrophy and reduced sperm counts in our investigation supported the report of the Carlsen and Toppari et al. [32,33]. Sloughing off the spermatogenic cell layer from the basement membrane of the seminiferous tubular lining and the accumulation of slough off and/or shedding off tissues in the lumen of the tubules clearly indicates the toxic effects of arsenic on the male gonad. The nucleoli in most of the leydig cells were found absent. Environmental persistent toxicant when exposed to the laboratory male animals in their earlier life causes reduced fertility and induced testicular atrophy [43,44,45,46]. Our present investigation in arsenic affected goats supported their report. It can be suggested here that like other environmentally persistent toxicants, arsenic in higher doses may produce severe testicular damage in arsenic affected Black Bengal goats if the animals left for a longer period of time and without proper curative measure in the area.

4. CONCLUSION

Toxic effects of environmentally persistent arsenic on the spermatogenesis of Black Bengal goats were observed. The mechanism of action of this compound on the male testicular tissues

was not clearly understood. Further study is underway to elucidate the possible mechanism of action of this compound on the testicular tissue in goats. Finally, it can be suggested here that Black Bengal goats (*Capra hircus*) can be used as laboratory animal model to elucidate the toxic effects of arsenic on the male reproduction.

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COMPETING INTERESTS

Authors have declared that no competing interest exists.

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