HISTOARCHITECTURAL STUDIES OF GONADS OF RAT PUPS FOLLOWING BRIEF EXPOSURE TO HYPERTHERMIA IN-UTERO

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ABSTRACT

This study investigated the effect of hyperthermia on the gonads of the pups of Wistar rats following maternal exposure to brief hyperthermia during pregnancy. Twenty-five pregnant adult female Wistar rats were randomly selected into two groups: Group A (Control) which consisted of 10 female rats and Group B (Experimental) which had 15 female rats. The pregnant dams in the experimental group were exposed to brief hyperthermia for 15 minutes twice daily at 8.00am and 4pm on gestational day (GD) 12-18. The pups produced by the rats were weighed, examined and sacrificed at 35days of post-natal life. Recorded were the microscopic appearances of the gonads while the luminal diameter and thickness of the gonadal vessels were measured and recorded. Data was analysed, mean and standard deviation were generated with student t-test, and $p < 0.05$ was taken as significant. Maternal exposure to brief hyperthermia during pregnancy significantly reduced the birth weight of the pups in Group B (3.86 ± 0.26g) compared to Group A (4.71 ± 0.18g). The luminal diameters of the testicular and ovarian arteries of the pups in Group B were significantly increased whereas the gonadal vascular arterial wall thicknesses were significantly reduced in comparison with the Group A. Histological examination of the gonads revealed fewer cell population with degeneration and damage to the germinal epithelium of the gonads of the pups in Group B which was more severe in the testes. Maternal exposure to brief hyperthermia during pregnancy has deleterious effects and subsequent destruction of the gonads of pups of Wistar rats and this may interfere with fertility of the pups later.
Key Words: brief hyperthermia, gonads, maternal, pregnancy, wistar rats

INTRODUCTION

The incidence of congenital anomalies due to maternal factors has been on the increase within the last few decades. Environmental and genetic factors have often been implicated as major causes of congenital anomalies and one of the leading environmental factors is maternal hyperthermia. There are several factors or activities that increase maternal body core temperatures in the tropics especially in sub-Saharan Africa where environmental temperatures are relatively higher. It is estimated that 10-15% of congenital structural anomalies are the results of the adverse effects of environmental factors during prenatal development (Brent, 2001).

Congenital reproductive system disorders and structural anomalies arise from disruption during embryonic development as a result of exposure to different environmental factors during critical period of development (Oyewopo and Togun, 2005).
This implies that approximately 1 in 250 newborn infants have structural defects caused by an environmental exposure and, presumably, a larger number of children have growth retardation or functional abnormalities resulting from non-genetic causes. Teratogenic exposures during prenatal development can cause disruptions regardless of the developmental stage or site of action. Most of the structural defects caused by teratogenic exposures occurs during embryonic period,

which is when critical developmental events are taking place and the foundation of organ systems are established (Gilbert, 2003).

Hyperthermia is an increase in the body temperature of at least 38.9_0C (Graham and Edwards, 1998). It is an antimitotic teratogen after exposure between the fourth and fourteenth week of gestation (Pleet et al., 2001). Hyperthermia is believed to be a teratogen in many mammalian species including humans (Sasaki et al., 1995). A little rise in the maternal body temperature due to heat stress of 0.8 to 2.5_0C above resting core body temperature may induce several teratogenic effects on growth and development in embryo of rats and several other species (Larry, 1986). It may result from febrile infections, hot and humid environments and heavy exercise especially in conditions of high heat and humidity. The average body temperature of most mammalian species falls between 37_0C-40_0C and the usual diurnal variation is approximately 1_0C (Graham et al., 1998). Hyperthermia from maternal infections and sauna bathing in the first trimester of pregnancy in humans have been reported to cause a variety of musculoskeletal and central nervous system anomalies like hypotonia, neurogenic arthrogryposis and anaencephaly in infants exposed to maternal heat stress (Smith et al., 1978 & Shiota 1982). Similarly, occipital encephalocele was observed to be associated with maternal hyperthermia in guinea pigs and rats suggesting the sensitivity of brain growth to elevated maternal core body temperatures (Fisher and Smith, 1980).

