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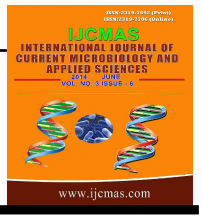

Original Research Article

Testicular hyperthermia in *Rattus norvegicus*: focus on gamatocytic alterations

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A B S T R A C T

Study was designed to investigate chronic effect of temperature (at 39 ºC, 41 ºC and 43 ºC) on histopathological features of testicular cells in *Rattus norvegicus*. Experimental setup was designed as control and experimental sets. After completion of five weeks exposure period, experimental animals were sacrificed for qualitative and quantitative analysis of male gametocytes. Result obtained showed depletion in sperm count, alteration in testicular architecture with morphological abnormalities in gametocytes as per heat exposure. After chronic temperature exposure we noticed that increased temperature found responsible for cellular hypertrophy of spermatogonium at the level of cellular kinesis which further found resign for infertility even in healthy and adult animal. The data obtained was discussed in relation to sperm abnormality and infertility of animals.

Keywords

Chronic heat exposure, *Rattus norvegicus*, Sperm morphometry, infertility.

Introduction

In numerous mammalian species, including man, testicular temperature found lower than core body temperature. In human scrotal temperature is 2-3°C lower than rectal temperature, where as optimum temperature required for human spermatogenesis is considered to be about 35°C. The deleterious effect of heat on spermatogenic function of the testes has been studied by several investigators. The elevation in intrascrotal temperature has suggested one of the major cause for deformities in spermatogenesis in these patient (Zorgniotti and Mc Leod, 1973). Elevated scrotal temperature in man leads to more alterations in their sperm morphometry (Mieusset et al 1987; Mieusset and Bujan, 1995). Increasing testicular or scrotal temperature induces a decrease in sperm count, percentage motility and sperm morphology (Robinson and Rock, 1967; Mieusset et al 1985). Laven et al (1988) reported that, the proportion of good motile spermatozoa was significantly reduced in warm workers (seated for an average working day of 6 hrs or more) and warm sleepers (sleeping with close fitting underwear or using electric blankets or down-filled quilts) than in men who were continuously exposed to a colder environment. A reversible decreased semen quality was also found in welders, who

were exposed to radiant heat with an average 1.4°C because of increased skin temperature (Bonde, 1992).

More recently, studies have documented adverse effects of hyperthermia on the normal adult testes among several species, including mouse, rat, pig and human. The reported effects include a temporary reduction in relative testicular weight, which can cause partial or complete infertility. Sperm quality also been shown to suffer with a reduction in progressive motility and has lowered significantly. Precoital testicular heating appeard not only to reduce the number of successful matings, but also produces a transient retardation in embryo growth and also seen to increase the rate of embryonic degeneration in rats (John et al 2001). The major physical factor of environment affecting sperm production and development include temperature, photoperiod, humidity, nutrition, drugs, stress and toxic substances (Hafez et al 2007; Oyeyemi et al 2006).

Among these factors, which interfere with fertility, varied temperature found most dangerous, since warmer temperature could randomly trigger reproductive activity. Conversely high temperature could restrict reproductive behaviour and gamatogenesis (Haim et al 2005) and increases risk of becoming infertile. Focus to the available literature and information, scientific approach is need in relation to study effect of hyperthermia on spermatogenesis of *Rattus norvegicus*. Purpose of present study was to determine effect of acute and chronic heat exposure. The problem was also selected to discus it with in relation to quantitative and qualitative abnormalities in reproductive physiology of animal.

Materials and Methods

For the present study, *Rattus norvegicus* was selected as experimental animal. Selected animals were reared in departmental animal house (CPC SEA/ 233), Department of Zoology, Shivaji University, Kolhapur, under maintained laboratory conditions. Animals were feed with pellets (feed from Pranav Agro Industries Ltd., Sangli) and provided with normal drinking water. Experimental animals were acclimatized for two weeks prior to the experiment. Healthy animals were selected for the study.

