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Abstract

Background: Indomethacin is commonly used as a nonsteroidal anti-inflammatory drug (NSAID) to treat inflammation, arthritis and joint pains. Unfortunately, it has a wide range of adverse effects on the physiological system, including gonads. This study aimed to assess possible beneficial effects of black tea extract (BTE) against indomethacin-induced alteration of gonadal hormone levels in male rats.

Materials and methods: Adult male rats were divided into Group I (control), Group II (indomethacin, 5 mg/kg body weight [bwt.]; i.p., 21 days), Group III (BTE, 2.5 g tea leaf/dL of water, i.e. 2.5% of aqueous BTE, orally, 21 days) and Group IV (indomethacin + BTE, 21 days). Sperm count and motility, serum luteinising hormone (LH), follicle-stimulating hormone (FSH) and testosterone, along with histopathology of testes were studied. One-way ANOVA, followed by post-hoc t-test were conducted.

Results: Indomethacin-treated rats showed significant decrease in testicular weight, sperm count, sperm motility, serum gonadotropins and testosterone concentrations. Histopathology of the testes showed tortuous and distorted seminiferous tubules, marked thickening of the tubular basement membrane, reduced spermatogenesis process (>30%) and marked decrease in the number of interstitial cells of Leydig in indomethacin-treated rats. Interestingly, rats supplemented with BTE showed remarkable improvements in testicular weight gain, sperm count and motility, serum gonadotropins and testosterone concentrations, along with testicular histopathology.

Conclusions: The results suggest that BTE might have potential ameliorative effects against sub-chronic indomethacin-induced alteration of gonadal hormone levels in male albino rats.

Keywords: black tea extracts; gonadotropins; histopathology; indomethacin; testes; testosterone.

Introduction

Indomethacin is a known nonsteroidal anti-inflammatory drugs (NSAID) that is routinely used to treat moderate to severe osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, musculoskeletal disorders and cancers [1]. It has many side effects, such as gastritis, ulcers, and cardiovascular problems, including high blood pressure and stroke as well as some adverse reports on male gonadal system possibly through the inhibition of the synthesis of prostaglandin [2]. Prostaglandins E and F play an important role in sperm metabolism and its functions; they also increase the contractility of the epididymal tubule smooth muscle layer [3]. Further, the judicious use of prostaglandins enhances circulatory levels of luteinising hormone (LH), follicle-stimulating hormone (FSH) and prolactins, which are essential for the maintenance of normal reproductive functions [4]. Das et al. have reported indomethacin-induced generation of oxidative free radicals and potential damages to biological functioning [5]. The oxidative free radicals may cause DNA damage, which may possibly impair spermatogenesis and sperm function and lead to male infertility [6].

Meanwhile, black tea is considered as a dietary source of antioxidant nutrients, such as theaflavins and thearubigins, along with unoxidised catechins. By virtue of their singlet oxygen quenching ability, they against oxidative stress and are thus effective in the maintenance of various physiological functions. Black tea polyphenols act as

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preventive agents against numerous physiological disorders by disrupting the electron chain and inhibiting the progression of various ailments [7]. Hence, the present study was undertaken to assess the effect of BTE supplementation on male gonadal hormone profile and testicular histopathology of rats treated with indomethacin in vivo.

Materials and methods

Experimental animals

Adult (aged 60–70 days) laboratory-bred male Wister strain rats (140 ± 5 g) were fed with laboratory stock diet and water ad libitum for 7 days. The animals were kept in air-conditioned animal house maintained at temperatures ranging from 22 °C to 24 °C. The acclimatised animals were divided into four groups of six animals each, and three animals were each kept in a metabolic wire cage (60 cm × 30 cm × 20 cm). Group I rats were healthy controls. Group II rats were treated intraperitoneally with indomethacin drug (Yerrow Chem Products, Mumbai, India) at a dose of 5 mg/kg of body weight for 21 days [8]. A dose of black tea extracts (BTE) consisting of 2.5 g tea leaf/dL of water (i.e. 2.5% of aqueous BTE) was orally administered to Group III rats for 21 days [9]. Group IV rats were treated with both indomethacin and BTE simultaneously for 21 days; indomethacin was given intraperitoneally and BTE was given orally.