Exposure of the pregnant uterus to ultrasound may lead to temperature elevation, depending on factors such as intensity of the scan, ultrasound frequency, dwell time among the beam axis, width of the beam, tissue properties, length of the scan and stage of pregnancy (Miller *et al.,* 2002). According to the Bio effects committee of the America Instistute of Ultrasound Medicine (AIUM) in 1987; foetal exposure from an in-situ temperature rise to greater than or equal to 41_0C is considered hazardous. They, therefore, recommended that diagnostic exposures should not result in a temperature rise of greater than 1_0C above the physiological levels and suggested that, provided the causative factors related to hyperthermia are controlled, the procedure remain safe (Repacholi, 1987, Jensh and Brent, 1999).

An embryonic or foetal organ is susceptible to hyperthermia only when the elevated temperature exceeds the threshold for that organ. The type of defect caused by heat in embryos is determined largely by the developmental stage at the time of exposure, while the severity and incidence of defects depend largely on the duration of the exposure. The central nervous system is mostly affected by hyperthermia suggesting a low threshold for increased maternal core body temperatures. It however remains to be seen whether hyperthermia has any effect on the gonads especially the testis which is believed to be affected by elevated body temperatures after delivery in humans. This study therefore aimed at examining the effect of hyperthermia on the gonads of the pups of Wistar rats following maternal exposure to brief hyperthermia during pregnancy.

MATERIALS AND METHOD

Twenty – five female Wistar rats, weighing 300-350g were obtained from the Central Animal House, College of Medicine, University of Ibadan, Ibadan, Nigeria. They were housed in well-ventilated separate cages with liberal access to food and water. They were weighed to assess their physical condition at 14:00h twice per week and made to acclimatize for two weeks.

The female rats were randomly divided into two groups: Group A (Control) and Group B (Experimental). The control group comprised

ten female rats whereas the experimental group comprised fifteen female rats. They were mated in the dark and females were checked for presence of vaginal plugs. The day on which the plug was seen was marked as day 0 of pregnancy. Fertilization was confirmed through vagina smear and was observed for the presence of sperm cells with the aid of microscope.

The pregnant dams in the experimental group were exposed to brief hyperthermia twice daily at 6am and 6pm from Gestational days (GD) 10-15. This timing was specifically selected as to when the animals would be most susceptible to hyperthermia. It should be noted that the larger number of pregnant animals in the experimental group was to make up for any losses that may come up during exposure to brief hyperthermia, such as death, foetal abortion and foetal death (Kline *et al.,* 1985).

Hyperthermia was induced at 8:00am and 4:00 pm daily (at this time, the thermoregulatory mechanism of the animals' internal environment is minimal, which helps the body temperatures to be compromised easily) on the dams in the experimental group from GD 12-18 using laboratory oven. This timing was specifically selected because it is the time the animals were most susceptible to hyperthermia. The core body temperature of rat is approximately 38.5_0C (Germain *et al.,* 1985), therefore, the oven was set at 46_0C and the animals kept in the oven for 15 minutes to raise their core body temperatures by $3-4_0C$. It has been reported that in all experimental rat species, a threshold elevation of approximately $2.0-2.5_0C$ above the normal body temperature is capable of causing congenital defects (Edwards 1969). The core body temperatures of the rats were measured with a rectal thermometer before exposure to hyperthermia and intermittently (every two minutes) after the exposure. Also, physical examination of the dams was carried out to observe their activities and other changes that might have taken place in them following the brief exposure to hyperthermia. The control

group was left at room temperature throughout the period.

The animals in both groups were allowed to deliver the pups at term. The pups were weighed at birth and at fifth week postnatally using a digital laboratory weighing scale (My weigh i201 cardinal scale, USA). They were sacrificed at 35 days post natal life, with chloroform fumes in a coplin jar and then abdominal wall incised. The testes and ovaries and their vessels were harvested and fixed in 10% formalin.