Experimental Design

Chronic heat stroke

Ten experimental animals of body weight 250 gm to 300 gm were divided in to two sets. First set-control and set second- experimental.

The nine animals from set second were divided in to three groups as per their exposed temperature as 39°C, 41°C and 43°C.

During exposure, nine animals from set second were lightly anesthetized with chloroform. Anesthetized experimental animals were vertically exposed in water bath so that, only scrotum (external reproductive structure) completely get embedded in to the water. Repeatedly the animals were subjected to temperature 39°C, 41°C and 43°C for 20 min up to five weeks respectively. In each week two days were selected for heat exposure by the interval of 24 hrs. Every time exposure period was increased by 4 min. up to 20 min. during the total experiment (five weeks). After completion of respective heat treatment, animals were sacrificed, to expose reproductive tract and targeted organ as testes were selected for further experimental procedure with semen analysis.

Parameters under study

Body and testes weight measurement

Healthy adult animals were selected for the present study. Whole body weight of experimental animal was measured initially and after completion each exposure period up to five weeks. After dissection testicular weight of experimental rat was recorded by using electronic balance throughout the experiment, the weight was expressed in gram.

Sperm morphology

For morphological study of sperm, semen sample was obtained from sacrificed experimental animal. The semen sample was obtained from epididymis of male reproductive tract. In semen sample 0.8% saline was added. Smear of semen was prepared on glass slide and allowed to air dry for 20 min. The slides were stained with 10% of eosin and observed under light microscope at 40 X. Morphometric results were described as per methods of Soleimanzaeh and Saberivant, (2013).

Sperm count analysis

Semen sample was obtained from epididymis of male reproductive tract. 1ml of semen sample was mixed with 1ml of 0.8% saline solution was this mixture was used for sperm count. For counting hemocytometer was used. The hemocytometer (Neubaur´s chamber) which has flat thick slide with two 0.1 mm deep chambers in the canter for counting cells. A coverslip of specific thickness was provided with the chamber. Each counting area was marked with lines, 5 such areas were subdivided in to 5 squares (separated by triple lines), each measuring 1 mm ^{3}, enclosing 25 small squares (or 16 small squares in the corners).

Thoroughly mixed semen sample was carefully introduced between the slide and the coverslip using pipette (by avoiding over spillage and air bubbles) and were counted under the microscope (inverted phase contrast microscope) using the 40 X objective. Sperms were counted in 5 squares on each side of the hemocytometer, using the formula given below.

Quantitative analysis of sperm was carried by a method of Parhizkr et al (2013), and was calculated by applying formula described by Rathje et al (1995), as:

Sperms (cells/ ml) count = Total number of sperm in five square × 50,000 × 100

Histological observation

Targeted organ testis was removed and they were fixed in the ice cold Calcium Acetate Formalin (CAF – 2% in 10% formalin) fixative for 24 hrs. The fixation of tissue was followed by washing in chilled distilled water and under running tap water, dehydration in alcohol, clearing in xylene and paraffin embedded. The sections were cut at 4 to 6 µ. For histological studies the sections were stained with Haematoxylene – eosin (HE).

Statistical analysis

The obtained data was statistically analysed by Graph Pad InStat application. The value of $P < 0.05$ was considered as statistically non-significant.

Results and Discussion

Chronic heat shock

a) Body weight

i) Alterations after exposure to 39°C

The body weight of control rat at first week was 276.66 ± 2.517, at second week 269.66 ± 5.033, at third week 273.66 ± 3.215, at fourth week 257.33 ± 39.57 and at fifth it was 282 33 ± 6.658. At first week for 39°C, the body weight was non-significant as compared to control which was 265.33 ± 1.528. Likewise at second, third and fourth weeks the body weight were non-significant which were 256.33 ± 2.082, 272.66 ± 3.215 and 264 ± 4.359. At fifth week, was highly significantly decreased which was 230 ± 11.136.

ii) Alterations after exposure to 41°C

At first and second weeks for 41°C, significantly decreased body weight was observed as compared to control which was 249.33 ± 8.145 and 257 ± 8.888. At third week, more significantly decreased body weight which was 204.66 ± 5.508. At fourth and fifth week, there was significantly decreased body weight which was 165.33 ± 10.06 and 246.66 ± 6.506 respectively.

iii) Alterations after exposure to 43°C

At first week, body weight was more significantly decreased which was 291 ± 1.000. At second and fourth week, there was non-significantly decreased body weight as 273.66 ± 3.215 and 266.66 ± 1.528. At third and fifth week, the body weight was significantly decreased as 291.33 ± 5.508 and 250 ± 6.557 respectively.

