Int J Clin Exp Pathol 2016;9(5):5346-5356
www.ijcep.com /ISSN:1936-2625/IJCEP0022500

Original Article

Microscopic structural alterations and p53 immune-expression in adult rat testis in response to intra-tunical versus intra-parenchymal injection of methylene blue

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Received December 22, 2015; Accepted March 13, 2016; Epub May 1, 2016; Published May 15, 2016

Abstract: Background: Methylene blue is used in varicocelectomy to identify lymphatic vessels. This study aimed to investigate microscopic structural alterations and immunohistochemical expression of p53 as a key regulator of apoptosis in rat testis in response to intra-tunical versus intra-parenchymal methylene blue injection. Material/Methods: Thirty adult rats were divided into; group I (control), group II injected with methylene blue in intra-tunical space between tunica albuginea and tunica vasculosa, and group III injected with methylene blue in intra- parenchymal testicular tissue. After 2 weeks, blood was collected for serum testosterone assay. Testes were prepared for light microscopy and p53 immunohistochemical study. Epididymal fluid was collected to assess sperm concentration. Results: Group I showed negative p53 expression. In group II, the area beneath the site of injection showed limited degenerative changes in seminiferous tubules, and inflammatory cells infiltration in interstitial tissue. Changes in serum testosterone level and epididymal sperm concentration were insignificant as compared to group I. Degenerated cells in affected seminiferous tubules showed a positive p53 immune-reaction. Group III showed massive degenerative changes in seminiferous tubules and cells of Leydig, interstitial inflammatory cells infiltration, and a significant increase in p53 immune-expression in seminiferous tubules and interstitial tissue. Serum testosterone level and sperm concentration were significantly decreased as compared to group I & II. Conclusion: Intra-tunical injection of methylene blue resulted in limited degenerative changes beneath the site of injection, while intra-parenchymal injection resulted in massive degenerative changes with an increase in p53 immune-expression, suggesting that the approach of intra-tunical methylene blue injection is much safer.

Keywords: Immunohistochemistry, light microscope, methylene blue, p53, testis

Introduction

One of the serious complications of varicocelectomy surgery is the development of hydrocele [1]. Partial or complete division of lymphatic vessels during varicocelectomy not only leads to hydrocele formation, but also results in a decline of testicular function [2]. Therefore, sparing of testicular lymphatics is an optimal goal to decrease the incidence of postoperative hydrocele formation [3]. Mapping of lymphatic vessels during varicocelectomy through dye-assisted technique represents an easily accomplished technique with an excellent surgical outcome sufficient to spare lymphatics [4].

Since it was first synthesised in 1876, methylene blue has been used in different areas of medicine. It is a commonly available dye used in general, urologic, and gynecological surgery for the identification of normal and abnormal tracts [5]. Furthermore, it has been incorporated in different surgical procedures for lymphatic mapping to direct visualize lymphatic vessels and lymph nodes [6, 7]. Methylene blue has been used to identify lymphatic vessels during varicocelectomy either through an intra-tunical injection of the dye in the space between tunica albuginea and tunica vasculosa [8, 9], or by a direct injection in the intra-parenchymal testicular tissue [10, 11]. Some clinical studies have

considered that methylene blue injected during varicocelectomy provides an easy, rapid, and cost-free technique for mapping of testicular lymphatic drainage that almost eliminates postoperative hydrocele formation and improves the surgical outcome [12, 13]. Yet, the microscopic structural alterations induced in the testis in response to methylene blue injection by both techniques are in need of investigation.

One of the most studied proteins to date is the tumor suppressor p53. It is a transcription factor, which has the ability to trans-activate various target genes implicated in the regulation of cell cycle arrest, apoptosis and the initiation of cell cycle repair mechanisms after cellular stress [14]. Under multiple physiological and pathological conditions, p53 acts as a key regulator of apoptosis. DNA-damaging agents, hypoxia and oxidative stress all induce p53-dependent cell death [15]. It has been shown that p53 aids in the removal of damaged germ cells during spermatogenesis [16].

Therefore, the aim of the present work was to investigate the light microscopic structural alterations and the immunohistochemical expression of p53 protein in the adult rat testis in response to intra-tunical versus intra-parenchymal injection of methylene blue.

Material and methods

Animal experiments

The study was conducted in November 2015. All the procedures in the study were performed in accordance with the institutional research board (IRB) committee in our institute. Thirty adult male albino rats (100-120 days old and 200-250 gm weight) were maintained with water and food *ad libidum*. Animals were equally divided into 3 groups (10 rats/group). *Group I* included control rats which were not subjected to any procedure. *In Group II*; both testes of each rat were injected with 0.25 ml of 1% methylene blue (319112 Sigma-Aldrich, St. Louis, MO, USA) in the intra-tunical space. *In group III*; both testes of each rat were injected with 0.25 ml of 1% methylene blue deep in the intra-parenchymal testicular tissue.

