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Detection of sperm DNA damage in a Benign Prostatic Hyperplasia model in rats

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Summary

The structure, function and size of the prostate gland affect fertility, so infertility may develop in benign prostatic hyperplasia (BPH). Androgen levels have an important role in BPH pathogenesis. We administered testosterone (3 or 5 mg/kg daily for 30 days) to rats to study the effects on sperm parameters and sperm DNA damage. We found sperm motility to be decreased in testosterone-treated groups compared to control groups ($p=0.006$) and testosterone significantly increased the proportion of abnormal sperm ($p=0.001$). There were no significant differences in sperm density between the groups ($p>0.05$) but there was significantly more sperm DNA damage in testosterone-treated groups ($p<0.001$). We conclude that testosterone can cause sperm DNA damage in a dose- and time-dependent manner.

Zusammenfassung

Erkennung von DNA-Schäden in Spermien im "Gutartige Prostatahyperplasie-Modell" bei Ratten

Struktur, Funktion und Größe der Prostata beeinflussen neben anderen Faktoren die Fruchtbarkeit. Daher kann sich bei benigner Prostatahyperplasie (BPH) eine Unfruchtbarkeit entwickeln. Die Androgenspiegel spielen eine wichtige Rolle bei der BPH-Pathogenese. In dieser Studie sollte untersucht werden, wie sich Spermienparameter und Spermien-DNA-Schäden in Ratten, denen Testosteron verabreicht wurde, entwickeln. Die Ratten wurden einer Kontrollgruppe und zwei Gruppen mit Gaben von 3 bzw. 5 mg/kg Testosteron täglich über 30 Tage zugeteilt. Die Spermienmotilität der Tiere, die mit Testosteron behandelt wurden, sank im Vergleich zu jener der Kontrolltiere ab ($p=0,006$). Zusätzlich wurde festgestellt, dass die Testosterongabe den Anteil abnormer Spermien signifikant erhöhte ($p=0,001$). Es gab keine statistisch signifikanten Unterschiede zwischen den Gruppen hinsichtlich der Spermiendichte ($p>0,05$). Die Schädigung der Spermien-DNA war in den Gruppen, die mit Testosteron behandelt worden waren, signifikant stärker ausgeprägt ($p<0,001$). Im BPH-Modell unter Verwendung von Testosteron wurden zwar eine erhöhte DNA-Schädigungsrate der Spermien, verringerte Beweglichkeit der Spermien und ein erhöhter Anteil abnormer Spermien nachgewiesen, die Spermiendichte blieb aber unverändert.

Abbreviations: BPH = benign prostatic hyperplasia; DHT = Dihydrotestosterone; GDI = Genetic Damage Index; PBS = phosphate-buffered saline; ROS = reactive oxygen species

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Introduction

The prostate has an important role in the success of fertilization in most mammalian species. Prostate fluid provides sperm viscosity in the female genital tract and increases sperm movement and fertilization by neutralizing the internal pH of the uterus (Guyton & Hall 1996). The structure, size and function of the prostate gland are important in fertility. Growth factors (Sreenivasulu et al. 2018), infectious agents (Breyer et al. 2016) and the amount and type of circulating androgens (Zhang et al. 2018) affect the size of the prostate gland and androgens have a central role in benign and malignant prostate enlargement (Knobil & Neill 1998). A certain level of prostatic androgen is required to control the growth of prostate tissue: when it falls below normal there is significant prostate involution (Yurdakul & Güven 2006; Ho & Habib 2011; Özden & Gökkaya 2015).

Benign Prostatic Hyperplasia (BPH) (Polat et al. 1997) is hyperplasia of the stromal and epithelial layers of the prostate. The levels of androgens in puberty and the senescent period have an important role in the pathogenesis of BPH. Testosterone and its more active form, Dihydrotestosterone (DHT), trigger the initiation of BPH process. DHT is involved in the development of normal prostate and the aetiopathogenesis of BPH. It is metabolized by 5 alpha-reductase enzymes bound to the nuclear membrane of the prostate. DHT and testosterone bind to the same androgen receptor (McConnell 1995) but DHT is more potent than testosterone. It has a higher affinity to the androgen receptor (Isaacs & Coffey 1989) and stimulates the expression of more genes involved in the development of BPH, eventually leading to BPH.

