Morphological and laboratory improvement of Cisplatin - induced azoospermia using Adipose Derived Mesenchymal Stem Cells in a rabbit model

Hamdy Y. Ismail[1*], Nora A. Shaker[1], Shaymaa Hussein [2], Mohamed Fathi[3], Hamdy Rizk[1], Y. R. Wally [1]

1. Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
2. Department of Cytology and Histology, Faculty of Veterinary Medicine, Cairo University Giza, Egypt
3. Department of Theriogenology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt

* Corresponding author: Hamdy Y. Ismail, Email: hamdy.yossef@cu.edu.eg , tel. +201159691074

1. Abstract

The testes are highly susceptible to the adverse effects of chemotherapy and radiation at all stages of life. Exposure to these threats mainly occurs during cancer treatment and as an occupational hazard in radiation centers. The present study investigated the regenerative ability of adipose-derived mesenchymal stem cells (ADMSCs) against the adverse effects of cisplatin on the structure and function of the testes. New Zealand white male rabbits (N = 15) were divided into three groups of five: a negative control group (no treatment), a cisplatin group (single dose of cisplatin into each testis), and a cisplatin with ADMSCs group (cisplatin was injected followed by an ADMSC injection three days later). On day 45 post-treatment, serum testosterone levels were evaluated, and the testes and epididymis were collected for histology, and epididymal sperm analysis. Cisplatin caused damage to the testicular tissue and decreased serum testosterone levels and epididymal sperm counts The ADMSC-treated group displayed a moderate epididymal sperm count, suitable hormone levels, and enhanced testicular tissue morphology. ADMSCs treatment enhanced damaged testicular tissue and modified pathological changes caused by cisplatin.

Keywords: testicular degeneration; sperm analysis; cisplatin side-effects

2. Introduction

Infertility is a significant medical, financial, and psychological issue in modern society affecting 60–80 million couples worldwide [1]. Over the past ten years, the prevalence of infertility has progressively risen to affect 10% to 5% of sexually active people [2]. Male factor infertility, due to defective spermatogenesis as a result of a failure in germ cell proliferation and differentiation [3], appears to be the cause of 25%–50% of infertility cases, according to several surveys [4].

Chemotherapy, which has shown significant efficacy in treating cancer, is considered one of the main causes of testicular toxicity and degeneration, especially with respect to the gonads, representing a serious cause of permanent or temporary infertility [5].

Cisplatin is a significant chemotherapeutic agent used to treat various cancers [6], but it has a toxic

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effect on many organs, especially gonads. This drug has been shown to result in depletion of germ cells, atrophy of the testes [7-9].

The primary objective of stem cell therapy is to repair damaged tissues that cannot heal on their own, giving many patients hope that their illnesses will be cured and dying cells will be replaced. The anti-inflammatory, anti-fibrotic, and regenerative properties of mesenchymal stem cells (MSCs) are responsible for their therapeutic potential [10, 11].

The current study aimed to investigate the healing and therapeutic potential of adipose-derived mesenchymal stem cells (ADMSCs) in cases of testicular injury caused by cisplatin in adult rabbits.

3. Materials and Methods

Ethical approval

Our study was performed in accordance with the regulations of the Veterinary Medicine Cairo University Institutional Animal Care and Use Committee (Vet-CU- IACUC) with approval number (**Vet CU 8/03/2022/404**).

3.1. Animal selection and housing

The present study was conducted with 15 healthy mature male New Zealand rabbits, 8–10 months of age, weighing 3–3.5kg. Rabbits were individually housed at the Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Cairo University. The animals were maintained at a controlled temperature (20–23°C) and had free access to a commercial standard rabbit diet (10-12% protein) and water.

3.2. Experimental design

Following a week of acclimatization, the animals were divided into three equal groups of five. Group I (negative control group) received no treatment. Group II (cisplatin group) received a single intra-testicular injection of cisplatin to induce testicular degeneration. Group III **(**cisplatin + ADMSCs group) testes were injected with cisplatin to cause degeneration, followed by ADMSCs three days later. After 45days, the testes and epididymis of all rabbits were surgically removed for histological evaluation and epididymal sperm analysis.