Methylene blue injection

Rats were anaesthetized by intra-peritoneal injection of 50 mg/kg pentobarbital [17]. A transverse skin incision (about 1-cm) was made directly over the scrotal covering of the testis. Using a 30-G needle, methylene blue was injected in the space between the tunica albuginea and tunica vasculosa of the testes in group II, and deep inside the parenchymal testicular tissue in group III. Gentle manipulation of the testis and hemiscrotum was done after injection for few minutes until lymphatic vessels were blue stained. Then, the subcutaneous tissue was closed with 4-0 Vicryl sutures and the skin was approximated using a running 4-0 white Vicryl stitch.

Sampling

After 2 weeks, animals were anaesthetized by intra-peritoneal injection of 50 mg/kg pentobarbital. Blood was collected from aorta for the hormonal assay of serum testosterone level. The testes were obtained and prepared for the light microscopic examination of the structural alterations and the immunohistochemical expression of p53. Epididymal fluid was collected from the caudal part of epididymis for the assessment of the sperm concentration.

Light microscopic study

Preparation of tissue samples

Small incisions were made in the tunica albuginea to allow the diffusion of the fixative inside the testis. The whole testis was fixed in Bouin solution for 4 hours until it became hard in consistency. Then the testis was cut transversely into tissue slices of 2-4 mm thickness that were further fixed in Bouin solution for 20 hours at a temperature of 4°C. Tissue samples were numerously rinsed in 70% alcohol to remove picric acid in a timely manner to improve the immunohistochemical detection of antigens in the tissue [18]. After that, the tissue samples were dehydrated in alcohols, cleared in xylol and embedded in paraplast. For the light microscopic examination, tissue sections of 5 µm thickness were stained with *haematoxylin and eosin stain* to study the structural light microscopic changes, and *Masson's trichrome stain* to demonstrate collagen fibres [19]. Berg method was used to stain spermatozoa inside the seminiferous tubules of the testes [20]. Sections of 4 µm thicknesses were used for the immunohistochemical study of p53.

Immunohistochemical (IHC) technique for p53

Kits used: Primary mouse monoclonal antibody to p53 [Anti-p53 antibody (PAb 1801)] was purchased from abcam (Cambridge, UK). The following kits were purchased from Dakocytomation (Glostrup, Denmark); ready-to-use target retrieval solution (S1700), ready-to-use antibody diluent with background reducing components (S3022), and universal detection kits (K0673).

Steps of p53 immune-staining: Sections were dewaxed in two changes of xylol for 20 minutes, and hydrated in descending grades of alcohol down to distilled water. Sections were immersed in citrate buffer (pH 6) at 95-99°C in a water bath for 40 minutes, removed from the bath, and allowed to cool at room temperature. Sections were rinsed with phosphate buffered saline (PBS). Hydrogen peroxide was applied to cover the specimen for 5 minutes, and then the slides were rinsed gently with PBS. Primary antibody at a concentration of 1 µg/ml (according to Manufacturer Company) was applied to specimens, and incubated for two hours in a humid chamber at room temperature. Slides were rinsed in PBS. Biotinylated link was applied for 10 minutes and sections were rinsed in PBS. Streptavidin HRP reagent was applied for 10 minutes and sections were rinsed in PBS. Freshly prepared DAB substrate chromogen solution (1 drop of DAB chromogen/1 ml of substrate buffer) was applied on specimens for 10 minutes. Slides were rinsed gently in distilled water, immersed in haematoxylin for 1/2 minute and rinsed in tap water until blue. Slides were dehydrated in ascending grades of alcohol, cleared in xylol, mounted by Canada balsam and covered with a cover slip. Human colon adenocarcinoma (obtained from the pathology department in our institution) served as *a positive control* according to Manufacturer Company. Positive reaction for p53 appeared in the form of brown cytoplasmic granules [21]. *Negative control slides* were prepared by the same steps except they were incubated with antibody diluent instead of the primary antibody.

The percentage (%) of the area positively immune-stained with p53 was estimated in all groups. Six p53 immune-stained slides were examined from each specimen and six high-power fields (× 400) from each slide were randomly chosen. The % area of p53 positive immune-staining were digitized using Olympus® digital camera installed on an Olympus® microscope with 1/2 × photo adaptor, using 40 × magnification of an objective lens.

Epididymal sperm concentration

The cauda epididymis was gently squeezed to discharge epididymal fluid on a slide, and then aspirated using the leucocyte pipette of the haemocytometer to calculate its volume. The viscid epididymal fluid was allowed to liquefy with 0.5 ml of washing media (FP10FL06-FertiPro NV, Belgium). Sperm concentration (million/ml) was assessed by a computerized assisted microscopic system (Auto Sperm, FertiPro N.V., Beernem, Belgium) [22].