Preparation of 2.5% aqueous BTE

The aqueous BTE (*Camellia sinensis*) was prepared from cut, torn and crushed orange pickoe grade black clonal tea. It was completely processed and supplied by Tocklai Experimental Station, Jorthat, Assam. A fresh 2.5% aqueous BTE solution was prepared every day following the method of Wei et al. [9]. To 500 mL of boiling water, 25 g of black tea leaves were added and steeped for 15 min. The infusion was cooled to room temperature and then filtered. The tea leaves were extracted for second time with 500 mL of boiling water and filtered; both the filtrates were combined to obtain 2.5% aqueous BTE (2.5 g of tea leaf/100 mL water).

Experimental protocols including hormonal assays

The body weights of all animals were recorded on the 1st day of the indomethacin treatment and on the day of sacrifice. At the end of the last dose, the animals were sacrificed after overnight fasting by decapitation between 0900 AM and 1130 AM to avert the circadian rhythm [10]. Blood was collected in centrifuge tubes, kept at room temperature for about 2 h and centrifuged at 1500 × *g* for 15 min to collect serum. Serum was then used for the estimations of LH, FSH and testosterone hormone concentration by using the CLIA method [11–13]. One side of each testis was dissected out, washed with ice-cold normal saline immediately, wiped clean and kept in a tissue container and stored at − 20 °C prior to the analysis. The testicular weight was determined after dissecting out, and washing in ice-cold saline in a single pan balance (ATCO. M. No. D2RS02-W). Testicular somatic index, the ratio of testicular weight to body weight of rats before sacrifice, was obtained.

All the experimental procedures were performed in accordance with the approval of the Institutional Animal Ethics Committee (1169/ac/08/CPCSEA) under strict compliance with the guidelines for experimental studies released by the Committee for the Purpose of Control and Supervision of Experiments on Animals.

Determination of sperm count and motility

Both the sperm count and motility were determined by the Neubour counting method [14]. Briefly, a sperm count was performed on sperm samples collected from the cauda epididymis. The cauda portion was separated and placed in 0.25 M sucrose solution. The epididymal tubes were punctured with a fine hypodermic needle and spermatozoa were extruded by squeezing. Sperm heads within a 10 square area were counted in the same manner as blood cells counted in a hemocytometer; their number represents their concentration in millions/mL.

Histopathological studies

The testes of the experimental rats were dissected out immediately and subjected to histopathological evaluations [15]. The microscopic study on routine stain (hematoxylin and eosin stain) was done, and the following changes of the tissues of control and various treated groups were observed; viz. atrophy, degeneration, necrosis, inflammation, tumorogenecity, and so on. Photomicrographs were taken out with the help of the Magnifying Image Processing System (MIPS) that was connected to personal computer.

Statistical analysis

Data were expressed as mean ± standard deviation of the mean. Statistical comparisons were performed by one-way ANOVA, followed by post-hoc t-test. The values were considered as statistically significant when $p < 0.05$.

Results

Gravimetric study

Among all the four groups, indomethacin-treated rats (Group II) and the BTE-treated rats (Group III) showed the lowest and highest percentage body weight gain, respectively (Table 1). The results also indicated the lowest testicular weight and testicular somatic index in indomethacin-treated rats (Group II), but interestingly, in BTE-supplemented rats (Group IV), remarkable improvements in both testicular weight and testicular somatic index were observed (Table 1).

Table 1: Effect of BTE (2.5 g tea leaf/dL of water; oral) on gravimetric parameters in male albino rats after indomethacin treatment (5 mg/kg bwt.; i.p.)

Gravimetry, Grams	Group I	Group II	Group III	Group IV	F-ratio	p-Value
IBW	139.6 ± 2.33^{a}	139 ± 3.28^{a}	138.3 ± 3.88^{a}	139 ± 5.17^{a}	0.122	0.946
FBW	221.3 ± 7.86^{a}	144.6 ± 3.32^{b}	224.8 ± 6.46^{a}	171.3 ± 7.76^{c}	210.212	0.00
% wt gain	57.05 ± 7.27^{a}	4.05 ± 0.77^{b}	62.69 ± 8.10^{c}	23.31 ± 6.25^{d}	117.921	0.00
Testicular wt	1.76 ± 0.22^{a}	1.20 ± 0.24^{b}	1.83 ± 0.22^{c}	1.63 ± 0.33^{d}	7.053	0.02
Testicular Somatic Index	0.0080 ± 0.0008^{a}	0.0076 ± 0.001^{b}	0.0081 ± 0.0007^{a}	0.0079 ± 0.001^{c}	0.136	0.938

bwt., body weight; IBW, initial body weight; FBW, final body weight. Group I, control; II, indomethacin; III, black tea extracts (BTE); IV, indomethacin + BTE. Each value is mean ± SEM of six observations in each group. In each row, values with different superscripts (a, b, c, d) were significantly different from one another ($p < 0.05$).