Briefly, the animals were injected with Xylazine (Xyla-Ject, ADWIA Co. SAE, Egypt) 1–3mg/kg IV as a pre-anesthetic, followed by induction of anesthesia with Ketamine 10% (Alfasan International, The Netherlands) 2mg/kg IV in the ear vein. The animals were restrained in dorsal recumbency, and the scrotum was disinfected using povidone-iodine. Through an incision in the scrotum, the testes were accessed by blunt dissection followed by ligation of the spermatic cord to prevent bleeding when it was transected. The scrotum was sutured using non-absorbable black silk 2-0, M-Natur material removed ten days after the operation. All animals were provided an analgesic (Ketofan®0.2Ml, Amrya industries) IM, once daily for three days postoperative to minimize pain. Systemic (Pentomycin® 0.2ml IM, Univet) and topical antibiotic spray (Bivatracin®) were used to prevent infections.

3.3. Induction of testicular degeneration

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Before induction, the testes of animals in groups II and III were immersed in a hot water bath at 43°C for 15 minutes by placing the rabbits on a plate containing the hot water so that only the testes are exposed to the hot water. Using an insulin syringe, cisplatin (0.7ml/kg) was injected as a single intra-testicular dose. The total dose of 1mL per testis was accomplished by inserting the 31G needle cranially into the tail of the testis parallel to the epididymal tail.

3.4. Isolation of ADMSCs [12]

The animal was placed in ventral recumbency, and the skin over the scapular area was sterilized with 70% ethyl alcohol. A small longitudinal incision in the midline was made, and fat over the scapular areas was dissected and placed in a sterile Petri dish containing pre-warmed phosphate-buffered saline (PBS). The tissue was processed in a laminar flow hood where the collected fat was washed several times in PBS and sliced into small pieces (0.1–0.3 mm). Afterward, it was poured into 50-mL falcon tubes and washed with PBS containing 1% antibiotic (Penicillin 100 IU, Streptomycin 100mg) It was then digested with an equal volume of warm, filtered, 0.1% collagenase type-I solution dissolved in PBS for 60min in a water bath with shaking at 37°C. After digestion and inactivation of the collagenase by adding an equal volume of Dulbecco's Modified Eagle Medium (DMEM) media containing 10% fetal bovine serum (FBS), the mixture was filtered through a 100-µm sterile nylon mesh filter and spun at 4000 rpm (JANETZKI) for 10 min. The resultant pellet was re-suspended in 10 ml of saline for washing, which was repeated in triplicate. The pellet was washed twice with DMEM/F12 + 10% FBS and plated in 10-cm plates in the same complete media. Stem cells were allowed to adhere for 3 h, and the culture medium was gently removed and replaced. Aspiration of the old media was performed to remove non-adherent cells after 48h, and subsequent media changes were made every 2-3days until confluence reached 80%–90%.

3.5. Flow cytometry characterization of stem cells

After the third passage, immunophenotyping was performed on cultured cells using a fluorescence-activated cell sorting analyzer (FACsort,Becton,Dickinson,Germany). The cell surface markers CD73, CD105, and CD44 were evaluated [13].

3.6. Differentiation capacity of ADMSCs

For in vitro differentiation of ADMSCs [14], a medium containing 10 µL/mL insulin (Sigma), 0.5mM isobutyl-methyl xanthine (IBMX) (Sigma), 0.5µg/mL dexamethasone (Sigma), and 50µM indomethacin (Sigma) was used to induce adipogenic differentiation. Adipogenesis was assessed by detecting intracellular lipid deposition. Differentiated adipose stem cell monolayers stained with Oil Red O. Using 10µg/mL transforming growth factor beta (TGF-b), 50mg/mL ascorbic acid 2-phosphate (Sigma), and dexamethasone, chondrogenic differentiation was achieved. Cells of passage three were plated in six-well plates, and differentiation media was applied for 21days, with the medium changed every three days.

3.7. Injection of ADMSCs

After the third passage, cells were washed twice with PBS and detached using 2 ml of 0.25% trypsin EDTA

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solution (Thermo Fisher) for 5 min at 37°C. The suspension was centrifuged, the supernatant discarded, and the pellet was suspended in saline for injection. The prepared solution containing 1×10^6 ADMSCs in 1 ml of PBS was injected, by insulin syringe (31G), into the tail of the testis on day three post cisplatin injection. The needle was inserted cranially into the tail of the testis parallel to the epididymal tail.