Specimens of testes and ovaries were excised and then immersed in normal saline. The tissues were passed further through the routine stages of tissue processing for histological studies i.e fixed in Bouin's fixative, dehydrated in various grades of ethanol, cleared in benzene, infiltrated and embedded in paraffin wax. The tissue blocks were mounted on a wooden block and trimmed to size at 20µ thick. They were sectioned on a rotatory microtome at 7µ thick. The sections were stained with Haematoxylin and Eosin, and Van Geisson & Van Hoeff stains for connective and elastic tissues. This revealed the general architecture of the testes and ovaries. Microscopic views of the testicular and ovarian arteries were performed to investigate the effect of brief hyperthermia on the lumen of the arteries. This procedure was carried out by measuring the luminal diameter and the thickness of the walls of the vessels with a software called "Image J". Measurements taken included the luminal diameter and thickness of the wall of the testicular and ovarian arteries, birth weight, and litter size. The measurements were made using a light microscope at x500 magnification and mitotic images software for spherical measurements. Data analysis was performed using the Graphpad prism Version 5.0 software. Mean and standard deviation were generated with student t-test, and $p < 0.05$ was taken as significant. The morphological measurements were compared between the experimental and control groups.

RESULTS

Overall, 17 female rats became pregnant. Of these 7 rats were in Group A and 10 in Group B. Following exposure of pregnant dams in Group B to brief hyperthermia, there were two maternal deaths and one rat had abortion. The temperature change profile of the pregnant dams revealed that it took about 20 minutes for the body temperature of the rats to return back to the initial temperature after removal from the oven (Figures 1 and 2).

Physical examination of the pregnant dams in Group B following exposure to hyperthermia revealed hyperaemia of the hands and feet, the skin around the abdominal region was wet, the whisker became straight, they became weak with reduced activity and their eye balls became reddish compared to the pink coloured eye balls of the dams in Group A.

The mean birth weight of the pups from dams in Group B (3.86 ± 0.26g) was significantly lower than the mean birth weight of the pups from Group A which was 4.71 ± 0.18g (p= 0.02). Table 1 revealed that the mean luminal diameter of testicular arteries in pups from dams in Group B (230.4±21.95μ) was significantly more than the mean luminal diameter of testicular arteries (117.1±15.76μ) of pups from dams in Group A (p= 0.01). The mean luminal diameter of the ovarian artery in pups from pregnant dams in Group B (216.7± 21.00μ) was significantly more than the mean luminal diameter of ovarian artery (118.7 ± 11.66 μ) of pups from dams in Group A (p= 0.01).

Table 1: Mean± SEM luminal diameter of gonadal arteries (μ)

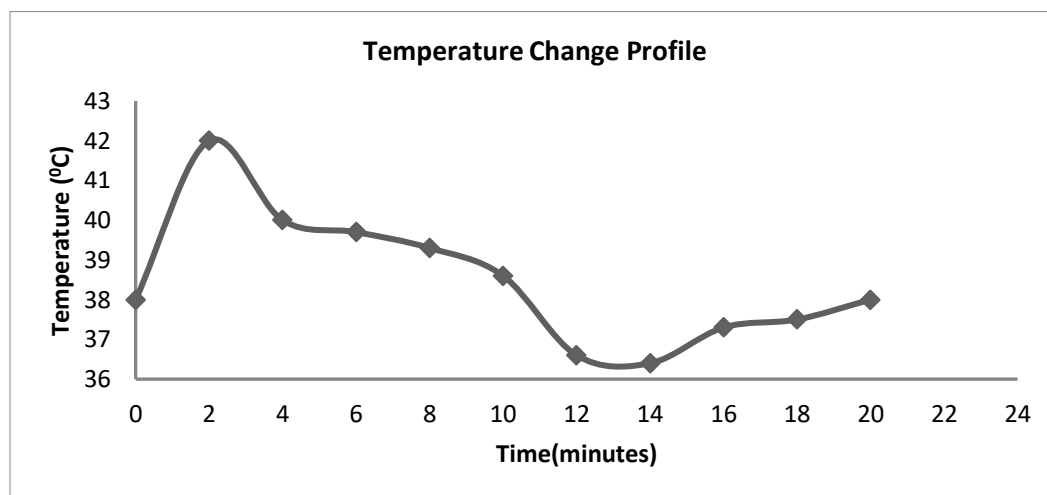| Gonadal artery	Group A (Control	Group B (Experimental)	p value
Testicular artery	117.1±15.76	230.4±21.95	0.0006
Ovarian artery	118.7±11.66	216.7±21.00	0.0008

Figure 1: The temperature change profile (Morning exposure) of pregnant dams exposed to hyperthermia from GD12-18 shows that it took 20 minutes for body core temperatures of rats to return to initial temperature after removal from the oven.