Patients with BPH have significant health problems, such as abnormally frequent urination, nocturia, urinary retention, difficulty in urination, urinary incontinence, urinary hesitancy and urinary tract obstruction (Jønler et al. 1994). All of them could potentially contribute to infertility. There are no data in the literature on the effects of BPH on sperm production, sperm quality and sperm DNA damage. We have used the BPH model created with testosterone for the first study of the effect of testosterone on sperm motility, sperm density, abnormal sperm rate and sperm DNA damage.

Materials and methods

Animals

Adult, pathogen-free, male Albino Wistar rats (average weight 200 g, 15–16 weeks old) were obtained from Van Yuzuncu Yil University. Animals were fed *ad libitum* and kept with 12 hours of light and 12 hours of dark per day. The living areas had an average temperature of 26 °C and 60 % relative humidity.

Groups

We used 18 Albino Wistar rats, randomly divided into 3 groups. The study was undertaken under agreement no. 2016/04 of Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee, dated 05/05/2016.

Group I (n=6): Control. No application was made to affect the physiological condition.

Group II (n=6): 3 mg/kg testosterone propionate (Sustanon; Aspen Pharma, Bridgewater, USA) administered intramuscularly daily for 30 days.

Group III (n=6): 5 mg/kg testosterone propionate administered intramuscularly daily for 30 days.

After the applications, blood was taken from the hearts of the rats under anaesthesia and the animals were sacrificed.

Spermatological examination

Motility examination: The sperm sample was obtained by epididymis puncture immediately after sacrifice and was placed on a glass slide on the heating table set to 38 °C. The coverglass was closed at an angle of 45° and motility (in %) detected by microscopy at 40x magnification (Hafez & Hafez 2013).

Density analysis: After epididymal puncture, 0.1 ml of sperm sample was added to Eppendorf tubes with 0.5 ml Hayem solution (Norateks, İstanbul, Turkey). Sperm count per ml was calculated on a Thoma cell counting chamber (Hafez & Hafez 2013).

Abnormal sperm ratio: The sperm obtained by epididymis puncture was transferred to Eppendorf tubes with 0.5 ml Hancock solution (Norateks). At least 400 sperm samples were examined at 40x magnification to determine the ratio (Hafez & Hafez 2013).

Sperm DNA damage (Comet assay)

The DNA damage in rat sperms was measured by single cell gel electrophoresis (Comet assay) at neutral conditions. The method was optimized according to our previous studies and resembled several published protocols (Li et al. 2008; Bucak et al. 2010).

When the experiment was completed, the sperm was collected and gave an approximate volume of 1 ml. It was centrifuged at 600 x *g* and 4 °C for 10 min. and the supernatant was gently discarded. The sperm at the bottom of the tubes was washed twice with phosphate-buffered saline (PBS) (Ca^{2+}-, Mg^{2+}-free) (Merck, Darmstadt, Germany) under the same conditions. PBS [(Ca^{2+}-, Mg^{2+}-free), 1 ml] was added to the tubes after the second washing.

Precleaned slides were pre-coated with 1 % normal melting agarose (Merck) and dried for 24h at room temperature. Roughly 100,000 cells were mixed with 0.75 % low melting agarose (Merck) at 37° C and the mixtures dropped onto slides and coated with cover-