3.8. Sperm collection and evaluation

The sperm was obtained from the tail of the epididymis by flushing the tail with pre-warmed physiological saline. Suspensions were obtained from each epididymis. A hemacytometer was used to determine the total epididymal sperm count.

Sperm motility was assessed in fresh samples. Only progressive forward motile sperm were evaluated in different microscope fields using 40 × magnifications. Briefly, 10 µl of flushed sperm was put on a pre-warmed slide on a hot stage microscope as previously described in the literature [15-17]. For assessment of sperm morphology, a 40µl sperm suspension was mixed with 10µl of eosin-nigrosin stain for 45min. Smears were produced on grease-free slides and allowed to air dry before being inspected under an oil immersion lens using a light microscope [18].

3.9. Serum hormonal level of testosterone

Blood samples collected from each experimental group were put in a blood tube containing a clotting accelerator and allowed to stand for 15min. The serum extracted from the blood through centrifugation at 3000 rpm for 10min was kept at −80°C until analyzed.

Testosterone levels were measured using rabbit testosterone ELISA kits (Monocent, Inc. 9237 Eton Ave, Chatsworth, CA 91311, United States.). The assay is based on a competitive ELISA in which the sample's testosterone competes with added testosterone-horseradish peroxidase (HRP) for antibody binding. Anti-testosterone antibodies were precoated on a 96-well plate. The wells were filled with samples and the testosterone-HRP conjugate, then washed, and 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to create a blue color. A stop solution was added to prevent the color from developing and producing a yellow color. The signal intensity was negatively correlated with the sample's testosterone level measured at 450 nm.

3.10. Anatomical evaluation

The whole testes were investigated morphologically. An anatomical description of the normal rabbit testes was compared to the other groups' morphometrical analysis.

3.11. Histopathological examination (Light microscope)

After 45days, the testes were removed. Specimens from all groups were carefully dissected at the end of the experiment, promptly fixed in 10% NBF for 48h, then washed and dehydrated in escalating ethyl alcohol dilutions. Xylene was used to clear the specimens before they were embedded in paraffin wax. Using a rotatory microtome, sections 5µm thick were cut, dewaxed, and stained with hematoxylin and eosin stain for light microscopic analysis [19].

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3.12. Statistical analysis

One-way ANOVA was applied to the data from random samples. When P ≤0.05, the post hoc test was used to compare the effects of the various treatments. The significant differences between the groups were indicated on the columns by the letters a, b, and c. The statistical software package OriginPro, version 2016, was used for data analyses.

4. Results

4.1. Morphology of ADMSCs

The adipose stem cells appeared round and of different sizes, and most floated on the dish's surface. After 24 h of culturing, a few ADMSCs were attached to the culture plate and appeared in an elongated spindle shape with long cytoplasmic processes with clear elliptical nuclei. The plated cells survived, divided, and multiplied enough to crowd the dish within seven days. The fields had many cells connected by interlacing processes (Fig.1).

4.2. Flowcytometric analysis of ADMSCs

The flow cytometric analysis of ADMSCs showed robust expression of CD105 and CD73 markers but weak expression of CD44 (Fig 2)**.**

4.3. Differentiation capacity of ADMSCs

After a 21-day incubation of ADMSCs in a specific differentiating medium, cells differentiated into chondrocytes and adipocytes. Chondrocytes (Fig.3/A) were confirmed by staining with Safranin O stain. Adipocytes were characterized by intracellular lipid droplets with Oil Red O stain (Fig.3/B)**.**

4.4. Sperm analysis

Cisplatin injection caused a significant decrease in sperm parameters and epididymal sperm count to the level of azoospermia. Treatment with ADMSCs led to a significant increase in sperm count, individual motility, and viability compared to the cisplatin group (Table 1).

4.5. Serum hormonal level of testosterone

Cisplatin injection in group II caused a significant decrease in serum testosterone level (0.7±0.01 ng/mL) compared to normal (2.4±0.03 ng/mL). In group III, stem cell treatment after cisplatin injection significantly increased the serum testosterone level (1.2± 0.02 ng/mL) compared to group II (Histogram1).