Table 2 revealed that the mean thickness of the wall of the testicular artery in pups from pregnant dams in Group B (141.9 ± 11.95µ) was significantly reduced compared to the mean thickness of the wall of testicular artery (224.5 ± 16.39µ) in Group A (p= 0.01). The mean thickness of wall of ovarian artery in pups from pregnant dams in Group B (98.83 ± 5.87µ) was significantly reduced in comparison to the mean thickness of wall of ovarian artery (127.5±7.45µ) in pups from dams in Group A (p= 0.05).

Microscopic examination of the walls of the testicular and ovarian arteries of pups in Group A shows distinctively the tunica intima, tunica media and tunica adventitia with visible endothelial lining of the lumen. The arterial wall maintained relative uniform thickness and the luminal diameter was normal. Whereas, the lumen of the testicular and ovarian arteries of pups from the pregnant dams in Group B showed severe congestion.

Table 2: Mean± SEM wall thickness of gonadal arteries (µ)

Gonadal Artery	Group A (Control)	Group B (Experimental)	p value
Testicular artery	224.5±16.39	141.94±11.95	0.0008
Ovarian artery	127.5±7.45	98.83±5.87	0.0054

There is considerable thinning of the tunical layers of the vessels, reduced concentric layers of the tunica media and considerably larger luminal diameter compared to the vascular walls of the gonadal vessels in Group A. The vascular thickness was reduced in all the pups from Group B in comparison to those of pups from Group A (Figure 3 A – D).
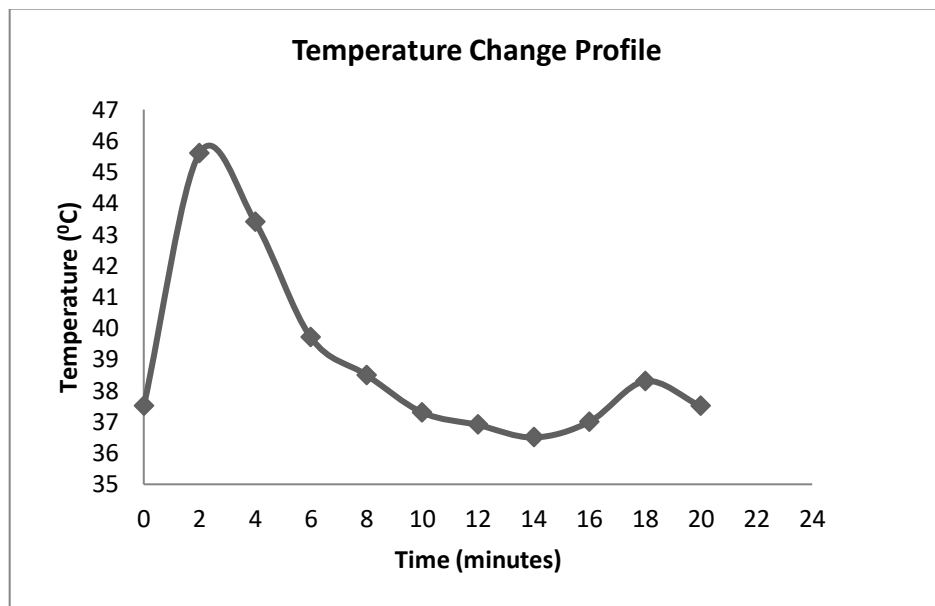