Endocrine assay

As shown in Table 2, there were significant reductions of LH, FSH and testosterone concentrations in Group II rats compared with the controls in Group I; however, simultaneous supplementation with BTE (Group IV) increased all the three hormone concentrations compared with Group II. Figure 1A–C depicts the percentage change decrease of serum LH, FSH and testosterone levels in Group II rats (indomethacin) compared with their respective controls (E-1). There were also remarkable improvements of percentage changes in all the three hormonal parameters of Group IV rats (indomethacin + BTE) (E-1 vs. E-3).

Sperm count and motility

Figure 2 depicts the percentage change reduction of sperm count (– 47.25%) and sperm motility (– 28.77%) in Group II (indomethacin, E-1) rats compared with their respective controls. Supplementation with BTE in Group IV rats clearly showed percentage change improvements in both sperm count (– 25.59%) and sperm motility (– 13.54%) (E-3). Hence, sperm count and motility were improved with percentage increases of 21.66% and 15.23%, respectively, in Group IV rats compared with those in Group II.

Histopathology

In Figure 3, Group I (control) rat testes show a normal structure, with normal seminiferous tubules, fibrovascular stroma and normal spermatogenesis process, whereas tortuous and distorted seminiferous tubules, decreased spermatogenesis (> 30%), and a marked decrease in the number of interstitial cells of Leydig and focal necrosis were found in Group II rats treated with indomethacin. In Group III (BTE) rats, testes showed densely packed seminiferous tubules, and spermatogenesis appeared normal with the involvement of the entire lumen of the tubules. In Group IV (BTE + indomethacin) rats, the testes showed near normal spermatogenesis (up to 60%), with a loss of germ cell layer and a slight thickening of basement membrane.

Discussion

Gravimetry

Testicular weight is a valuable index of reproductive health. The toxic effect of indomethacin induces necrotic and degenerative changes, tortuous seminiferous tubules, marked thickening of basement membrane and inhibition of spermatogenesis and steroidal hormones. These may

Table 2: Effect of BTE (2.5 g tea leaf/dL of water; oral) on endocrine levels in male albino rats after indomethacin treatment (5 mg/kg bwt.; i.p.)

Sr. no	Hormones	Group I	Group II	Group III	Group IV	F-ratio	p-Value
1	LH, mIU/mL	0.106 ± 0.029^{a}	0.045 ± 0.025^{b}	0.105 ± 0.033^{a}	0.096 ± 0.042^{c}	91.834	0.000
2	FSH, mIU/mL	0.25 ± 0.054^{a}	0.2 ± 0.089^{b}	0.25 ± 0.054^{a}	0.2 ± 0.089^{c}	118.543	0.000
3	Testosterone, ng/dL	293.76 ± 13.48^{a}	136.31 ± 12.00^{b}	295.63 ± 16.81^{a}	252.10 ± 9.67^{c}	350.109	0.000

Group I, control; II, indomethacin; III, black tea extracts (BTE); IV, indomethacin + BTE. Each value is mean ± SEM of six observations in each group. In each row, values with different superscripts (a, b, c) were significantly different from each other ($p < 0.05$).