4.6. Anatomical evaluation of rabbit testis

Testis of the negative control group appeared as elongated ovals with a laterally compressed range from 2.8 to 3.2cm in length with a sharp caudal pole and a blunt cranial pole located in the inguinal region inside two thin hairless scrotal sacs just cranial to the penis. The testes had a marbled appearance (Fig. 4/A&Fig.5/A), slightly firm in consistency, with the tunica vasculosa running longitudinally on the ventral free border. The internal structures (Fig. 4) of longitudinal sections of testes were characterized by centro-axial mediastinum toward the cranial pole.

Three days after cisplatin injection, the testes appeared swollen, firmer in consistency, with engorgement of the tunica vasculosa. Then, the swelling disappeared, and the consistency became

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soft with patches of redness and decreased in size from 1 to 1.5cm in length **(**Fig. 5/B**)**. The ADMSC-treated testes' color and consistency nearly returned to normal **(**Fig. 5/C**).**

4.7. Histopathological examination (Light microscope)

The mature seminiferous tubules in the negative control group had normal architecture according to histological sections. The connective tissue septum entered the testicular parenchyma from the tunica albuginea, separating it partially or completely into lobules. Four to six seminiferous tubules in each lobule were separated by a thin band of areolar connective tissue. The cross-sections appeared round or oval with regular outlines. Each tubule was lined by the stratified epithelium of spermatogenic cells and Sertoli cells that protruded into the lumen. Polyhedral and interstitial or Leydig cells were present in the connective tissue separating the seminiferous tubules. Spherical nuclei and the characteristically foamy acidophilic cytoplasm of Leydig cells were used to identify them. (Fig. 6/ a-c).

In group II (cisplatin group), the seminiferous tubules appeared irregular in outline with an elongated shape, degeneration of the stratified germinal epithelium, and severe vacuolation. There were fewer sperm or none in the seminiferous tubule lumen. Edema was also seen in the interstitial area and dilatation in the blood vessels (Fig. 6/ d-f).

In group III (cisplatin + ADMSCs), the architecture of the seminiferous tubules returned to being nearly normal, much like in the control group. Layers of spermatogenic cells lined the interior of each seminiferous tubule separated by interstitial connective tissue that contained blood vessels and interstitial cells (Fig.6/ g-i).

5. Discussion

The present study confirms the therapeutic capacity of MSCs based on their regenerative effect on damaged tissues and their ability to prevent adverse effects from chemotherapeutic drugs on gonads. Similar findings were reported by Sutton and Bonfield [11], Fazeli, Abedindo [20], Kalra and Tomar [21], Lodi, Iannitti [22]**.**

In the present study, we isolated the ADMSCs from the adipose tissue over the scapular area of rabbits, as described by Mazzetti, Oliveira [23]**.**

The current investigation revealed that the ADMSCs appeared round in shape and of different sizes. After 24 h in culture, few cells adhered to the culture plate, showing long cytoplasmic processes and elongated, spindle-shaped nuclei. This result agreed with Meligy, Abo Elgheed [13] in rats.

One of the main features of stem cells is their ability to give rise to specialized cells by a process called differentiation that is controlled by signals inside and outside cells. Regarding the present study, after a 21-day incubation of ADMSCs in specific differentiation medium, cells differentiated into adipocytes and chondrocytes as recorded by Hassan and Alam [24], Kobayashi and Suda [25], Watt and Driskell [26].

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This study shows the adverse effects of Cisplatin injection on testicular tissue, blood, and semen parameters, in accordance with the findings of Bakalska, Atanassova [27], Andriana, Tay [28], Hou, Chrysis [29], Sawhney, Giammona [30], Habermehl, Kammerer [31] who reported that chemotherapeutic drugs could not differentiate between cancerous and normal cells and therefore cause damage to different organs, especially gonads.

We documented the adverse effects of cisplatin injection, an important platinum-based chemotherapeutic drug, on testis tissue and germ cells. Our results were similar to the findings of Azu, Duru [32], Sherif, Abdel-Aziz [7], Cheng, Rai [8].