In second week, all three heat shock treated group were found non-significant as compared with control, while in third and fourth week, 39°C heat shock treated group was found non-significant and 41°C and 43°C heat shock treated group was significantly affected and in fifth week, all three heat shock treated groups were found significantly affected as compared with control. The detailed data of body weight in heat shock treated and control group was shown in Table No. – 1

b) Testis weight

i) Alterations after exposure to 39°C

When rats, *Rattus norvegicus* were exposed to temperature, it was noticed that, the testis weight was significantly reduced as compared to control. The weight of left testis was 6.226 ± 0.1825 and that of right testis was 6.1383 ± 0.1741. At 39°C, the weight of left testis was 4.837 ± 0.0468 and weight of right testis was 4.6716 ± 0.03055.

ii) Alterations after exposure to 41°C

At 41°C, the weight of left testis was 4.1066 ± 0.0992 and that of right testis was 3.9933 ± 0.02082.

iii) Alterations after exposure to 43°C

At 43°C, the weight of left testis was 3.7016 ± 0.1077 and that of right testis was 3.5966 ± 0.1845. The most significant changes in weight of testes was found in 41°C and 43°C heat shock treated group ($P < 0.001$). The testes weight was found to half the normal value in 41°C and 43°C heat shock treated group. The weight of the right testis in each experimental group was decreased significantly than the control. The changes which occurred in testes (both left and right) in response to the heat treatments are shown in Table No.-2.

c) Sperm count analysis

The mean value of sperm count from control group was 1546.66 ± 48.045 millions cells/ml. The mean of sperm count from heat shock treated groups were significantly decreased after five weeks of exposure period. The mean sperm count from 39°C was 1063.66 ± 43.132 millions cells/ml where as,903.33 ± 41.633 millions cell/ml from 41°C and 518.33 +137.69 millions cell/ml from 43°C after five weeks of exposure period. The result indicates that, there was highly significant difference as compared with control ($P < 0.001$). The data of sperm count from control and three different temperature groups were represented in Figure – 1.

d) Abnormalities of sperms-

In the present study sperm morphology was significantly affected in 41°C and 43°C heat shock treated group. After five weeks of exposure period, some morphological abnormalities were observed in the all exposed groups. 39°C heat shock treated group showed about 37.16% morphological abnormalities, where as 41°C and 43°C heat shock treated group showed, significantly altered morphological features in sperm (43.33% and 67.5% respectively). The morphological abnormalities of sperm in 39°C , 41°C and 43°C heat shock treated groups were represented in Figure - 2. Abnormalities include headless sperms, curved structure, tail -less sperms, absence of both head and tail were observed in some of the sectional view (Fig. 3 A - F).

Histological observations

1) Normal histology of testis

The testis of control rat appeared in normal

structure and seminiferous tubules were associated with germ cell at different stages of spermatogenesis with abundant spermatozoa in it. Testis of control rat showed normal tunica albugenia and supplying blood vessels. Seminiferous tubules were richly populated and showed healthy appearance. All the cells such as spermatogonia, spermatocyte, spermatids, spermatozoa and sertoli cells could be identified in the tubules. Majority of tubules contain mature spermatozoa. Interstitial cells of Leydig were appeared in between the tubules (Fig. 4 A, B).

