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Randa M. Breikaa, Hisham A Mosli, Ashraf B. Abdel-Naim

Onabotulinumtoxin A (onabotA) is gaining wide medical use in children. However, little is known about its potential testicular effects. The present study was planned to investigate the influence of its injection on the maturing testicular structures in rats. Immature rats were injected in the peritesticular area by onabotA with three doses of (10, 20 and 40 U/kg) three times in a two-week interval. The effect of these injections on fertility indices (sperm parameters, semen quality and testosterone levels) was examined. In addition, levels of antisperm antibodies and several apoptosis parameters were investigated. DNA content in form of ploidy was compared to control group via flow cytometric analysis. Histopathological examination was carried out to confirm the findings. OnabotA-injected groups showed decreased sperm count and semen quality, while sperm vitality, morphology and testosterone levels were not significantly affected. Furthermore, DNA flow cytometric analysis confirmed delayed sperm maturation. Apoptosis markers were significantly increased by the injections. In conclusion, onabotA use in growing rats adversely affected sperm count and maturation. However, it had no significant effects on sperm morphology or vitality. OnabotA testicular effects are mediated, at least partly, by apoptosis.

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Short title: Effects of OnabotA on young rat testes

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Abstract

Onabotulinumtoxin A (onabotA) is gaining wide medical use in children. However, little is known about its potential testicular effects. The present study was planned to investigate the influence of its injection on the maturing testicular structures in rats. Immature rats were injected in the peritesticular area by onabotA with three doses of (10, 20 and 40 U/kg) three times in a two-week interval. The effect of these injections on fertility indices (sperm parameters, semen quality and testosterone levels) was examined. In addition, levels of antisperm antibodies and several apoptosis parameters were investigated. DNA content in form of ploidy was compared to control group via flow cytometric analysis. Histopathological examination was carried out to confirm the findings. OnabotA-injected groups showed decreased sperm count and semen quality, while sperm vitality, morphology and testosterone levels were not significantly affected. Furthermore, DNA flow cytometric analysis confirmed delayed sperm maturation. Apoptosis markers were significantly increased by the injections. In conclusion, onabotA use in growing rats adversely affected sperm count and maturation. However, it had no significant effects on sperm morphology or vitality. OnabotA testicular effects are mediated, at least partly, by apoptosis.

Keywords

Onabotulinumtoxin A, rats, testes, spermatogenesis, apoptosis

Introduction

Since the introduction of Onabotulinumtoxin A (onabotA) into clinical urological use, its applications have been steadily expanding (*Fortuna et al., 2011*)**.** When injected intramuscularly at therapeutic doses, the toxin targets receptor binding sites on motor nerve terminals, inhibiting the release of acetylcholine, thus, reducing muscle activity. Most of the current usages in urological practice center on relaxing muscle spam and relieving painful conditions.

In adults, OnabotA injections are well established treatment for neurogenic detrusor overactivity, furthermore it was approved to be used for the more prevalent idiopathic over-active bladder and idiopathic detrusor over-activity, painful bladder syndrome and intestinal cystitis (*Chancellor, 2010*). These conditions mostly affect women at any age. Some of them can be in the child bearing period and unaware of the possibility of pregnancy at the time of treatment with onabotA injections. The performance of pregnancy test prior to onabotA injections was neither mentioned by the drug label nor by the Food and Drug Administration (FDA).

There is no indication in the literature to dispute the possibility of onabotA of reaching the intra-testicular environment either via direct diffusion or carried to the testis by blood when injected in a nearby or distant organ. Distant spread of the toxin was mentioned in the drug label, warning on the adverse effect on skeletal muscles and paralysis with repeated injections of the toxins (*FDA, 2011*), in addition the drug label stated the precaution of the concomitant use of other drug that affect the neuromuscular junction such as aminoglycosides and curare. Crossing the blood-central nervous system barrier was mentioned in the mechanism of the use of this drug in the treatment of migraine. It is thought that onabotA may prevent central sensitization in wide dynamic range neurons in the trigeminal nucleus caudalis (*Ramachandran & Yaksh, 2014*). In

rats, the blood-testis barrier is established between 16 and 19 days of age. The mechanism by which onabotA can cross the blood-testis barrier needs to be further explored.