Serum testosterone level analysis

The serum was obtained from collected blood and frozen at -20°C. Testosterone level was assessed by enzyme-linked immunosorbent assay (ELISA) kit (IB79174, IBL-AMERICA). The values were expressed as ng Testosterone/ml serum [22].

Statistical analyses

Statistical data included the assessment of; the percentage (%) of the positive immune-stained area for p53, the serum testosterone level and the epididymal sperm concentration in all the groups of the experiment. Statistical data were expressed as arithmetic mean ± standard deviation (SD). Student t-test was used to test the significant change in the parameters in different groups of the study in comparison to control group I as well as to test the significance between groups II & III. Statistical analysis of the data was done by MedCalc software for medical statistics with a statistical significance realized at probability $P < 0.05$ [23].

Results

Light microscopic examination

Group I

In sections stained with *haematoxylin and eosin*, the testis was surrounded by a fibrous

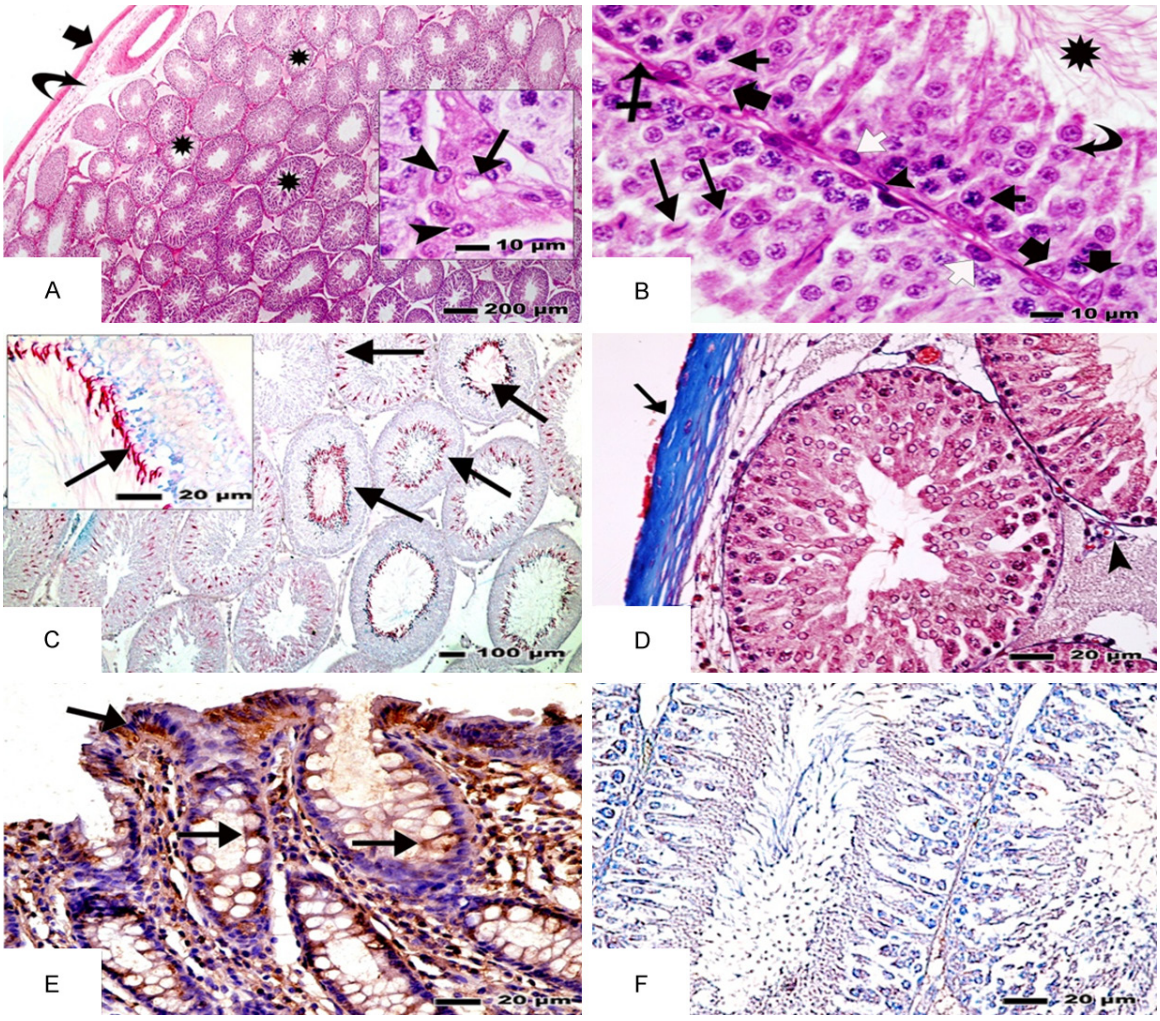

Figure 1. The testis in group I. A: The testis is surrounded by tunica albuginea (thick arrow) and an underneath tunica vasculosa (curved arrow). It contains seminiferous tubules (asterisks) separated by interstitial tissue (as seen in the inset at a high magnification) showing blood vessels (arrow) and interstitial cells of Leydig (arrow heads) with finely granular acidophilic cytoplasm and large vesicular nuclei [Hx&E × 50, inset × 1000]. B: Two seminiferous tubules surrounded by basement membrane (crossed arrow) and myoid cells (arrow head). Tubules contain Sertoli cells (thick arrows) and spermatogenic cells including; spermatogonia (white short arrows), primary spermatocytes with large nuclei containing dispersed chromatin (black short arrows), rounded early spermatids (curved arrow), elongated late spermatids (arrows), and mature spermatozoa with their flagella projecting into the lumen (asterisk) [Hx&E × 1000]. C: Red stained spermatozoa project into the lumen of the majority of seminiferous tubules (arrows). The inset is a high magnification [Berg stain × 100, inset × 400]. D: Collagen fibers are seen mainly in the tunica albuginea (arrow). Few collagen fibers are seen in the interstitial connective tissue (arrow head) [Masson's trichrome × 400]. E: Human colon carcinoma as a positive control for p53 demonstrates the positive immune-reaction (arrows) [IHC for p53 × 400]. F: Seminiferous tubules and interstitial tissue show a negative immune-reaction for p53 [IHC for p53 × 400].

tunica albuginea and an underlying loose vascular connective tissue of tunica vasculosa. The testis was formed of seminiferous tubules separated by interstitial tissue containing blood vessels and clusters of Leydig cells with finely granular acidophilic cytoplasm and large vesicular nuclei (**Figure 1A**). Seminiferous tubules were enclosed by a basement membrane and myoid cells. Seminiferous tubules were lined with: Sertoli cells and spermatogenic cells at different stages of spermato- and spermiogenesis. Sertoli cells showed pale cytoplasm and vesicular nuclei. Spermatogenic cells included spermatogonia resting on the basement membrane, large primary spermatocytes with large nuclei containing dispersed chromatin, early or