Figure 2: The temperature change profile (Evening exposure) of pregnant dams exposed to hyperthermia from GD12–18 shows that it took 20 minutes for the body core temperatures of rats to return to initial temperature after removal from oven.
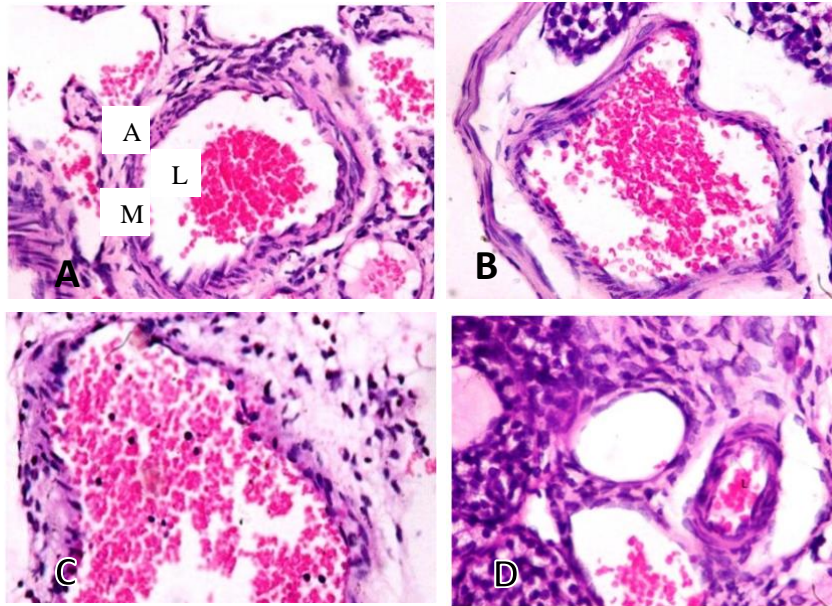
Figure 3 A − D (x400): Photomicrographs of sections of gonadal artery of pups of Wistar rats. **A −** Section of testicular artery of a pup of pregnant dam in Group A (Control) showing distinct tunica intima and concentric layers of tunica media with congestion of blood cells in the lumen (L; lumen, M; tunica media, A; adventitia). **B -** Section of testicular artery of pup of pregnant dam exposed to brief hyperthermia (Group B) showing severe congestion and thining of the tunica intima and tunica media. **C −** Section of ovarian artery of a pup of pregnant dam in Group A (Control) stained by haematoxylin and eosin showing distinct tunica intima and concentric layers of tunica media with congestion of blood cells in the lumen (L; lumen, M; tunica media, A; adventitia). **D -** Section of ovarian artery of pup of a pregnant dam exposed to brief hyperthermia showing mildly thickened wall and congested and narrowed lumen (L).
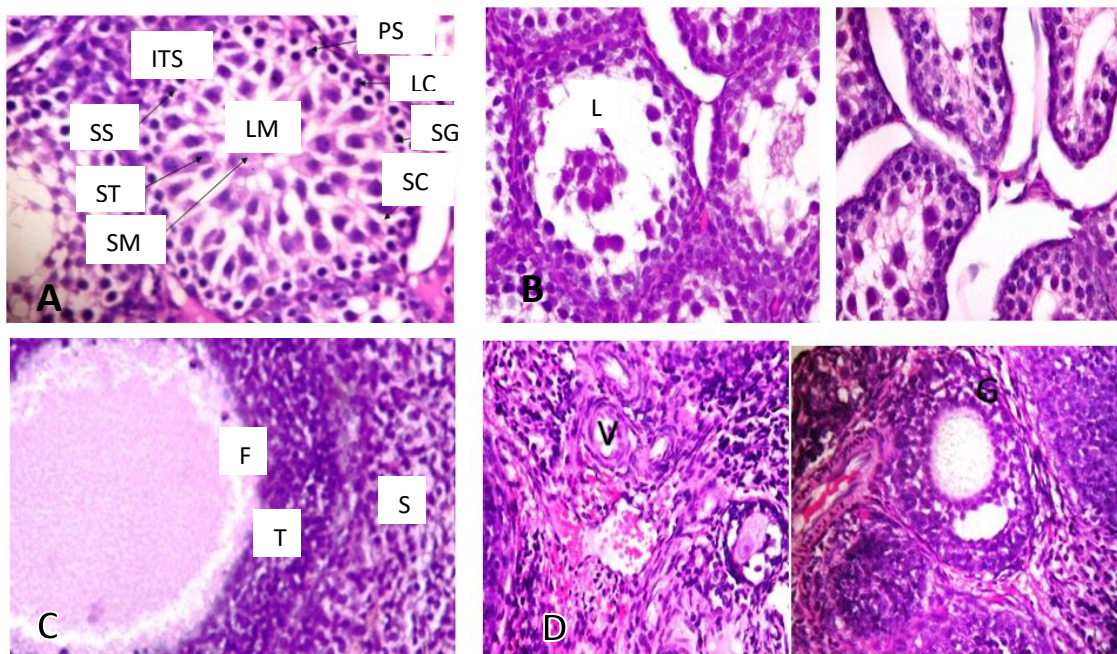