Similar to adults, children with refractory detrusor overactivity associated with spina bifida and those suffering from cerebral palsy are nowadays candidates for the treatment with intradetrusal onabotA injections (*Chancellor & Smith, 2011*). Among the applications of onabotA in the pediatric age group, a potential application that has been suggested is the use of onabotA for retractile testes and cremasteric synkinesia *(Mori et al., 2011*). This can be explained by the fact that onabotA relaxes the cremasteric muscle, allowing proper descending of the testes. The use of onabotA for this purpose seems very promising, since then; hormonal therapy and a complex surgical procedure would be avoided. However, its safety in children must be considered first, especially because the consequences of treating relatively immature muscles are not known *(Pascual-Pascual & Pascual-Castroviejo, 2009*)**.** In fact, in 2008, reports of children with cerebral palsy dying after the injection of the toxin were made public, prompting physicians and patients to question the safety of this medication (*Apkon & Cassidy, 2010*). Furthermore, a treatment option that might affect future fertility or cause apoptosis of testicular structures would not be suggested. The present study was planned to investigate the influence of onabotA injections on the maturing testicular structures in rats.

Materials and Methods

Chemicals

OnabotA (100 U) was purchased from Allergan, CA, USA. All other chemicals used were of highest grade commercially available.

Animals and experimental protocol

The work was conducted on immature male Sprague-Dawley rats (1 month-old), maintained on a 12 h light-dark lighting schedule, under controlled temperatures (22°C ± 2°C) and provided with food and water *ad libitum*. The rats were divided into four groups (6 rats per group). The first group served as control (peritesticular saline), and the other groups were injected with three doses of onabotA peritesticularly (10, 20 and 40 U/kg, respectively). These doses were selected as representatives for the minimum effective doses used for urologic indications *(Cakmak et al., 2003; Schurch & Reitz, 2004)*. The repetition of injections was dictated by the fact that ONABOTA needs multiple injections due to its finite duration till acquiring therapeutic effect (*Mori et al., 2011*). OnabotA was reconstituted with 4 ml 0.9 % saline and used within 1 h. All injections were given three times in a two-week interval. After two weeks from the last injection, blood was collected and the serum separated. The animals were then sacrificed by decapitation and testes collected. Part of the testes was kept in 10% formalin for histopathological and immunohistochemical examinations, part homogenized in saline, and part stored as such in -80°C for flow cytometric analysis. Animals were obtained from King Fahd Medical Research Center and the protocol was approved by the Unit of Biomedical Ethics Research Committee, Faculty of Medicine, King Abdulaziz University.

Sperm parameters and semen quality

The epididymis was dissected, freed from fat, minced and used to examine sperm parameters. Count and motility were examined using a hemocytometer and light microscope after staining with hematoxylin and eosin (H & E). Estimations were performed in three different fields per sample, and then the mean value was used as the final score. Sperm morphology was examined using Spermac stain and sperm vitality using negrosin-eosin stain.

Semen quality was estimated by assessing resazurin reduction, which depends on the ability of active sperms to reduce the blue dye to a pink compound, both measured spectrophotometrically at 615 and 580 nm, respectively. Results are expressed as resazurin reduction (RRT) ratio (i.e. 580 nm to 615 nm).

Levels of testosterone and antisperm antibodies

Total testosterone and antisperm antibodies were determined using enzyme-linked immunosorbent assay (ELISA) kits from BioCheck and Biocompare, CA, respectively.

Immunohistochemical examination

Apoptosis markers were examined immunohistochemically using rabbit polyclonal IgG to rat Fas (Santa Cruz, Cat#sc-834), Bax (Abcam, Cat#ab7977) and caspase 3 (Cas3, Thermo Scientific, Cat#RB-1197-B). Quantitative analysis was made by calculating the area percentage using Image J software.