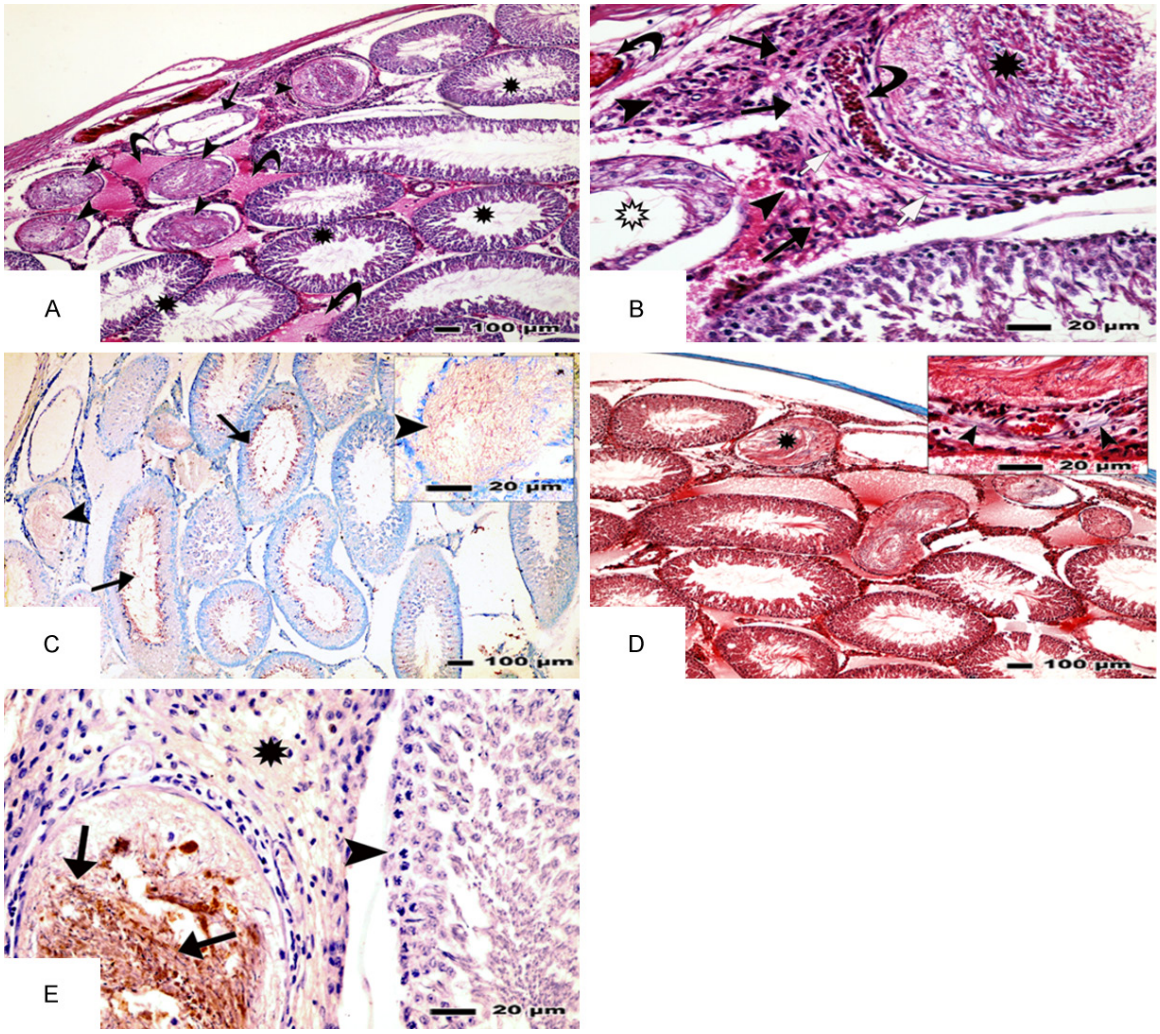

Figure 2. The testis in group II. (A) Beneath the site of intra-tunical methylene blue injection, few seminiferous tubules appear degenerated (arrow heads) with cells filling their lumen. One tubule is just lined with few layers of spermatogenic cells (arrow). Note the acidophilic exudates among the tubules (curved arrows). The rest of the tubules are not affected (asterisks) [Hx&E × 100]. (B) A higher magnification of figure (A) showing degenerated cells filling the lumen of one seminiferous tubule (black asterisk), and the other tubule is lined with few layers of degenerated cells (white asterisk). Interstitial tissue contains congested blood vessels (curved arrows), and inflammatory cells infiltration (arrows) among the interstitial cells of Leydig (arrow heads). Note the nuclei of fibroblasts (white short arrows) [Hx&E × 400]. (C) Some red stained spermatozoa (arrow heads) are present in the lumen of an affected tubule (the inset is a high magnification). Non affected tubules show numerous spermatozoa (arrows) [Berg stain × 100, inset × 400]. (D) The interstitium surrounding a degenerated tubule (asterisk) contains some collagen fibers (arrow heads) as seen in the inset at a higher magnification [Masson's trichrome × 100, inset × 400]. (E) A positive immune-reaction for p53 is detected in the degenerated cells (arrows) filling the lumen of an affected seminiferous tubule. Note the negative reaction in a non affected tubule (arrow head) and in the interstitial tissue (asterisk) [IHC for p53 × 400].

rounded spermatids, late or elongated spermatids, and mature spermatozoa with their flagella projecting into the lumen of the seminiferous tubules (**Figure 1B**). Sections stained with *Berg stain* demonstrated red stained spermatozoa in the majority of seminiferous tubules (**Figure 1C**). *Masson's trichrome stain* showed collagen fibers mainly in the tunica albuginea. Few collagen fibers were seen in the interstitial connective tissue (**Figure 1D**).