2) Histology of testis after chronic induction of heat

After five weeks of exposure period at 39°C, seminiferous tubules were showed shrunken and had wavy outline. In these seminiferous tubules, vacuolization were prominently appeared (Fig. 4 C, D). At 41°C, seminiferous tubules were ruptured at some places, number of spermatozoa were declined as compaired to control (Fig. 4 E, F). At 43°C heat shock, more vacuoles were appeared and the tubules contained scanty number of spermatozoa. The interstitial cells of Leydig were reduced (Fig. 4 G, H). After chronic heat treatment, number of spermatozoa appeared in the seminiferous tubules were more scanty as compared to acute heat treatment. In the acute heat treatment, outline of the seminiferous tubules was wavy, while in the chronic heat treatment, wall of the seminiferous tubules were ruptured at some places along with number of vacuoles in it.

Present studies focus on the effect of different temperature on sperm characteristics of adult rats *Rattus norvegicus* after 96 hr of exposure period. The results of the present investigation have shown that, heat shock treatment to scrotum in experimental rats at different temperature (39˚C, 41˚C and 43˚C) for acute period has resulted to severe alterations in weight of testes, morphology of sperm and also sperm count. In the present investigation, body weight of rat *Rattus norvegicus* was non-significant from 39˚C and 41˚C treated group, whereas 43˚C treated group showed significantly reduced weight of rats. Chong, (2011) reported that, the treatment of *Phaleria macrocarpa* (Scheff Boerl fruit) has significantly reduced the body weight gain and total cholesterol. The weight of testes showed significantly decreases in the entire heat shock group (39˚C, 41˚C and 43˚C) as compared with control after 96 hr of exposure period. Gomes et al (1971) reported the evaluated temperature reduces the weight of testes in rats. Bowler, (1971) also noted that, there was significantly decreased weight of testes after 43-5˚C, heat treatment for 6 week. Khan and Reddy, (2002) reported effect of phosphorothionates and parathion pesticides which has decreased weight of testes in rat. Ahmad et al (2008) noted dose dependent decrease in the weight of testes and assessory sex organs of male reproductive tract.

John *et al*(2001), reported the effects of hyperthermia on testis in which they reported reduction in spermatid and numbers of spermatozoa, due to failure of spermatocyte during their maturation cycle. Purushottam *et al* (2012), observed acute and chronic exposure to traffic noise with intensity 80 dB to 90 dB and recorded adverse effect on testicular weight, sperm count and sperm morphology of male albino rat *Rattus norvegicus* which leads to disturbances in regulation of spermatogenesis. Gomes, (1971) reported that, rams had significantly smaller testis after heat treatment as compared to controls. Nirupama *et al*(2013) reported that, chronic exposure of albino rats to stress resulted in a significant duration of exposure dependant decrease in weight of testis and counts of germ cells. Ram *et al*(2009) reported that, weight of the right testis was more affected by the pesticides than the left testis. Ramazan *et al*(2012) studied reduction in the weight of testis and epididymis of male rats treated with insecticide Imidacloprid. The other parameters such as sperm count, motility, and its morphology were also assessed. The result showed, mean sperm count of *Rattus norvegicus* from all experimental groups (39˚C, 41˚C and 43˚C) found significantly decreased as compared

with control ($p < 0.001$). The measure significant count of sperm found in 43˚C heat shock group was less than the 39˚C and 41˚C. Venkatachalam and Ramanathan, (1962) also showed significantly decreased in sperm count of rat and monkey after moderate heat exposure. Morfologicas et al (2009) reported reduced sperm count in wistar rats subjected to prolonged treatment of Chloromphenicol. Parhizker, (2013) studied reduction in sperm count of rats treated with *Phaleria macrocarpa* which can be due to increase in mounting frequency of rats in same group.