DNA flow cytometric analysis

DNA analysis was carried out by removing the tunica albuginea from the testes, followed by immersing the decapsulated testes in ice cold Dulbecco's modified eagle medium and mincing gently. After centrifugation, samples were transferred into nutrient medium containing 0.25% collagenase and shaken for 30 min in a water bath at 32.5°C. A single cell suspension was

prepared and ploidy of cells determined with a flow cytometer (FACS Calibur, Becton-Dickinson, USA) and analyzed.

Histopathological examination

One part of each formalin-fixed testis was processed by standard histopathological methods, stained with H & E, and analyzed by light microscopy for apoptosis, necrosis and other histopathological changes.

Statistical analysis

Data are presented as mean ± standard deviation (SD) and multiple comparisons were performed using one-way ANOVA followed by Dunnett as a post-hoc test. Differences between groups were considered significant at $p < 0.05$ and highly significant at $p < 0.01$.

Results

Sperm count and motility

Sperm count and motility were significantly decreased by all onabotA doses when compared to control, reaching 24% and 56% of the control value by the highest dose, respectively (Table 1). Fig. 1 shows the prominent difference in sperm count between the groups. On the other hand, sperm vitality and morphology were not greatly affected by the toxin, showing insignificant changes between the groups (Table 1).

Testosterone

Data from the present study showed that there was no significant difference in testosterone levels in all groups (Table 1).

Semen quality

OnabotA injections significantly decreased the quality of semen after testes maturation. The first two doses decreased the quality to about 78% of the control, while the highest dose decreased it to 40% of control (Fig. 2).

Antisperm Antibodies

Antisperm antibodies were higher in onabotA injected groups, especially with the two higher doses, showing a 60% and 84% increase than the control (Fig. 2).

Apoptosis markers

As shown in Fig. 3, injection of the toxin caused a drastic increase in Fas expression with all three doses of onabotA. The increase was 2-fold with the first two doses and 3.1-fold from control with the upper dose.

Expression of Bax showed a similar pattern as Fas, where there was a dramatic change with the three doses. There was about a 3-fold increase from control with the lowest dose, while the increase reached 11-fold with the 40 U/kg dose (Fig. 3).

Different from the former apoptosis markers, increase of Cas3 expression was significant with the two upper doses only. They caused a 3-fold and a 9-fold increase from control, respectively (Fig. 3).

DNA flow cytometry

All groups showed two different peaks, one for haploid cells representing mature spermatids, and one for diploid cells representing secondary spermatocytes and spermatogonia. The difference between the groups was in the relative proportion of those two peaks. In the control group, the haploid part was about 87.6% of total cells, while in the onabotA-injected groups, it decreased to 86%, 68% and 67%, respectively (Fig. 4).

Histopathology

The 10 U/kg group showed a histopathology similar to control with little noticeable changes. The tubules were uniformly sized and lined by regularly arranged rows of sperms at different stages of maturation, spermatogonia at the outer layer of the tubule, followed by primary and secondary spermatocytes, and finally spermatids and spermatozoa in the middle of the tubule. On the other hand, with the dose 20 U/kg dose, marked increase in apoptotic and necrotic cells was observed. In addition, disruption and atrophy of testicular structures were beginning to be noticed with thickening of basement membranes. The 40 U/kg dose caused prominent disruption and atrophy. The basement membranes were disintegrated and the tubules showed non-uniform arrangements (Fig. 5).

Discussion

OnabotA is a powerful tool and a promising solution for many disorders nowadays. As a muscle paralytic agent, it is useful for testicular disorders especially that the procedure is minimally invasive and avoids a complex surgery and side effects of common therapies. However, when the patients involved are of young age, the benefits and risks must be weighed and assessed thoroughly.