Human colon carcinoma was used as a positive control for p53 (**Figure 1E**). In group I, a negative immune-reaction for p53 was seen in the seminiferous tubules and in the interstitial tissue (**Figure 1F**).

Group II

Sections stained with *haematoxylin and eosin* showed few degenerated seminiferous tubules

Table 1. Mean values ± S.D of different parameters measured in all groups

Groups of rats	Group I	Group II	Group III	Significance
% area of positive p53 immune-stain	0.2200 ± 0.1398	5.9000 ± 1.5239	44.4000 ± 5.3996	P1 < 0.0001*
				P2 < 0.0001*
				P3 < 0.0001*
Serum testosterone (ng/ml)	3.3176 ± 0.4228	3.1255 ± 0.3812	1.9938 ± 0.4356	P1 = 0.3000
				P2 < 0.0001*
				P3 < 0.0001*
Epididymal Sperm concentration (million/ml)	85.7420 ± 24.5911	81.6909 ± 11.7187	53.8438 ± 11.8497	P1 = 0.6438
				P2 = 0.0017*
				P3 = 0.0001*

* = significant change (P < 0.05) by student t-test. P1 = comparison between group I and group II. P2 = comparison between group I and group III. P3 = comparison between group II and group III.

beneath the site of intra-tunical methylene blue injection containing degenerated cells filling the lumen. Occasional tubules were just lined with few layers of degenerated cells. Away from the site of methylene blue injection, seminiferous tubules were not affected (**Figure 2A**). Interstitial tissue surrounding affected tubules showed acidophilic exudates, congested blood vessels, inflammatory cells infiltration, and fibroblasts among the interstitial cells of Leydig (**Figure 2A**, **2B**). *Berg stain* revealed the presence of some spermatozoa in the affected tubules beneath the site of methylene blue injection, while in the non affected tubules away from the site of the injection numerous spermatozoa were seen (**Figure 2C**). *Masson's trichrome stain* showed some collagen fibers in the interstitium surrounding degenerated tubules (**Figure 2D**).