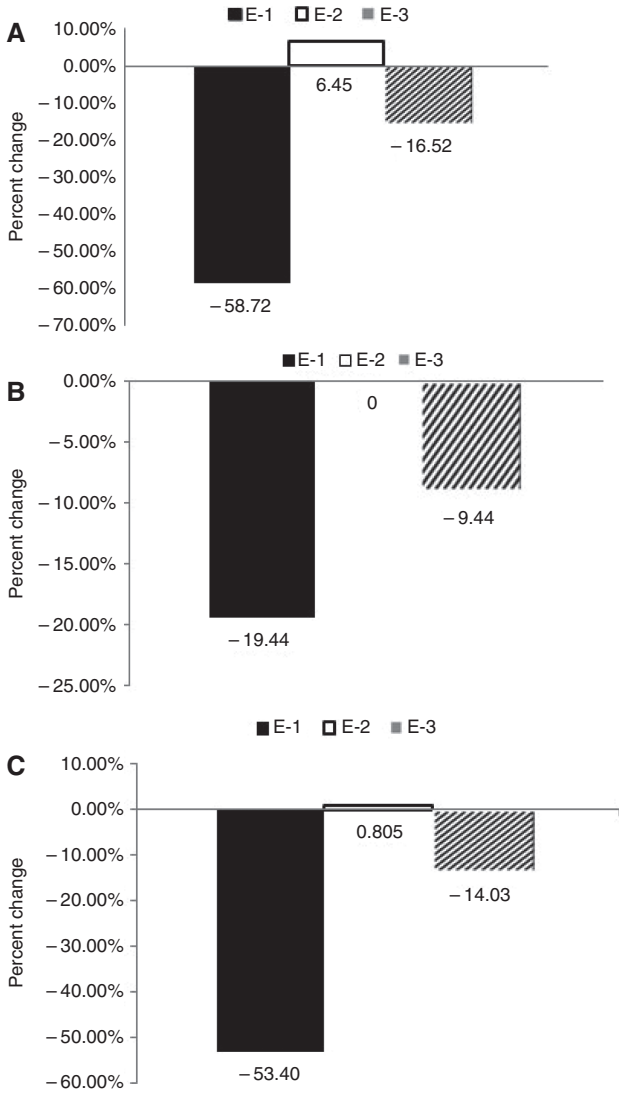

Figure 1: % Change effects of BTE.
(A) Percentage change effect of BTE on LH hormone concentration in Indomethacin-treated rats. E-1, Group I vs. Group II; E-2, Group I vs. Group III; E-3, Group I vs. Group IV. (B) Percentage change effect of BTE on FSH hormone concentration in Indomethacin-treated rats. E-1, Group I vs. Group II; E-2, Group I vs. Group III; E-3, Group I vs. Group IV. (C) Percentage change effect of BTE on testosterone hormone concentration in indomethacin-treated rats. E-1, Group I vs. Group II; E-2, Group I vs. Group III; E-3, Group I vs. Group IV.

lead to massive cellular loss from seminiferous tubules and reduced morphological parameters, such as tubular volume, length and diameter [16]. Diminution in testicular somatic index may be due to apoptotic cell death caused by low serum testosterone level as it is the prime regulator of normal growth of male reproductive organs [17]. The reduction of testicular weight indicates adverse morphological alterations in the seminiferous tubules [18]. The protective role of BTE in the indomethacin-induced

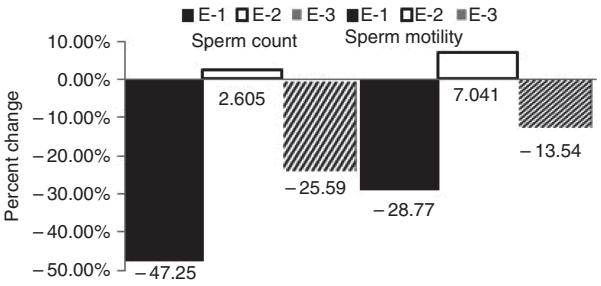

Figure 2: Percentage change effects of BTE on sperm count and motility in indomethacin-treated rats. E-1, Group I vs. Group II; E-2, Group I vs. Group III; E-3, Group I vs. Group IV.

alteration of testicular weight and testicular somatic index may be attributed to the antioxidant properties of polyphenol compounds of BTE, which may have protected the oxidatively sensitive testicular protein targets and prevented the formation of reactive oxygen species (ROS), thus altering the testicular protein metabolism [19].