Figure 4 A − D (x400): Photomicrographs of the gonads of pups of Wistar rats. **A −** Section of testis of pup of a pregnant dam in Group A (Control) showing normal spermatogonium (SG), primary spermatocytes (PS), secondary spermatocytes (SS), spermatids (ST), spermatozoa (SM), interstitial space (ITS), sertoli cells and leydig cells (LC). **B −** Section of testis of pup of a

pregnant dam exposed to brief hyperthermia in Group B (Experimental) showing seminiferous tubules with necrosed germ cell layer within the lumen (L), the seminiferous tubules have moderately thickened propria enveloping the tubules, there are few tubules showing eosinophilic fluid within their lumen. **C –** Section of an ovary of pup of a pregnant dam in Group A (Control) stained with haematoxylin and eosin showing several normal follicles (F). The theca cells (T) appear normal and the stroma (S) shows normal fibroblastic tissues and no inflammatory cells. **D –** Section of an ovary of pup from a pregnant dam exposed to brief hyperthermia (Group B) showing ovarian stroma with mild fibrosis and increased vascularization (V) and normall lutenization within the granular cells. There are several follicles noted with degenerated granullosa cells (G).

Histological sections of the testes of pups in Group A revealed normal histoarchitecture of the testicular tissue with normal seminiferous tubules, germ cell layer normal spermatogonia and normal sertoli cells. The interstitial spaces and leydig cells appeared normal. The histological section of the testes of pups in Group B showed severely sloughed germ cell layer and exfoliation of degenerated spermatogonia on the basement membrane.

Also, there is a slight increase in connective tissues surrounding the tubules. Histological sections of the ovaries of pups in Group A revealed normal follicles, normal thecal cells with the stroma showing normal fibroblastic tissues. The histological sections of ovary of pups from dam in Group B showed ovarian stroma with mild fibrosis and degenerated granulosa cells (Figure 4 A – D).

DISCUSSION

The general reduction in the birth weight of the pups exposed to hyperthermia in-utero in Group B suggests that there may be a general reduction in tissue growth from disruption of uteroplacental blood flow and necrosis of the placenta as a result of maternal exposure to increased temperature (Nilsen, 1985, Edwards, 1986). A finding that is similar to observations earlier reported by Martinez-Avarez *et al.,* (2000).
The physical changes observed in pregnant dams immediately after exposure to hyperthermia could be as a result of increased vasodilation of the blood vessels in the hands and feet caused by the increase in temperature. The pregnant dams became sluggish in movement and their response to tapping was reduced compared to pregnant dams in control group. This reduced motor activity may be due to the effect of temperature on the motor area of the brain (Edwards, 1967). Body temperature is regulated by the thermoregulatory centre in the anterior hypothalamus. Hyperthermia has been reported to alter the functional connectivity of the brain. The changes in the connectivity network might be a possible explanation for the cognitive

performance and work behaviour alteration (Liu *et al.,* 2007; Sun *et al.,* 2013).

The core body temperatures of the pregnant dams were monitored immediately after exposure to hyperthermia. The body temperatures of the pregnant dams were elevated by 3-4 $_{0}$C above core body temperature. However, it returned back to initial temperature 20 minutes after removal of the rats from the laboratory oven.