A positive immune-reaction for p53 was detected in degenerated cells filling the lumen of affected seminiferous tubules, while non affected tubules and interstitial tissue showed a negative reaction (**Figure 2E**). The % area of the positive immune-stain for p53 was significantly (P < 0.05) increased as compared to group I (**Table 1**).

Group III

The testes in this group were not easily dissected as they were adherent to the surrounding tissues. Tunica albuginea was very thick (**Figure 3A**). The underlying seminiferous tubules contained degenerated cells filling the lumen. Deeper seminiferous tubules were irregular in shape and were lined with few layers of degenerated cells with small dense nuclei. Some cells coalesced together forming multinucleated spermatogenic cells. Interstitial tissues contained congested blood vessels, degenerated interstitial cells of Leydig with small dense nuclei, inflammatory cells infiltration and numerous fibroblasts (**Figure 3B**, **3C**). In sections stained with *Berg stain*, few tubules contained red stained spermatozoa, while the majority of tubules did not show any spermatozoa (**Figure 3D**). *Masson's trichrome* staining revealed excess collagen fibers in the thickened tunica albuginea and in the interstitial tissue surrounding seminiferous tubules (**Figure 3E**).

A positive immune-reaction for p53 was detected in the cells of degenerated seminiferous tubules and in interstitial cells (**Figure 3F**). The % area of the positive stain for p53 was significantly (P < 0.05) increased in this group as compared to group I and II (**Table 1**).

Epididymal sperm concentration

No significant (P > 0.05) difference was found in the mean value of the sperm concentration in group II subjected to intra-tunical injection of methylene blue as compared to control group I. Rats in group III receiving an intra-parenchymal injection of the dye showed a significant (P < 0.05) decrease in this parameter as compared to group I & II (**Table 1**).

Serum testosterone level

There was no significant (P > 0.05) difference in the mean value of the serum testosterone level in group II as compared to group I. In group III, the mean value of serum testosterone level was significantly (P < 0.05) decreased as compared to group I & II (**Table 1**).