Endocrine profile

Reduction of pituitary gonadotropin (FSH and LH) secretions in indomethacin-treated rats may be due to the functional disturbances in the hypothalamo-pituitary axis. The decreased FSH and LH leveld in the present study also demonstrates the extra-testicular targets of indomethacin. LH stimulates Leydig cells to produce the testosterone hormone; thus, a decrease in LH may also be a contributing factor explaining the low level of testosterone. Results indicate a possible hypogonadotropic hypogonadism due to sub-chronic indomethacin treatment [20]. FSH and testosterone are extremely essential for spermatogenesis, that is, testosterone and FSH bind to Sertoli cell receptors and facilitate target receptors on the Sertoli cells and help keep germ cells functional [21]. Testosterone plays a key role in the development of male reproductive tissues, such as the testes and prostate. Testosterone is needed for the continued production of different generations of germ cells in the seminiferous tubules. Therefore, the reduced testosterone levels in the present study may lead to the separation of germ cells from the epithelium of the seminiferous tubules [22].

One of the possible reasons behind the decrease in serum testosterone level in the present study may be the direct effect of indomethacin on the testicular tissue. The findings of the current study indicate that the accumulation of indomethacin content in testicular tissue enhanced oxidative stress. In turn, the high oxidative stress resulted in decreased cell viability of all types of

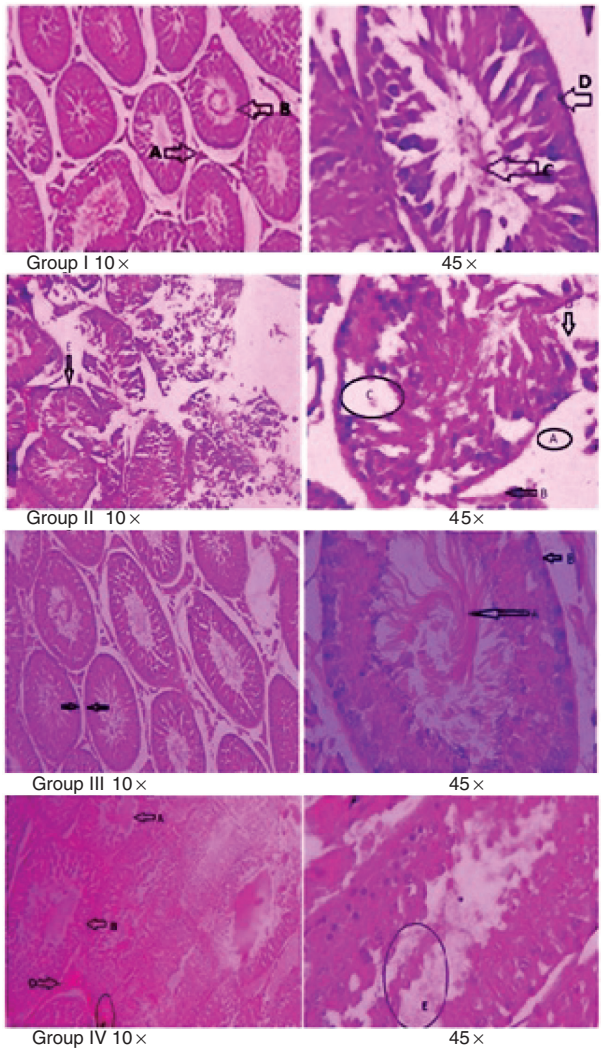

Figure 3: Testis section of Group I to Group IV rats.
Group I, testicular tissue showed normal architecture. The fibrovascular stroma (A) present between the seminiferous tubules contain the varying number of interstitial Leydig cells. The seminiferous tubules (B), spermatogenesis process (C) and basement membrane (D) appeared normal. Group II, (indomethacin-treated rats) showed distorted normal architecture with decreased Leydig cells (A), foci of fibrosis (B), loss of spermatogenesis process (C), foci of interstitial edema (D) and tortuous seminiferous tubules (E). Group III (BTE-treated rats) showed normal architecture with densely packed seminiferous tubules. Spermatogenesis filling up the entire lumen of the tubules (A) and basement membrane (B) appeared normal. Group IV (indomethacin + BTE treated rats) showed near normal architecture with interstitial edema (A), normal structure of seminiferous tubules (B), interstitial fibrosis (C), mild decrease in number of Leydig cells (D), and normal spermatogenesis process (E).