In rabbits, the single intra-testicular injection of cisplatin to the testes caused a severe reduction in epididymal sperm concentration to the level of azoospermia and decreased sperm motility and viability. Similar results were reported in rats [33-36] and humans [37].

In accordance with the results of [34, 38, 39] in rats, ADMSCs treatment in group III after cisplatin injection led to a significant improvement in the live percentage of epididymal sperm count and motility.

According to our investigation, cisplatin treatment caused a decrease in serum testosterone in the damaged testes. Testosterone promotes protein synthesis in all spermatogenic cells, so it plays a significant role in the formation of sperm. As a result, a decrease in testosterone impairs the production of proteins in germ cells, leading to degenerative effects. These results were similar to those reported in rats [33, 35, 36] and humans [40].

A marked elevation of serum testosterone was observed in the treated group compared to the untreated group, similar to the result reported in rats by Meligy, Abo Elgheed [13].

The anatomy of rabbit testes appeared elongated and oval with a blunt rounded cranial pole and sharp narrow caudal pole located within the membranous hairless scrotal sacs in the inguinal region just cranial to the penis. A similar description was provided by Sohn and Couto [41] and [42] in the rabbit. In agreement with the last author, the testis length ranged from 2.8–3.2 cm.

The testes appeared slightly firm in consistency, while those of cisplatin-injected group II appeared swollen in the first three days and firmer with engorgement of the tunica vasculosa. The swelling gradually disappeared, and the consistency became soft with patches of redness. Such results are in line with that of [33, 34, 36, 39] Moreover, the ADMSC-treated group III showed therapeutic improvement due to the ability of stem cells to differentiate into the cells that need to be replaced, resulting in the gradual restoration of testicular weight and sizes [13, 34, 39].

Regarding the microscopic evaluation, cisplatin exposure caused significant alteration and damage to the testicular structure. The seminiferous tubules appeared deformed with irregular outlines, and the germinal epithelium's

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thickness decreased to the point of being depleted. These changes caused defects in spermatogenesis. The decreased germinal epithelium was related to germ cell sloughing and the inability of the basal germ cells to mature. Furthermore, cisplatin exposure caused dilatation in the blood vessels in the interstitium and under the capsule of the testes, similar to what was reported in rats exposed to cisplatin [13, 34-36, 39] and also with Busulfan exposure [38].

ADMSCs treatment promoted tissue regeneration by releasing growth factors and cytokines that encouraged the remaining spermatogenic cells to proliferate and complete their division [43]. Moreover, ADMSCs protect the testis by various mechanisms, including the ability to modulate the immune/inflammatory status caused by the administration of cisplatin and by having anti-apoptotic effects [10]. We recorded that the structure of the testis returned to nearly normal after 45 days of stem cells treatment, with more than one layer of germ cells lining each seminiferous tubule [13, 34, 39].

6. Conclusion

ADMSCs have a potential role in regenerative medicine as an effective therapy for many medical conditions due to their capacity to differentiate into various cell types and their sensitivity to their environment. ADMSCs have anti-apoptotic properties that can reverse cisplatin-induced testicular toxicity. In addition to their role in spermatogenesis, the current study highlights evidence that ADMSCs can restore the structural efficiency of rabbit testes after single intra-testicular Cisplatin injection cisplatin exposure.