Our data highlight potential risks for onabotA injection into a maturing testis. OnabotA significantly decreased the sperm count, where the testes were unable to restore their spermatogenic capacity during maturation. So, the overall semen quality was significantly affected. Furthermore, results of flow cytometric analysis confirm that there is a delay in the maturation process of the sperms, where the percentage of diploid cells (secondary spermatocytes and spermatogonia) increased markedly. On the other hand, examining the vitality and morphology of the sperms, they were able to be preserved. Testosterone levels were also not affected by the injection. This shows that although the maturation of sperms was delayed and the count decreased, the sperms that were produced finally in the maturing testes were normal. In addition, the decreased sperm count with normal testosterone levels suggests that the most prominent damage occurred in the seminiferous tubules, while the Leydig cells, which secrete testosterone, were minimally affected.

When investigating the immunologic status of testes, onabotA injections significantly elevated the antisperm antibody titer, a finding that can affect proper spermatogenesis (*Kollin et al., 2007*). Another problem that arises with onabotA is the induction of apoptosis, where a treatment that causes degeneration of the testicular structures would be problematic. Classically, there are two main apoptotic pathways leading to effector caspase activation: the Fas/tumor necrosis factor death receptors and the mitochondrial pathway. In the first pathway, binding of

the ligand to the death receptor recruits initiator caspases (2, 8 and 10), leading ultimately to activation of effector caspases (3,6 and 7). The mitochondrial pathway is thought to be triggered by translocation of a proapoptotic Bcl-2 family member such as Bax into the mitochondria (*Elmore, 2007*). In the testes, increased testicular Fas content has been associated with germ cell apoptosis. Furthermore, the co-localization of Fas ligand and Fas receptor in spermatocytes during meiosis supports a potential involvement of the Fas pathway *(Billig et al., 1995; Miura et al., 2002)*. Fas system in the testes is particularly interesting because it involves an intimate paracrine interaction between Sertoli cells and germ cells during spermatogenesis, where Sertoli cells express Fas ligand, initiating the killing of Fas receptor-expressing germ cells. So, the Fas system is a key regulator of germ cell apoptosis in normal and injury-associated conditions *(Lee et al., 1999)*. As both Fas and Bax were significantly increased by onabotA injections, it is obvious that onabotA initiates a permanent germ cell death process. This is accompanied by the increase of the effector caspase, which is upstream of caspase 6 and 7, therefore, representing a critical point in transmission of the apoptotic signal *(Omezzine et al., 2003)*. These alterations may represent one of the molecular events involved in the impaired spermatogenesis observed. The presence of many apoptotic cells in the histopathological sections confirms this finding.

The results of the current study are of particular importance, since they address the safety of the pediatric population, where the consequences of treating relatively immature muscles are not known. Analyzing the data indicated that onabotA had different effects on the maturing testes, where sperm count and maturation were affected, while morphology and vitality were not. This differs greatly from our previous work *(Breikaa et al., 2014)*, where already mature rats were injected. The different effects observed in mature and immature testes suggest a difference in sensitivity or responsiveness to onabotA. In the case of the mature rats, the damage was much

more severe and permanent, and all sperm parameters were greatly affected. Herein, there was an observable decrease in count and delay in maturation. Yet, testosterone levels and other fertility parameters were normal or at least not significantly affected. It appears that negative effects on fertility indices can be recovered during maturation of the testes. However, it is important to point out that rats have a high reproductive efficiency *(Working, 1988)*, unlike humans. Therefore, the outcome in humans could be different.