cells in the testicular tissue. The low cell viability and accelerated cell death not only resulted in decreased cell and tissue mass but also led to various abnormalities in sperm structure and function [17]. Steroidogenesis in Leydig cells need oxidative enzymes located in the mitochondria and endoplasmic reticulum. Hence, the entire process of steroidogenesis always remained in danger of ROS-induced assault [23]. The low levels of testosterone, FSH and LH also inhibit effective spermatogenesis and the development of seminiferous tubule, resulting in decreased functional sperms and low fertility [6]. Treatment with BTE reverses the testicular malfunctions due to the fact that indomethacin induced oxidative stress. BTE treatment also brought back normal gonadal homeostasis resulting from the presence of a large number of nutritional antioxidants, including ascorbic acid, α-tocopherol and large polyphenol compounds that are stable at stomach pH and pass on to the small intestines relatively unchanged. A past study reported that both testicular steroidogenesis and spermatogenesis are sensitive to oxidative stress; interestingly, the testes normally remain in low oxygen tension and prevent itself from free radical assault [24]. The polyphenolic compounds in BTE reduce stress-induced oxidative stress and stimulate testosterone within the testis. Vitamin E sensitises Leydig cell to LH and increases testosterone synthesis [25]. Hence, we can conclude that BTE containing polyphenols and antioxidant vitamins might augment the antioxidant defence system in the testes and mitochondrion protective nutrients and keep healthy redox balance in Leydig cells, thus preventing such cells from carrying out the oxidative inhibition of testosterone biosynthesis and increasing the rate of testosterone secretion [26].

Sperm count and motility

The decrease in both sperm count and motility in the indomethacin-treated (Group II) rats may be due to the destruction of testicular germ cells by membrane damage or macromolecular degradation, which are incurred by the ROS generated in the testes by indomethacin, leading to a significant decline in sperm count [27]. Oxidative stress disrupts steroidogenesis in Leydig cells and spermatogenesis, especially spermiogenesis [28]. The present study demonstrated that supplementation with BTE can have a positive role in improving sperm quality by protecting testicular cell membrane and mitochondria from indomethacin-induced oxidative stress [29].

Histopathological studies

We observed in histological studies that indomethacin can cross the blood testes barrier and facilitate the

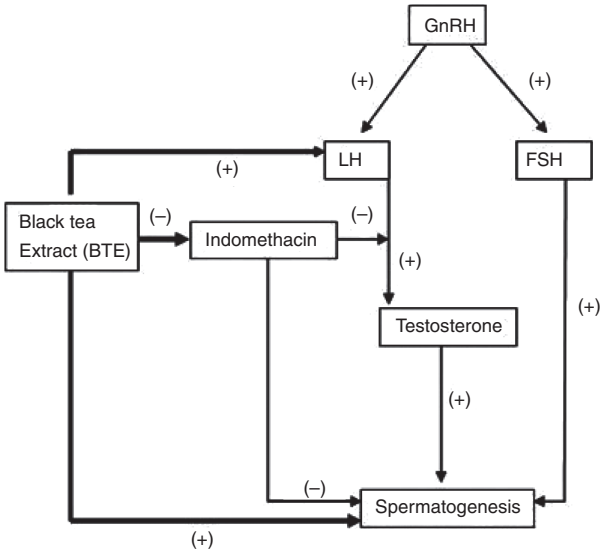

Figure 4: Possible mechanism of BTE on indomethacin-induced alteration of the pathophysiology of testes.

degeneration of seminiferous tubules, focal necrosis, basement membrane thickening, decreased spermatogenesis, degeneration of testes through the shrinkage of seminiferous tubules and decreased number of basal spermatogonia in the testes. Reduced epididymal sperms in the indomethacin-treated Group II rats indicate some interruption in spermatogenesis, which may have led to functional testicular impairments [7, 30]. However, the remarkable improvement of testicular architecture in Group IV rats supplemented with BTE may have protected the testes against indomethacin-induced testicular damages.

Conclusions

The possible mechanism of indomethacin-induced gonadotoxicities through the alterations of gonadotropins, testosterone secretion, and spermatogenesis with subsequent actions of BTE is depicted in Figure 4. Sub-chronic indomethacin treatment clearly led to changes in testicular microenvironment through the disruption of pituitary gonadal axis with altered histopathology of the testes. Simultaneous BTE supplementation showed remarkable protection against indomethacin-induced alteration of testicular pathophysiology.

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