Gasco *et al*(2005) reported, hypobaric hypoxia induced partially reversible quantitative changes with decrease in semen volume, sperm count and sperm motility. Orth *et al*(1988) reported, anabolic androgenic steroids (AAS) induced reduction in sertoli cell number which is resulted in a subsequent reduction in the number of spermatogonia which has eventually decreased sperm count. Matthew and Deborah (2009) reported prolonged treatment of Chloramphenicol in wistar rats resulted in a decreased in a volume, motility and live ability of sperms. This study was found that, morphologically, sperm cells of rat *Rattus norvegicus* specified with a hook shaped head, neck, mid - piece and a long tail. Similarly Morfologicus et al (2009), observed normal sperm cell of wister rats consist of a hook shaped head (Qustion marke shape) a thin neck, mid-piece and long tail. Garner and Hafez (1993) noted that, only in mice and rat the heads of spermatozoa terminate in distinct hook shape.

Nallela et al suggested that, normal sperm morphology has essential characteristics for in vitro fecundity and in vitro fertilization. The different morphological abnormalities of sperm were found in the present study as head less sperm, tail less, double headed, double tailed. The maximum abnormalities of sperm were observed when animals were exposed to 43˚C temperature as compared with 39˚C and 41˚C. Dalsenter et al (2003) reported, increased morphological abnormalities in sperm of Wister rats due to toxic effect of Endosulfan. Sperm head abnormalities after treatment of differentiating spermatogenesis were reported in mice (Alavantic et al 1988). Soleimanzadeh, (2013) reported, cryopreservation has increased percentage of dead or membrane damaged sperm. Wan et al (2006) studied, effect of fluoride on sperm quality which has declined in sperm viability and a significantly increased when sperm abnormality after 50, 80, 100 and 120 days in male rats when administered by 68 mg of fluoride ion/L through drinking water. This study was found that, histology of testis, after chronic heat treatment showed various histopathological changes, which have included shrunken and wavy appearance of seminiferous tubules, and reduction in spermatid and spermatozoa numbers. After chronic heat treatments, wall of seminiferous tubule′s was irregular, less compact and vacuolated, with reduced height. John (2011), reported reduction in spermatid and spermatozoa numbers due to hyperthermia, our results coincides with Bowler (1972), where he noted, heat treatment has caused severe damage to seminiferous tubules and resulted to less number of gametocytes. Imran et al (2003) noted, shrunken and wavy outline of semeniferous tubules due to lead poisoning, in some cells, the nuclear membrane had been ruptured with declined number of interstitial cells also recorded. Ram (2009), noted more cytoplasmic vacuolization of germ cells and sertoli cells in heigh dose of Phosphamidon treatment.

In the investigation, preliminary finding showed that, chronic heat treatment has greatly affected on testis weight, sperm count, sperm morphology and overall histology of testis in selected experimental animal *Rattus norvegicus*. The present study provides experimental evidences about, long term heat exposure and its impaired testicular activity which has resulted to infertility in experimental animals. Our findings supports previous work and strengthen the investigation related to reproductive physiology of animals., if supported by quantitative, histological biochemical and immunocytochemical applications for the better achievement in the field of reproductive biology.

Week	Body weight			
	Temperature (°C)			
	Control	39	41	43
1st	267.66 ± 2.517	265.33 ± 1.528 Ns	249.33 ± 8.145 **	291 ± 1.000 ***
2nd	269.66 ± 5.033	256.33 ± 2.082 Ns	257 ± 8.888 **	273.66 ± 3.215 Ns
3rd	273.66 ± 3.215	272.66 ± 3.215 Ns	204.66 ± 5.508 ***	291.33 ± 5.508 **
4th	257.33 ± 39.57	264 ± 4.359 Ns	165.33 ± 10.06 **	266.66 ± 1.528 Ns
5th	282.33 ± 6.658	230 ± 11.136 ***	246.66 ± 6.506 **	250 ± 6.557 **

Fig.1 Body weight (mean ± SD) of control and experimental rats. ns - $p < 0.05$ (non significant), * - $p > 0.05$ (partially significant), **- $p < 0.01$(significant), ***- $p < 0.001$ (highly significant).