Conclusions

It can be concluded that onabotA had different adverse effects on rat maturing testes. These include decreased sperm count and delayed sperm maturation. However, sperm morphology and vitality were not significantly affected. The mechanism of onabotA testicular toxicity is attributed, at least partly, to induction of apoptosis. The current data present an experimental warning for the pediatric use of onabotA that warrants further investigations.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

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FIGURE LEGENDS

Fig. 1. Representative images of sperms in the various groups after H & E Staining

(I) Control; (II) 10 U/kg onabotA; (III) 20 U/kg onabotA and (IV) 40 U/kg onabotA

(A) Abnormally flat head; (B) detached head; (C) coiled tail

Fig. 2. Semen quality (RRT ratio) and levels of antisperm antibodies after onabotA injections in the testes of the growing rat

* $p<0.05$ compared to control group

** $p<0.01$ compared to control group

Fig. 3. Representative images of the testes of the growing rat after immunohistochemical examination of various apoptosis markers (x100)

(I–III): Immunohistochemical detection of Fas, Bax and Cas3

Control gruop: minimal expression (brown color); onabotA groups: dose-dependent increase in expression. The bar charts represent results of the image analysis, performed by examining 6 fields per slide.

* $p<0.05$ compared to control group

** $p<0.01$ compared to control group

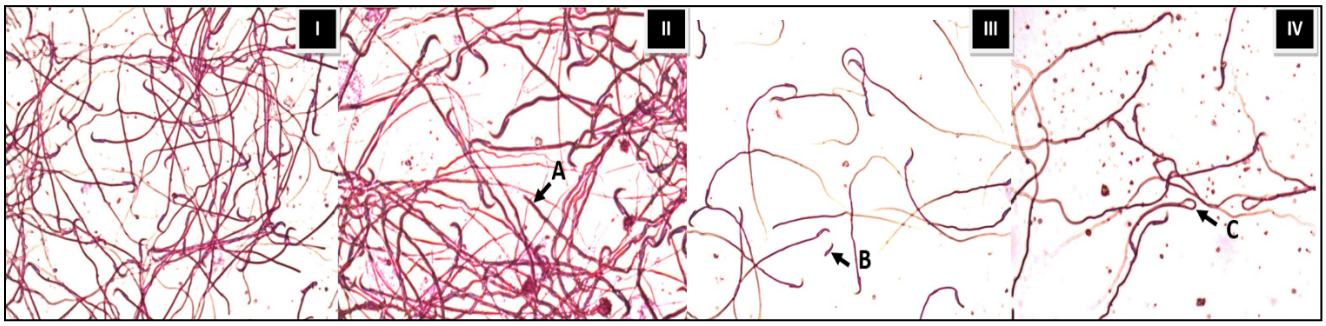
Fig. 4. Changes in DNA content (ploidy) after onabotA injection

311 **Fig. 5. Histopathological findings in the testes of the growing rat after H & E staining**
312 **(x100)**

313 (I) Control: uniformly sized tubules, lined by regularly arranged rows of spermatogenic cells at
314 different stages of maturation; (II) 10 U/kg dose: similar to control; (III) 20 U/kg dose: marked
315 increase in apoptotic (A) and necrotic cells (N). Begin of disruption and atrophy of testicular
316 structures with thickening of basement membranes (T); (IV) 40 U/kg dose: prominent disruption
317 and atrophy with disintegrated basement membranes and non-uniform arrangements of the
318 tubules.

319 (-) absent; (+) mild; (++) moderate; (+++) severe

Fig. 1. Representative images of sperms in the various groups after H & E staining



(I) Control; (II) 10 U/kg onabotA; (III) 20 U/kg onabotA and (IV) 40 U/kg onabotA
(A) Abnormally flat head; (B) detached head; (C) coiled tail

Fig. 2. Semen quality (RRT ratio) and levels of antisperm antibodies after onabotA injections in the testes of the growing rat