Conflict of interest

There are no conflicts of interest to declare

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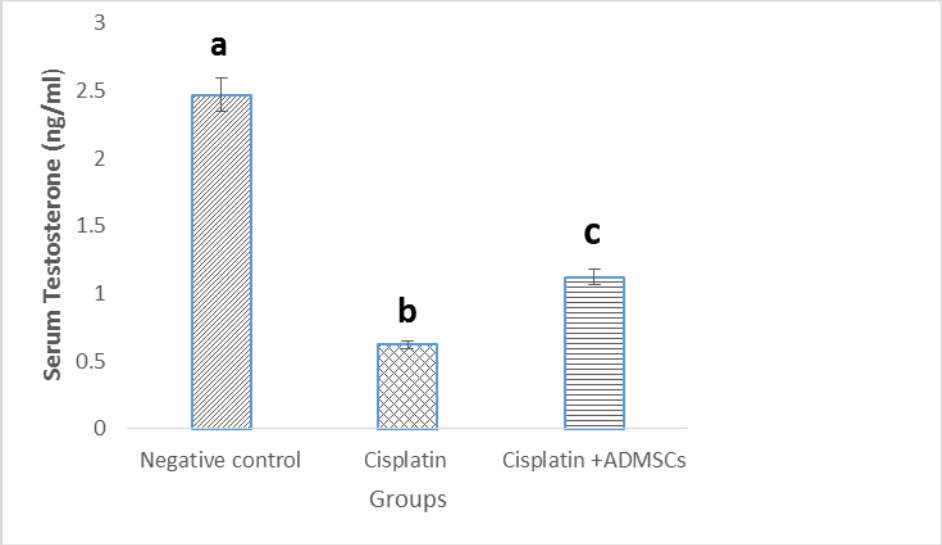
Table 1: Epididymal sperm analysis

Aspect	Control negative	Cisplatin group	Cisplatin + ADMSCs group
Individual motility (%)	65	-	30
Epididymal Concentration/mL	300× 10^6/mL	Epididymal azoospermia	50 -60×10^6/mL
Abnormal sperm percentage (%)	16	-	35
Live Percentage (%)	85	-	40 -50



Histogram 1 Bar chart between groups according to testosterone level (ng/ml),

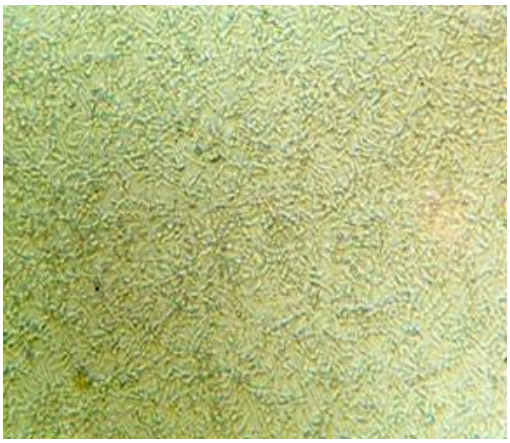


Fig. 1. ADMSCs morphology 7 days' culture 4X (Scale bar 50µm)

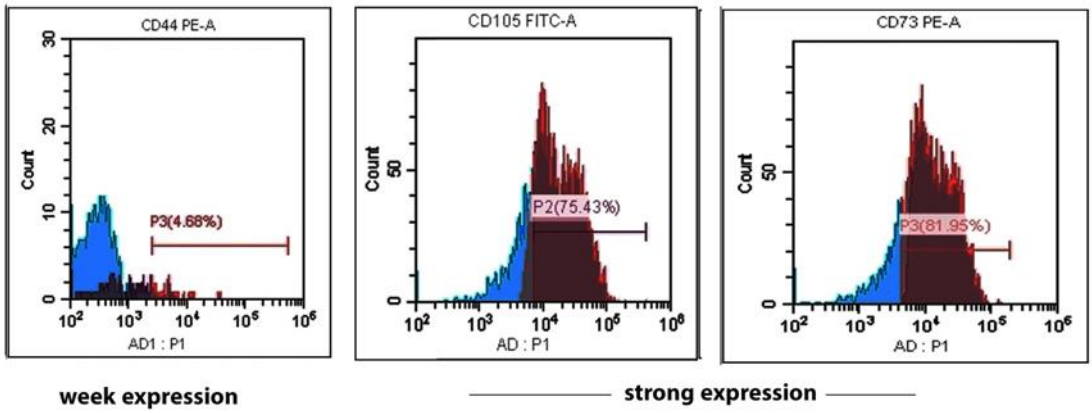


Fig. 2. Flow cytometry analysis of ADMSCs at passage 3 against surface markers CD 44, CD 105 and CD 73