The effects of hyperthermia combined with hypoxia from vascular compromise in vital organs may cause destruction of these organs with consequent foetal loss and maternal death (Graham *et al.,* 1988). This may account for the high incidence of hyperthermia-induced maternal mortality observed in the experimental group which corroborated an earlier report (Edwards, 2006). Foetal losses can also result from severe dehydration as hyperthermia can also interfere with the body's thermoregulatory mechanisms (Arora *et al.,* 1979). The reduced number of pregnant rats in the experimental group that reached full term may be attributed to the teratogenic effects of maternal hyperthermia. Kline et al., (1985) confirmed a significant association between fever during

pregnancy and spontaneous abortion, with some febrile episodes resulting in foetal deaths and expulsion, while other febrile episodes resulted in premature uterine contractions with expulsion of the foetus. Similar findings of induced embryonic death, tissue resorption, and embryonic wastages during sensitive stages have been reported (Matsuzuka et al., 2005, Tereza et al., 2007). The premature uterine contraction is believed to result from hyperthermia induced release of prostaglandins in both foetal and maternal tissues with consequent stimulation of uterine motility (Morishima et al., 1975, Andrianakis et al., 1989, Graham et al., 1989).

Prenatal death and abortions can also result from mild exposure to hyperthermia during the preimplantation period and this is worse with severe exposures during the embryonic and foetal development (Edwards, 2006).

The hyperthermic effects on the testicular and ovarian arteries of the experimental group revealed that the luminal diameter was significantly increased, and the vascular arterial thickness was reduced compared with the control group. The luminal diameter was highly significant in the testicular artery than the ovarian artery. It is generally accepted that heat causes a quick increase in the blood flow accompanied by dilation of the vessels and increase in the permeability of the vascular wall in normal tissue (Paul et al., 2009). The extent of the pathophysiological changes in the vascular system of normal tissue is dependent on temperature and duration of heating. An excessive exposure of tissues to heat can cause a breakdown of vasculature followed by necrosis of the tissues (Setchell and Breed, 2006). Previous studies with cutaneous tissue revealed that the increase in the blood flow upon heating is caused, at least in part, by dilation of vessels (Gabbiani and Badonnel, 1975). The general vasodilatation and increase in vascular blood flow to tissues exposed to hyperthermia could be the body compensatory mechanism to increase cellular blood flow that will supply more oxygen and nutrients to support the hypercatabolism that is always associated with hyperthermia.

The results of this study show that the gonads are quite delicate and susceptible to maternal hyperthermia in-utero, though the testicular tissue showed higher significant histoarchitectural changes than the ovary. This is supported by finding that reveals that vasculature of the testis is exceptionally thermo-sensitive and a little rise in the body temperature can cause considerable damage (Durairajanayagam *et al.,* 2014). The morphology of the ultastructure of the the germinal epithelium of the testes were significantly decreased following induced hyperthermia which resulted in desquamation and reduction in cell population of the germinal epithelium (Figure 4b). The cell desquamation and reduction in the cell population may be attributed to germ cell apoptotic response, damage and degeneration of Sertoli and Leydig cells that follows heat stress (Lue et al., 2002, Ren et al., 2006).

An embryonic or foetal organ is susceptible to hyperthermia only while passing through a sensitive stage of development during the exposure and when the elevation of temperature exceeds the threshold for that organ. This study suggests that an elevation of 3-4 $\,^\circ$C above the core body temperature for 15 minutes exposure twice daily on pregnant rats on GD12-18 is lethal to the development of the gonads especially the testis. This is similar to previous studies reported by Edwards (1995) that a threshold of 1.5- 2.5 $\,^\circ$C is required for alterations in development.

In this study, hyperthermic insult disrupted the germinal epithelium of gonads which was more pronounced in the testes and had negative effects on the vascular walls of the testicular and ovarian arteries. Histologically, the plates of the testes of rats in the experimental group show reduction of the cell population, desquamation of the germinal epithelium, necrosis of germ cell layer and damage to the leydig and sertoli cells, which suggest an arrest

of spermatogenesis. The photomicrograph of ovaries of rats exposed to hyperthermia in-utero shows increased vascularization, mild fibrosis and degeneration of granulosa cells compared to the control group. However, the hyperthermic insult had more damaging effects in the testes than ovary in the experimental groups.

In conclusion, exposure of pregnant Wistar rat to brief hyperthermia in early pregnancy has resulted in disruption of histoarchitecture and cellular degeneration of the gonads especially the testes of the resulting pups. It remains to be seen whether these effects could be replicated postnatally in the pups and juveniles of Wistar rats. A finding that could interfere with the reproductive abilities of these rats.

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