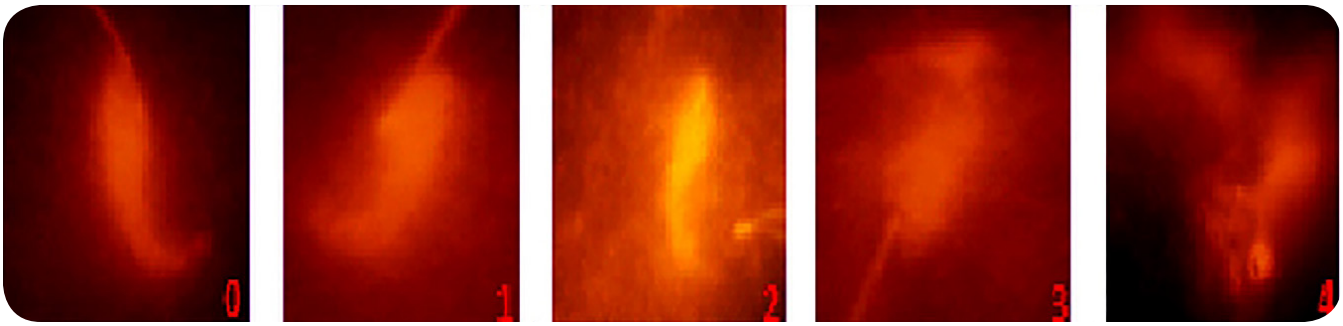

Fig. 1: Assessment of sperm DNA damage, fluorescence microscopy, 0 = no damage; 1 = low, 2 = moderate, 3 = high, 4 = ultra high damage / Bewertung der DNA-Schädigung in Spermien, Fluoreszenzmikroskopie; 0 = kein Schaden; 1 = gering-, 2 = mittel-, 3 = hoch- und 4 = höchstgradiger Schaden

slips. Three slides were used for each sample. Slides were solidified for 30 min at 4 °C, then the coverslips were discarded and the slides treated with fresh cold lysis buffer for 60 min. Proteinase K (20 µg/ml) (Merck) was added after 60 min and the slides were transferred to an incubator for 2 h at 37 °C before being transferred to the electrophoresis unit at 4 °C for 25 min. Electrophoresis was conducted at 26 V for 25 min, then the slides were dried at room temperature and stained with ethidium bromide (8 µl/ml) (Merck).

In the randomly chosen samples, 2,000 cells of each group were examined with a fluorescence microscope. DNA damage was classified into five groups according to the fluorescence intensity (UD = undamaged, 0; Type 1 = low damage, 1; Type 2 = moderate damage, 2; Type 3 = high damage, 3; and Type 4 = ultra high damage, 4) (Figure 1).

The Genetic Damage Index was calculated as:

GDI = [(0 x Type 0) + (1 x Type 1) + (2 x Type 2) + (3 x Type 3) + (4 x Type 4)] (Tables 4, 5).

Total testosterone

Total testosterone levels of the rats were determined with the ACCESS Testosterone Reagent kit 33560 (Beckman Coulter, Brea, USA) according to the manufacturer's instructions.

Statistical analysis

Before the significance tests, all variables (Sperm Motility Rate, Abnormal Spermatozoa Rate, Sperm Density, Sperm DNA Damage Index, Total Testosterone) were examined by the Shapiro-Wilk test for normal distribution and by the Levene's test for homogeneity of variance. The Kruskal-Wallis test was used to analyse the differences between the variables. The Mann-Whitney U test was used as the advanced test for the variables in which differences between groups were significant. The level of significance was set at 5 % for all statistical analysis. We used Statistical Package for Social Sciences (SPSS) 14.01 (IBM Corporation).

Results

Sperm motility rate

We found significant differences in sperm motility rates between the groups, as shown by chi-squared analysis (Table 1).

Abnormal spermatozoa rate

We also found significant differences in the rates of abnormal spermatozoa rates between the groups, as shown by chi-squared analysis (Table 2).

Sperm density

We found no significant differences in sperm density between the groups by chi-squared analysis (Table 3).

DNA damage in sperm

We compared sperm DNA damage according to the Genetic Damage Index (GDI) in the control group and the 3 mg/kg and 5 mg/kg dose groups and the paired

Tab. 1: Sperm Motility Rate / Spermienmotilitäts-Rate

Group	Mean	Standard Error	Standard Deviation	Median	p-value*	**
Control	83.33 %	2.11	5.16	80.00 %		a
3 mg/kg	66.67 %	3.33	8.16	65.00 %	0.006	b
5 mg/kg	48.33 %	10.46	25.63	50.00 %		b

* Kruskal-Wallis test; ** different letters indicate statistically significant differences between groups (Mann-Whitney test; p<0.001). / * Kruskal-Wallis Test; ** unterschiedliche Buchstaben bedeuten statistisch signifikante Unterschiede zwischen den Gruppen (Mann-Whitney Test; p<0,001).