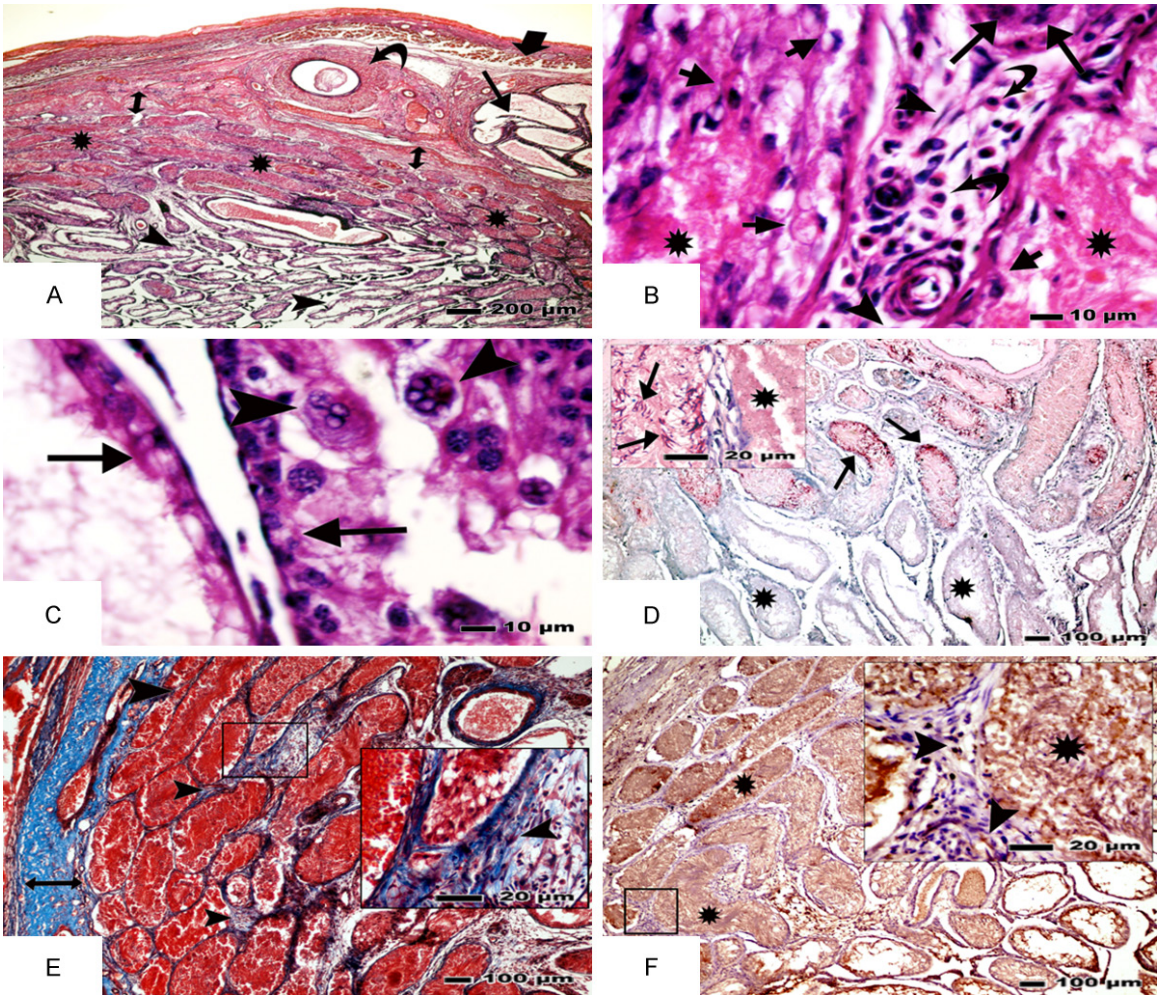

Figure 3. The testis in group III. A: The testis is adherent to surrounding tissues as; Cremaster muscle (thick arrow), epididymis (arrow) and vas deferens (curved arrow). Under the thickened tunica albuginea (double head arrows), the lumen of degenerated seminiferous tubules is filled with degenerated cells (asterisks). Deeper tubules are irregular in shape and are lined with few layers of cells (arrow heads) [Hx&E × 50]. B: Two seminiferous tubules (asterisks) contain degenerated cells (short arrows). Interstitial tissue shows; degenerated interstitial cells of Leydig with small, dense nuclei (arrows), inflammatory cells infiltration (curved arrows), and nuclei of fibroblast (arrow heads) [Hx&E × 1000]. C: Two deeper seminiferous tubules are lined with few layers of degenerated cells with small dense nuclei (arrows). Note the multinucleated spermatogenic cells (arrow heads) [Hx&E × 1000]. D: Few seminiferous tubules contain red stained spermatozoa (arrows). The rest of the tubules reveal no spermatozoa (asterisks). The inset is a high magnification [Berg stain × 100, inset × 400]. E: Excess collagen fibers are seen in the thickened tunica albuginea (double headed arrow) and in the interstitial tissue (arrow heads) surrounding seminiferous tubules. The inset is a higher magnification of the boxed area [Masson's trichrome × 100, inset × 400]. F: A positive immune-reaction for p53 is seen in degenerated cells in the seminiferous tubules (asterisks) and in interstitial cells (arrow heads). The inset is a higher magnification of the boxed area [IHC for p53 × 100, inset × 400].

Discussion

The present study, investigated the microscopic structural alteration in the testis of adult rats in response to intra-tunical versus intra-parenchymal injection of methylene blue dye. The testis in group II receiving an intra-tunical injection of methylene blue showed inflammatory cells infiltration in the interstitial tissue and degenerative changes in seminiferous tubules limited to the area beneath the site of injection, while seminiferous tubules away from the site of the injection were not affected. The serum level of testosterone and the epididymal sperm concentration in this group were insignificantly different than control group I. On the other hand, the testes in group III subjected to intra-parenchymal injection of methylene blue dis-

played massive degenerative changes. In this group, the testes showed extensive adhesion to surrounding tissues and contained degenerated seminiferous tubules. Similar pathological changes were previously demonstrated after intra-testicular injection of methylene blue and other dyes in the testis [24]. The tubules in group III contained multinucleated cells. Such cells are produced when the cytoplasmic bridges between dividing cells open and allow the fusion of the cellular contents of conjoined cells. They are believed to be associated with seminiferous tubular degeneration [25]. Degenerated seminiferous tubules in group III were surrounded by excess collagen fibers. Methylene blue has been found to induce fibrosis. Stromal fibrosis has been reported after using methylene blue for lymph node localization in cases of breast cancer [26]. The interstitial tissue surrounding degenerated tubules in group III showed inflammatory cells infiltration. Methylene blue has been reported to induce inflammatory cells infiltration in different tissues [27].