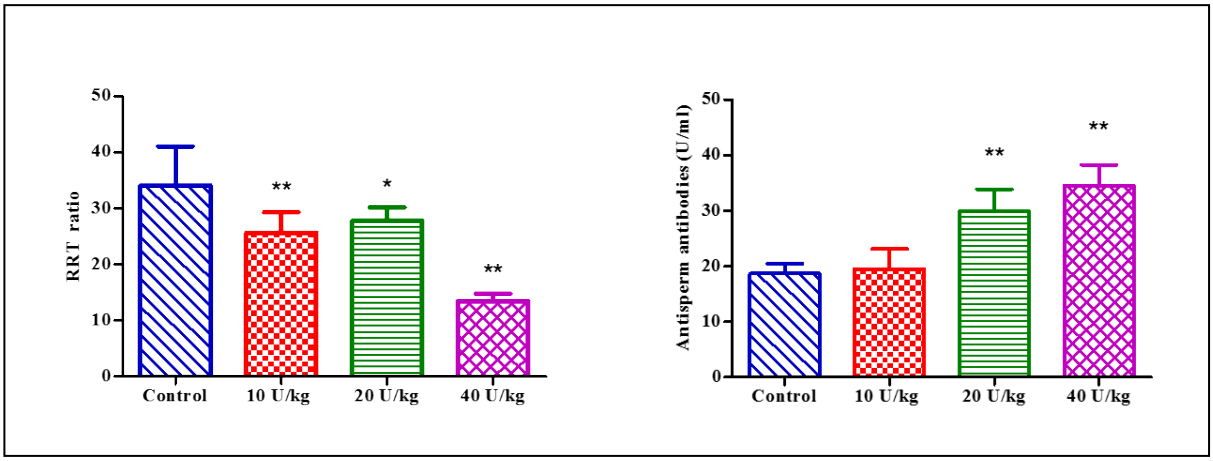


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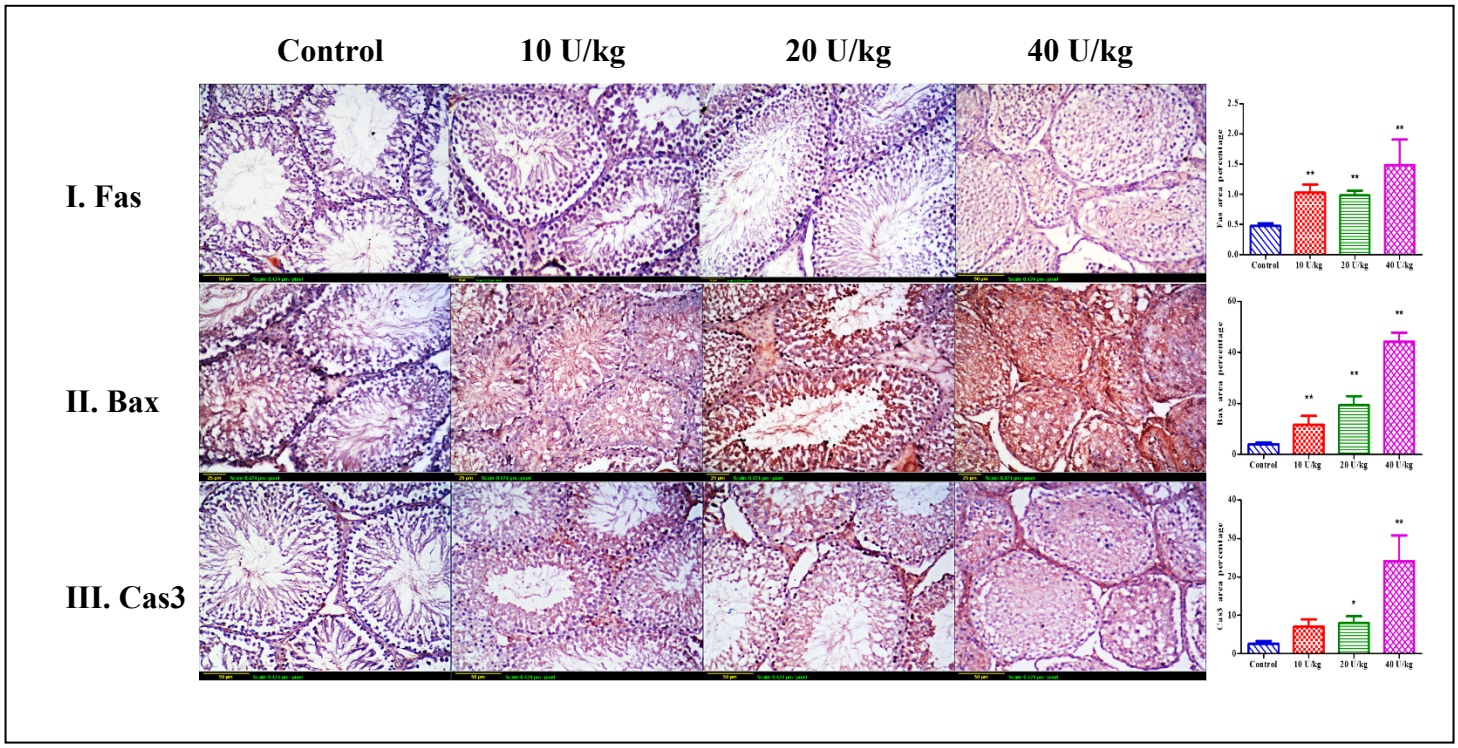
Fig. 3. Representative images of the testes of the growing rat after immunohistochemical examination of various apoptosis markers (x100)