Testes weight		
Temperature °C	Testes Position	
	Lt.	Rt.
Control	6.226 ± 0.1825	6.1383 ± 0.1741
39	4.837 ± 0.0468 ***	4.6716 ± 0.03055 ***
41	4.1066 ± 0.0992 ***	3.9933 ± 0.02082 ***
43	3.7016 ± 0.1077 ***	3.5966 ± 0.1845 ***

Fig.2 Testes weight (mean ± SD) of control and experimental rats. ns - p001 (highly < 0.05 (non significant), **- $p < 0.01$ (significant), ***- $p < 0$. significant) of chronic heat treatment

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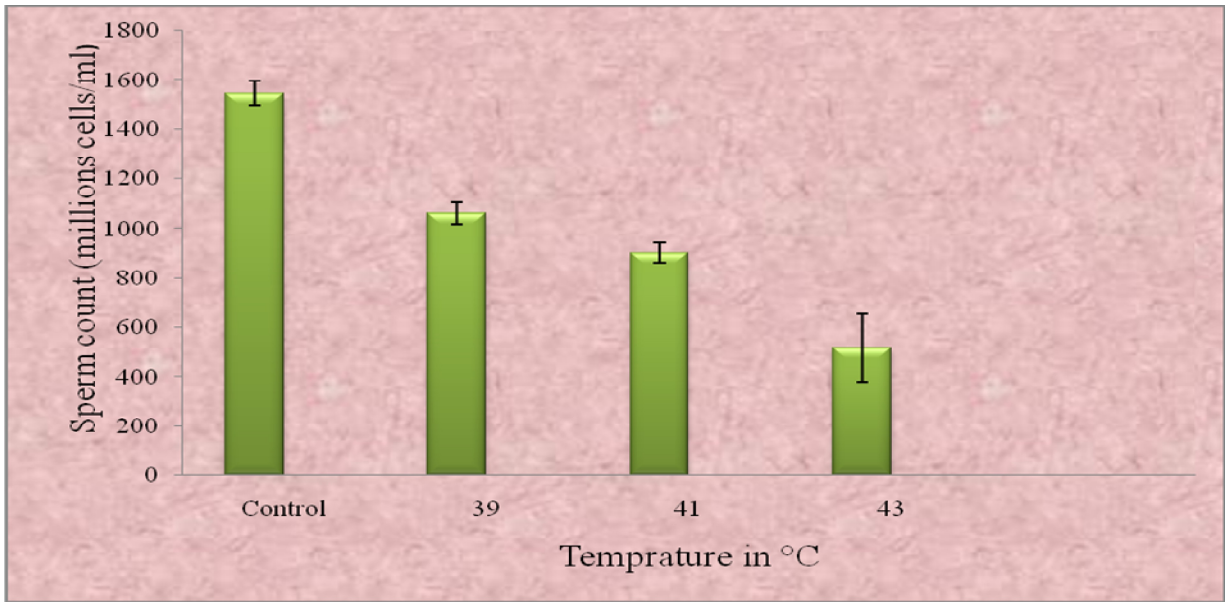

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Figure.1 Alterations in rat sperm count in control and experimental group at chronic exposure of temperature. *** - p < 0.001 (highly significant)