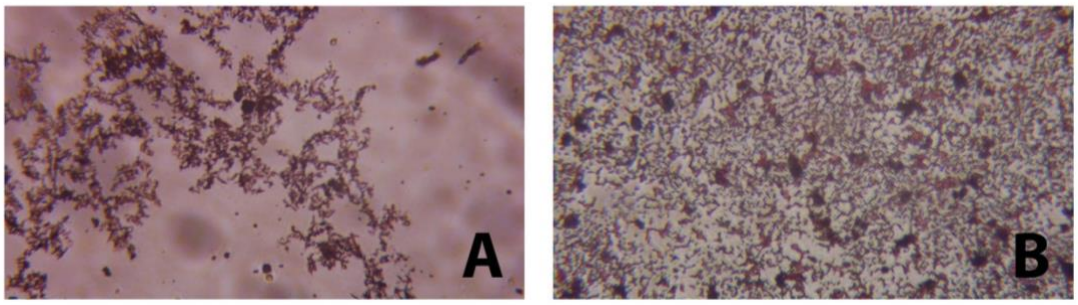


Fig. 3. ADMSCs differentiation potential A) Adipocyte differentiation Oil red O staining 4X. B) Chondrogenic differentiation, Safranin-O 4X (Scale bar 100µm)

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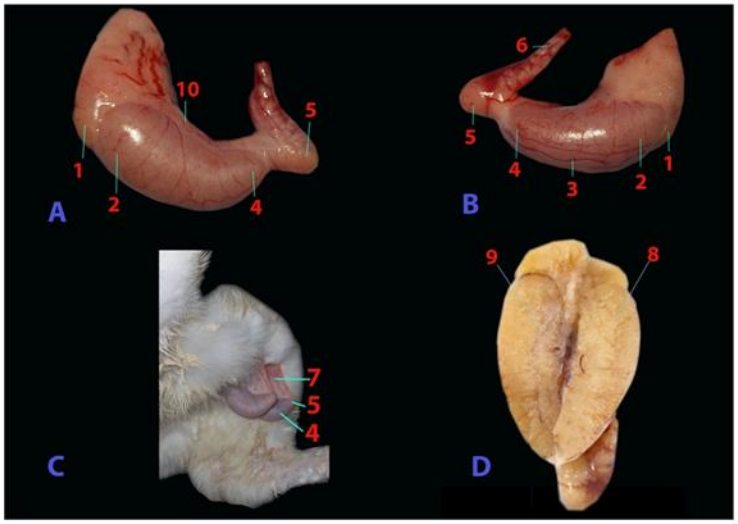

Fig. 4. Photograph of the negative control rabbit testis: A. Lateral surface, B. Medial surface, C. Position, D. Longitudinal section, 1. head of Epdidymis ,2. head of the testis, 3. tunica vascuolsa, 4. tail of the testis, 5. tail of the Epdidymis, 6. Ductus deferens, 7. Scrotum, 8. parenchyma of testis, 9. mediastinum, 10. body of Epdidymis

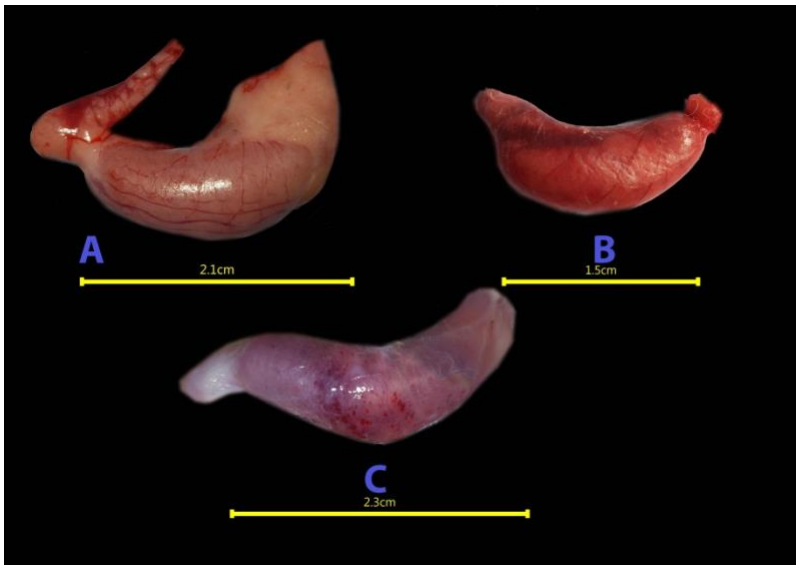

Fig. 5. Photograph of the three experimental groups: A. Negative control, B. Cisplatin group, C. Cisplatin + ADMSCs