(I–III): Immunohistochemical detection of Fas, Bax and Cas3
Control gp: minimal expression (brown color); BTX gps: dose-dependent increase in expression. The bar charts represent results of the image analysis performed by examining 6 fields/slide.

* $p<0.05$ compared to control group
** $p<0.01$ compared to control group

Fig. 4. Changes in DNA content (ploidy) after BTX injection

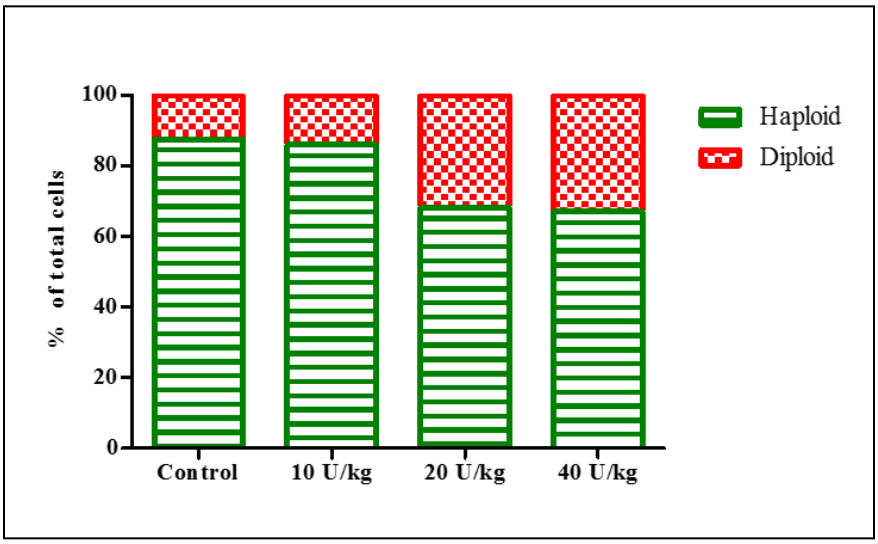
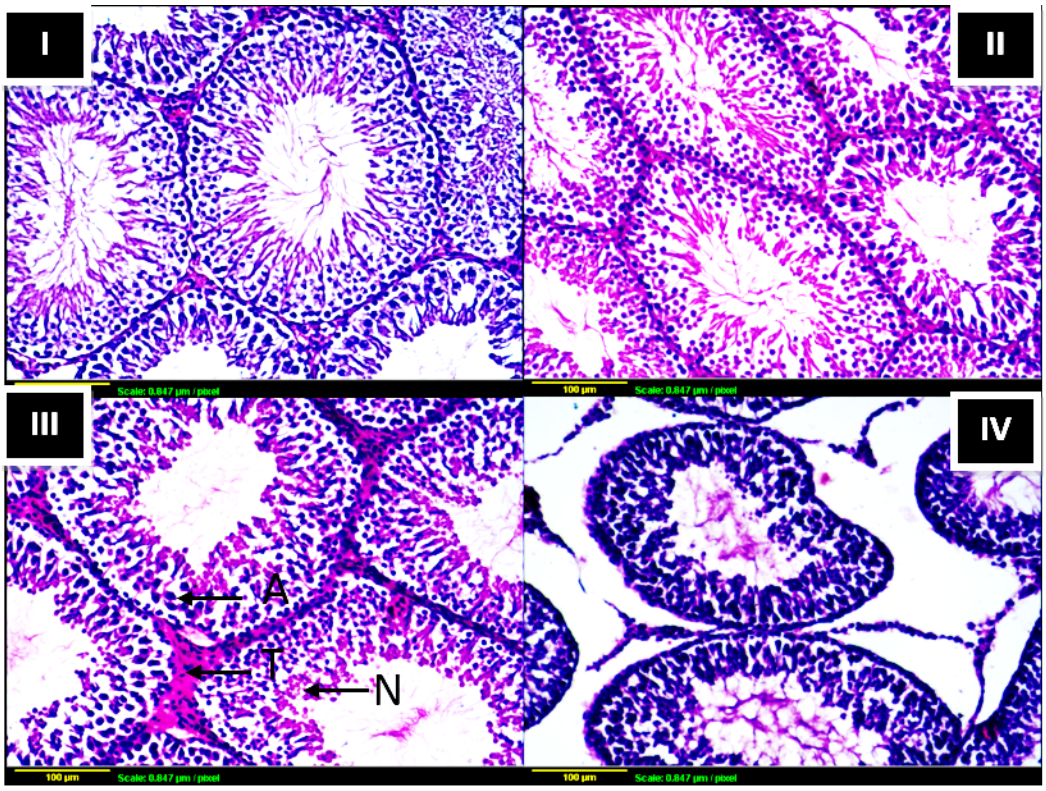