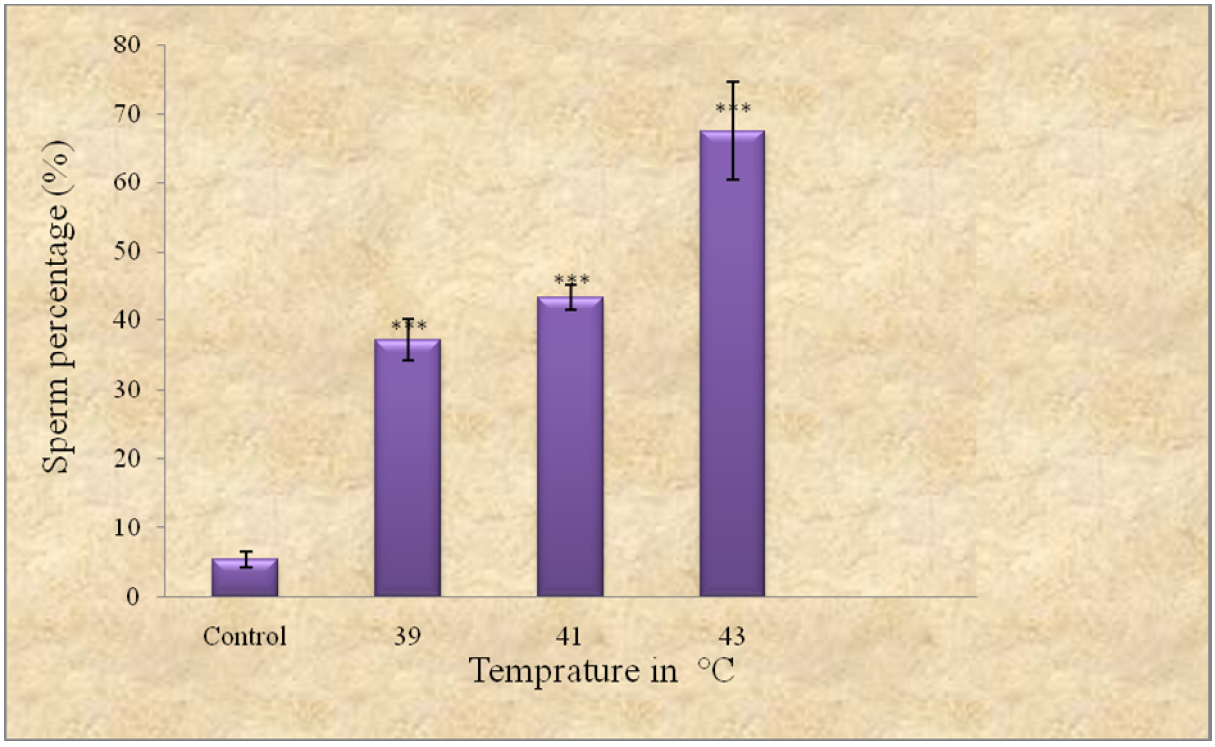

Figure.2 Percentage of abnormal sperm in rats Rattus norvegicus in different temperature at chronic exposure. *** - p < 0.001 (highly significant)