Tab. 2: Abnormal Spermatozoa Rate / Rate abnormaler Spermatozoen

Group	Mean	Standard Error	Standard Deviation	Median	p-value*	**
Control	5.79 %	0.22	0.53	5.75 %	0.001	c
3 mg/kg	11.75 %	1.02	2.51	11.88 %		b
5 mg/kg	16.96 %	1.01	2.47	17.00 %		a

* Kruskal-Wallis test; ** different letters indicate statistically significant differences between groups (Mann-Whitney test; p<0.001). / * Kruskal-Wallis Test; ** unterschiedliche Buchstaben bedeuten statististisch signifikante Unterschiede zwischen den Gruppen (Mann-Whitney Test; p<0,001).

Tab. 3: Sperm Density (/ml) / Spermiendichte (/ml)

Group	Mean	Standard Error	Standard Deviation	Median	p-value*
Control	2.37×10^9	0.22	0.54	2.35×10^9	0.645
3 mg/kg	2.92×10^9	0.44	1.09	2.75×10^9	
5 mg/kg	2.82×10^9	0.34	0.83	2.67×10^9	

group comparisons showed a statistically significant difference at p<0.001 (Table 4).

Total testosterone

Total testosterone ratios were compared by chi-squared analysis. We found no statistically significant difference (p>0.05) between the groups in which testosterone had been administered, although there was a statistically significant difference between the control group and the two testosterone-treated groups (p<0.005) (Table 5).

Benign Prostatic Hyperplasia

Macroscopically, volumetric enlargement was observed in the prostates of rats that had received administered testosterone. In the microscopic view, it was determined that the epithelial cell layer and stromal cell spaces were enlarged in the testosterone-treated groups (Fig 2).

Discussion

The structure, size and function of the prostate gland play an important part in fertility. Androgens such as testosterone are involved in the development of BPH (Ho & Habib 2011) but the effects on sperm production, sperm quality and sperm DNA damage are not known. We have investigated the effect of testosterone on sperm motility, sperm density, abnormal sperm rate and sperm DNA damage in a rat BPH model.

Various agents can be used to establish a model of BPH in the rodent prostate, of which testosterone propionate is the most commonly used (Hieble 2011). Auger-Pourmarin et al. (1998) reported that Sprague-Dawley rats treated daily with testosterone propionate (3 mg/kg per day) for 15 days developed BPH, with similar results reported by Julia-Guilloteau et al. (2006). Cho et al. (2007) created a BPH model in Wistar rats by treating them with testosterone propionate (3 mg/kg) for 4 weeks. We administered testosterone propionate daily at 3 mg/kg and 5 mg/kg for 30 days to induce BPH. Macroscopic examination showed an increase in prostate volume depending on the testosterone dose. Our finding of BPH is compatible with the results of Auger-Pourmarin et al. (1998), Julia-Guilloteau et al. (2006) and Cho et al. (2007).

Sperm DNA damage has two main causes (Aitken & De Iuliis 2010). The first is a defect in spermiogenesis resulting from the premature release of dysfunctional cells from the germinal epithelium due to the presence of excess chromatin, abnormal protamination and high levels of unsaturated fatty acids in the nucleohistone. The second cause is oxidative stress.

Testosterone causes abnormal protamination, the presence of excess chromatin (Bennetts & Aitken 2005) and oxidative stress (Shokri et al. 2014). The

Tab. 4: Sperm DNA Damage Index / Spermien-DNA-Schäden Index

Genetic Damage Intensity						Total	GDI	Damage Rate %	p-value*	**
	Undamaged	Damaged								
	0	1	2	3	4					
Control	2,084	58	18	6	2	2,168	120	5.5351	<0.001	a
3 mg/kg	2,063	120	38	28	27	2,276	388	17.0475		b
5 mg/kg	1,749	185	48	44	38	2,064	565	27.374		c

* Kruskal-Wallis test; ** Different letters indicate statistically significant differences between groups (Mann-Whitney test; p<0.001). / * Kruskal-Wallis Test; ** Unterschiedliche Buchstaben bedeuten statistisch signifikante Unterschiede zwischen den Gruppen (Mann-Whitney Test; p<0,001).

Tab. 5: Total Testosterone / Gesamt-Testosteron

Group	Control (ng/ml)	3 mg/kg (ng/ml)	5 mg/kg (ng/ml)	p-value
Average Testosterone Level	167.78[a]	> 1,600[b]	> 1,600[b]	<0.005

a, b: Different letters demonstrate significant differences (p<0.005). / a, b: Unterschiedliche Buchstaben bedeuten statistisch signifikante Unterschiede (p<0,005).