The impact of methylene blue on tissues is controversial. Some authors demonstrated a positive effect of the dye in the reparation of ischemia-reperfusion injury in different tissues [28]. However, this was not the case in the testis, as methylene blue has been found to exacerbate oxidative tissue damage in the contralateral testis after unilateral testicular torsion [29]. Likewise, the oxidative interaction properties of methylene blue are debatable. Some studies reported a protective antioxidant effect of methylene blue in some tissues [30-33]. On the contrary, methylene blue at a concentration range of 5 μM, which are easily obtained with a clinical dose of 1.5-2 mg/kg, was found to increase the intracellular oxidative stress and generates intracellular reactive oxygen species (ROS) in cultured endothelial cells [34]. Oxidative stress has been found to inhibit the process of androgenesis by Leydig cells in testis tissue [35]. This may explain the degenerative changes in interstitial cells of Leydig and the concomitant significant decrease in the serum level of testosterone encountered in group III of the present study. In some publications, methylene blue has been considered as a prime promoter of lipid peroxidation [36, 37]. It has been reported that the plasma membrane of the mammalian sperm is sensitive to lipid peroxidation related to ROS that can damage cellular structure, survival and metabolic functions of sperm [38, 39]. This may explain the significant decrease in the epididymal sperm concentration encountered in group III of the present study as compared to group I & II. Oxidative stress has been also associated with pathological levels of apoptosis in germ cells and Leydig cells [40]. ROS may initiate the propagation of a series of reactions by activating caspases that ultimately trigger apoptosis [41]. Lipid peroxidation has been directly associated with increasing p53 expression and induction of germ cell apoptosis in vitro [42].

The immunohistochemical expression of p53 was assessed in the present study. In control group I, p53 showed a negative immune-reaction. Similar results were demonstrated in previous studies [43]. It has been reported that p53 protein is present in normal cells in such a low quantity that is undetectable by immunohistochemistry [44], while under cellular stress, p53 can be highly expressed in the testis [45, 46]. In the latter condition, the intracellular concentrations of p53 robustly increase to a level that can be detected by immunohistochemistry [44]. In the present study, a positive immune-reaction for p53 was detected only in the degenerated cells of affected seminiferous tubules beneath the site of intra-tunical methylene blue injection in group II, while in group III subjected to intra-parenchymal methylene blue injection a positive p53 immune-reaction was detected in degenerated seminiferous tubules distributed all over the testis and in the interstitial cells. Those interstitial cells are most probably the cells of Leydig rather than being inflammatory cells as it has been reported that p53 is not expressed in inflammatory cells [47]. A significant increase in the % of positive immune-stained areas for p53 was detected in group III in comparison to both group I & II. It is believed that under the condition of cellular stress, p53 becomes activated and stabilized [48].

The response of p53 to different stimuli by activation and rapid stabilization occurs through phosphorylation or other posttranslational modifications in the cells [49, 50]. Different publications demonstrated that p53 is involved in apoptosis of testicular germ cells [51, 52]. Both extrinsic and intrinsic pathways of apoptosis are regulated by p53. It mediates cell death

primarily signals through the intrinsic pathway involving the mitochondria, while the extrinsic pathway involving death receptors plays a minor role. In response to stimuli, p53 can be localized to mitochondria to promote the transcription of a number of genes involved in apoptosis [46, 53, 54]. In most cases, p53-induced apoptosis proceeds through mitochondrial release of cytochrome c, which leads to caspase activation [55]. It was further shown that targeting p53 to the mitochondria was sufficient to induce apoptosis, just by the mere localization of p53 to mitochondria, in a transcription-independent manner [53].

A brief comparison between the two approaches of methylene blue injection in the testis accomplished in this study displays that the intra-tunical approach was much safer with limited pathological insults than the intra-parenchymal approach. Referring to the lymphatic drainage in murine testis, it is known that the seminiferous tubules are bathed in a sea of lymph that drains into irregular lymphatic capillaries incompletely bounded by endothelial cells just beneath the tunica albuginea but not in the interstitium between the seminiferous tubules. Such capillaries then drain into lymphatic vessels in the tunica albuginea [56]. Therefore, in the intra-parenchymal approach of methylene blue injection, the dye diffuses in the interstitial tissue fluid that surrounds the seminiferous tubules before being absorbed into lymphatics, while in intra-tunical technique; the dye reaches the large lymphatics to map them without being in direct contact with the testicular parenchyma. The latter technique, therefore, can provide a protection for the developing testicular germ cells from the hazards of being exposed to a substance that can affect their development.

Conclusion

The present study demonstrated that the microscopic structural changes induced by intra-tunical injection of methylene blue in the testis of adult rat was limited to the site of injection, while the intra-parenchymal injection of methylene blue resulted in massive degenerative changes in the testis accompanied with an increase in the immune-expression of p53, suggesting that the intra-tunical approach of methylene blue injection is much safer.

Acknowledgements

We thank Dr. Adel A Zalata, professor of medical biochemistry in Mansoura faculty of Medicine, Egypt, for providing kits and technical help in assessment of testosterone and epididymal sperm concentration.

Disclosure of conflict of interest

None.

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