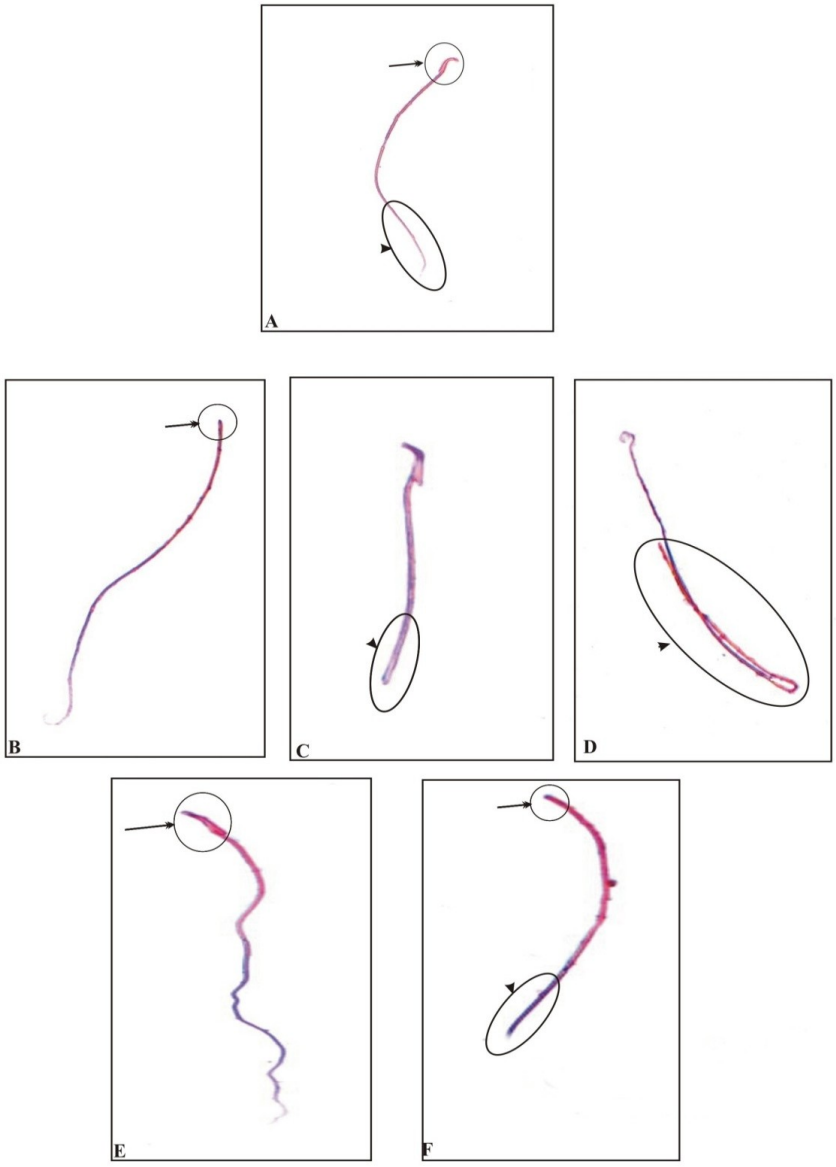

Figure.3 Normal and abnormal sperms in the experimental animal *Rattus norvegicus* in chronic heat shock. A- Normal sperm, B- Head less sperm, C- Tail less sperm, D-Curved sperm, E-Straight headed sperm.

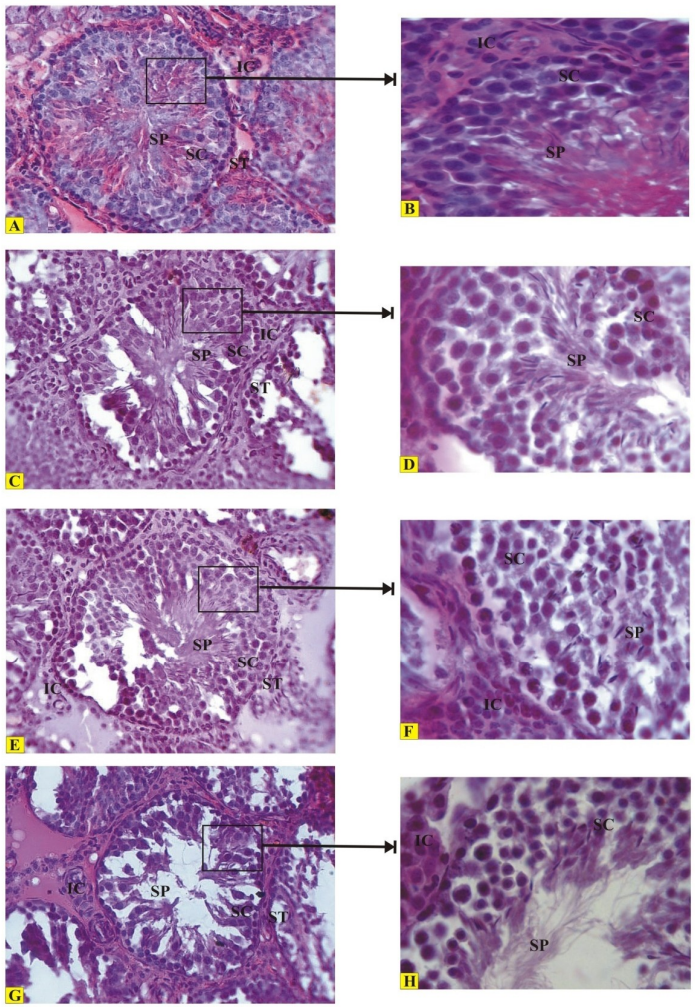

Figure.4 Transverse sections of normal and chronic exposed testes of experimental animal *Rattus norvegicus*. A - B: Normal testes, C - D: 39°C exposed testes, E - F: 41°C exposed testes, G - H: 43°C exposed testes.

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