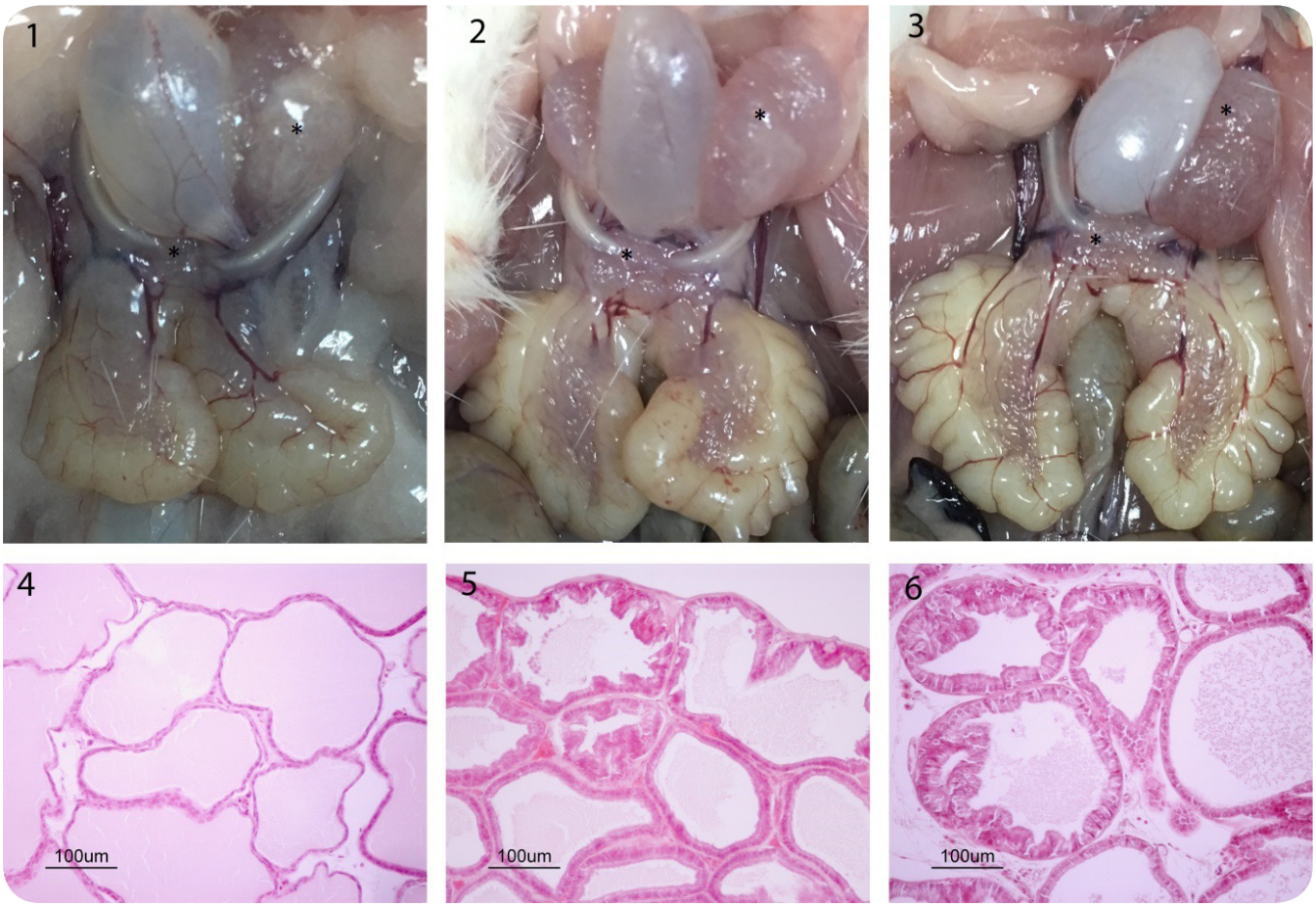

Fig. 2: Macroscopic and microscopic view of BPH. 1. Control group prostate macroscopic view; 2. 3 mg/kg dose testosterone prostate macroscopic view; 3. 5 mg/kg dose testosterone prostate macroscopic view; 4. Control group prostate microscopic view; 5. 3 mg/kg dose testosterone prostate microscopic view; 6. 5 mg/kg dose testosterone prostate microscopic view. The dorsal lobe, ventral lobe, and coagulating glands of the prostate are indicated by asterisks. / Makroskopische und mikroskopische Ansicht von BPH. 1. Makroskopisches Bild der Prostata bei der Kontrollgruppe; 2. Makroskopisches Bild der Prostata bei der Gruppe mit Gabe von 3 mg/kg Testosteron/Tag; 3. Makroskopisches Bild der Prostata bei der Gruppe mit Gabe von 5 mg/kg Testosteron/Tag; 4. Mikroskopisches Bild der Prostata bei der Kontrollgruppe; 5. Mikroskopisches Bild der Prostata bei der Gruppe mit Gabe von 3 mg/kg Testosteron/Tag; 6. Mikroskopisches Bild der Prostata bei der Gruppe mit Gabe von 5 mg/kg Testosteron/Tag. Die Prostataanteile sind mit Sternchen markiert.

increased sperm DNA damage we observe may be caused by the effects of testosterone on protamination and chromatin but further studies are needed to resolve the factors responsible.

Hydrogen peroxide, an oxidative stress agent, significantly increases DNA damage in fish, human and mussel sperm (Lee & Scott 2003; Zilli et al. 2003; Nasr-Esfahani et al. 2005). The endogenous aministration of testosterone, a highly potent anabolic agent, reduces gonadotropin levels and total testosterone levels and thus adversely affects the reproductive system (Torres-Calleja et al. 2001). Increased DNA damage may result from the effect of increasing testosterone hydrogen peroxide or from the alteration of gonadotropin and total testosterone levels (Lee & Scott 2003; Zilli et al. 2003; Nasr-Esfahani et al. 2005).

Sperm DNA damage is present in control groups at approximately 2–5 % (Suresh et al. 2010; Trivedi et al. 2010; Shokri et al. 2014), which accords well with the rate of 5 % in our control group.