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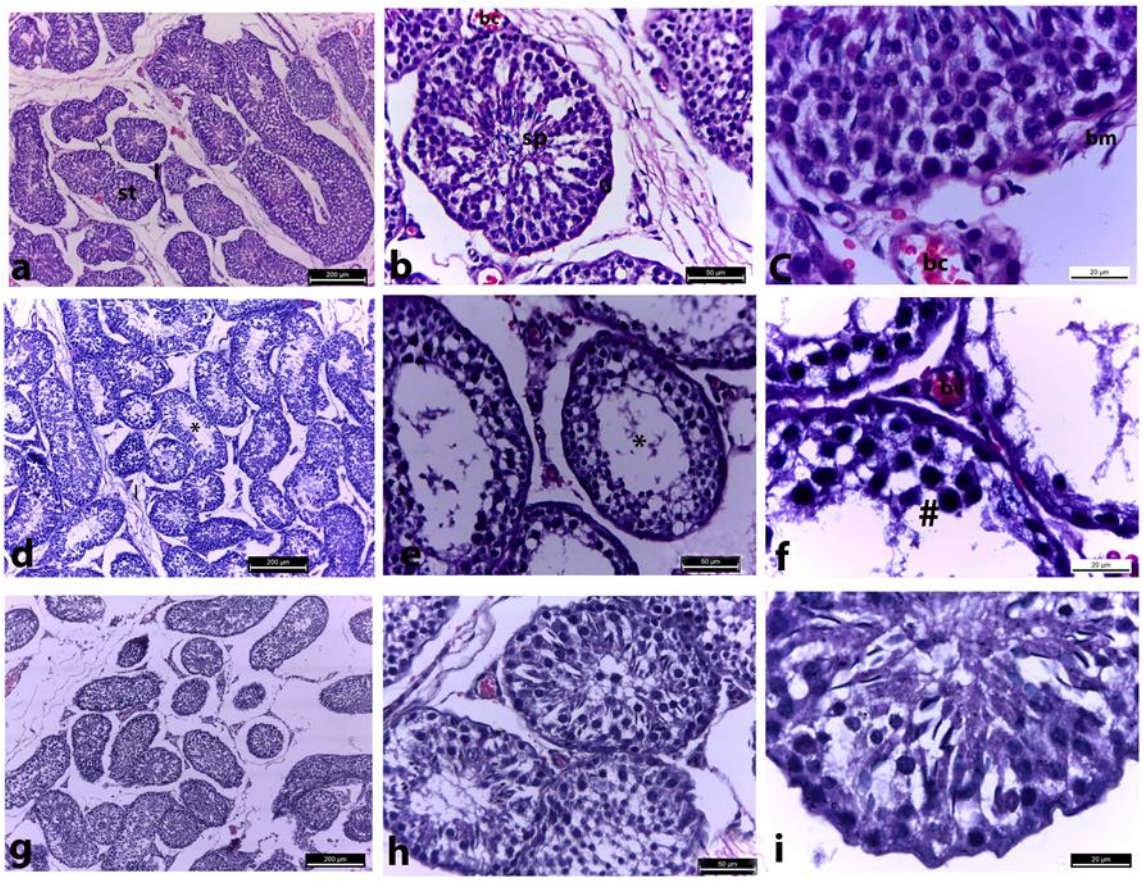

Fig. 6. H & E stained sections in the testis of New-Zeeland white rabbit, (a–c) for (G I), (d–f) for (G II), (g–i) for (G III), showing: image (a 200 µm) seminiferous tubules (st) and interstitial tissue (I), image (b 50µm) spermatogonia (G) Sperm (sp) and blood capillary (bc), image (c 20 µm) basement membrane (bm) and blood capillary (bc), image (d 200 µm) distortion and reduction in the thickness of the germinal epithelium (*) and wide interstitial spaces (I), image (e 50 µm) distortion and irregularity of seminiferous tubules (*), image (f 20 µm) thickening and congestion of blood vessels (bv) and sloughing of degenerated spermatogenic cells (#), image (h 50µm) restoration of the epithelial thickness (arrow).

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