Fig. 5. Histopathological findings in the testes of the growing rat after H & E staining (x100)



	Control	10 U/kg onabotA	20 U/kg onabotA	40 U/kg onabotA
Disruption of spermatocytes	-	+	++	+++
Apoptosis of spermatocytes	+	+	++	+++
Necrosis of spermatocytes	-	+	++	++
Testicular atrophy	-	-	+	+++

(I) Control: uniformly sized tubules, lined by regularly arranged rows of spermatogenic cells at different stages of maturation; (II) 10 U/kg dose: similar to control; (III) 20 U/kg dose: marked increase in apoptotic (A) and necrotic cells (N). Begin of disruption and atrophy of testicular structures with thickening of basement membranes (T); (IV) 40 U/kg dose: prominent disruption and atrophy with disintegrated basement membranes and non-uniform arrangements of the tubules.

(-) absent; (+) mild; (++) moderate; (+++) severe

Table 1

Effects of the various onabotA injections on fertility indices of the growing testes

Group	Sperm count (million/ml)	Motile sperms (%)	Live sperms (%)	Normal sperm Morphology (%)	Total testosterone (ng/ml)
Control	38.33 ± 11.26	89.36 ± 1.60	92.67 ± 3.72	92.67 ± 1.03	3.80 ± 0.39
10 U/kg BTX	23.41 ± 4.90**	61.33 ± 8.12**	88.67 ± 6.77	86.67 ± 5.16	3.68 ± 0.47
20 U/kg BTX	10.10 ± 0.21**	60.24 ± 7.38**	81.21 ± 14.14	85.67 ± 16.01	3.99 ± 0.26
40 U/kg BTX	9.33 ± 2.70**	50.28 ± 3.08**	80.00 ± 10.95	85.50 ± 0.55	3.69 ± 0.29

n = 6

** $p<0.01$ compared to control group