The daily administration of 50 mg/kg clomiphene citrate to infertile men for 3 months increases FSH, LH and testosterone and average sperm density from 13.3×10^9 to 28.7×10^9 /ml (Rönnberg 1980), while administering zinc sulphate increases testosterone level and testosterone sperm density from 8×10^9 /ml to 20×10^9 /ml in human oligospermia cases (Netter et al. 1981). We found no statistically significant difference in sperm density rates between the groups ($p>0.05$). The spermatogenesis cycle in rats is approximately 50 days. Our failure to detect a change in sperm density may relate to the fact that we administered testosterone for 30 days instead of 50 days.

We found that administration of testosterone increased sperm DNA damage and abnormal sperm rate in a dose-dependent manner ($p<0.001$). Testosterone accelerates cell metabolism and causes an increase in the level of reactive oxygen species (ROS). The main target of ROS is the lipid membranes in testicular tissue and sperm cells. (Alonso-Alvarez et al. 2007;

Erdemir et al. 2012). ROS-mediated lipid peroxidation is thought to cause damage to the lipid matrix, deterioration of sperm morphology and increased sperm DNA damage.

Sperm DNA integrity is important for proper fertilization, successful transmission of genetic material to future generations, development of a high-quality embryo and continuity of pregnancy and helps minimize the risk of congenital anomaly. The rate of sperm DNA damage is inversely related to fertilization potential, pregnancy and live birth rates (Sawyer et al. 2003; Simon et al. 2013; Duarte et al. 2017). Our data suggest that testosterone may cause sperm DNA damage. This effect should not be ignored in testosterone therapies in infertility clinics to support spermatogenesis.

In our BPH model, the exogenous administration of testosterone to rats resulted in increased sperm DNA damage, an increased proportion of abnormal sperm and decreased sperm motility, although sperm density was not affected.

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Fazit für die Praxis
Im "Gutartige Prostatahyperplasie-Modell" bei Ratten waren nach Gabe von Testosteron, abhängig von der Dauer und der Dosis, die DNA-Schädigungsrate der Spermien und die abnormale Spermienrate erhöht, die Motilität war verringert. Die Spermiendichte wurde hingegen nicht beeinflusst.

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