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Studying effect of heat stress on DNA damage exposure in sertoli cells

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ABSTRACT

In order to determine how heat stress affects Sertoli cells and study the type of caused damages in these cells affected by these conditions , this study aims at evaluating the response indicators of oxidative and the amount of DNA damage by using the TUNEL method in Sertoli cells affected by heat stress at 42°C . Testes of 9 lambs of 3 to 10months provided in slaughters and then isolation and culture of sertoli cells was performed on these samples.DNA damage and amount of reactive oxidative were studied in control groups under mild heat stress and extreme heat stress by using the experimental Random design and statistical test (one way ANONA) in a statistical software SPSS. the Results of present study showed that by increasing temperature , the amount of living cells decreases and the difference between the percentage of living cells and cells of the control group at 32 °C with 39°C groups and 42 °C groups is significant (P < 0.05) . Assessing by TUNEL staining showed that applying the heat stress caused an increase in the number of damaged cells from DNA and this increase was significant in 42 °C group compared with control group.

Key words: heat stress, oxidative reaction, DNA damage exposures, sertoli cells.

INTRODUCTION

Temperature of mammalian testes should be lower than body temperature to function properly. Anatomical features of the testis and scrotum enables the adjustment of testicular temperature. Thermal receptors in the scrotum skin can develop some reactions that make whole body temperature lower, and triggers breathing and sweating. Testicle is an organ that its natural function depends on temperature. So that in many mammals testicles placement inside the scrotum and outside the abdominal area causes that the testes be at a temperature between 2 to 8 degrees lower than body temperature [1]. Regulating body temperature is very important because the slight increase in the temperature can impair the process of sperm production and can cause some fertility problems. For example, heat stress in rat testis is causing the loss of reproductive cells, low sperm quality and appearance of some sperms damaged by DNA and associated with premature death of embryos from fertilization of such sperms [2]. Other studies on mice have shown that the occurrence of transient heat stresses (usually over 40 centigrade) or placing the testes and Epididymis inside the body by surgical procedure causing prolonged contact with body temperature is led to testicles weight loss, increasing cell death, loss of reproductive cells and decreases fertility of sperm cells [3 and 4]. Due to this sensitivity, there are different mechanisms in body to maintain the proper temperature for testes function [5]. The results of these studies and other related studies show the importance of heat stress effect on disorder of efficient re-productivity of males. Given the crucial roles of Sertoli cells in the procedure of sperm producing in testes, any kind of conditions that can damage the cells or disturb their function can affect the procedure of sperm production and ultimately male fertility. One of these destructive situations is heat stress exposure. By now, few studies have examined how heat stress can affect Sertloi cells. In this case clinical findings of sheep species indicate destructive effects of heat stress on fertility parameters of male in these species and show the negative effect of heat stress on reproductive efficiency in ram. But the reasons of such changes in sheep are not known.

MATERIALS AND METHODS

All procedures of Sertoli cells culture were performed in vitro in the Institute of Embryo Technology of Kurd city. Testicles of 9 lambs of 3-10 months were provided from of Kurd slaughter located in Jongan area and were transferred to laboratory inside an ice pack.

A) After moving the samples, testes were washed and disinfected several times by 70% ethanol after 3-4 hours and then Tunica Albojina layer was cut by a sterile scalpel.

B) After removing required and enough tissue, testicle tissue was sliced by using two scalpels. These slices were moved to Konical tubes. If the amount of sperm is high in the sample, it can be decreased by twice wash. So that, add a little phosphate-buffer Saline (5mL), then suddenly centrifuge will be short that by this sperm will be removed from the tissue at the bottom of the tube, and would be floated on phosphate buffer Saline .

C) Phosphate buffer Saline containing sperm was removed and this time tube's contents were centrifuged for 4 minutes at 400 g round. By performing second centrifuge the residues of phosphate buffer Saline in sample were removed.

D) A two-step enzymatic digestion: initially collagenase 1% was added to the sample during this procedure and was incubated for half an hour at 37 centigrade. The sample of tissue along with enzyme was shaken every 10 minutes to digest the enzyme in a best way. After this time, the samples were removed from the oven and were centrifuged for 4 minutes at 400 g round. After completing the centrifuge, the supernatant containing Leydig cells was digested by collagenase and discarded.

After accomplishing this procedure, the phase II of enzymatic digestion was initiated. Over this procedure, the next enzymatic solution containing Trypsin 0.25% and DNAse enzyme 20 was added. The sample containing these two enzymes were incubated for 20 minutes and so before it was shaken every minutes for proper enzymatic digestion. After fulfillment of second enzymatic digestion, the sample was centrifuged for a while, to deposit the large pieces of tissue. Supernatant was used at this procedure to continue the culture. Enzymes of second series functioned to separate the sperm-producing tubes. It should be noted that in order to inactivate the second enzymatic series from medium 10% fetal calf serum is used because calcium neutralizes Trypsin.

E) The sample was centrifuged for 4 minutes at 400g round. The supernatant was discarded and the deposited cells on the floor were used. The medium DMEM 4.5mLwere added to it to be quite uniform.

F) The resulted mixture was screened through a filter with holes of 7μm diameter then was centrifuged for 1 minute at 60g round. Then it was centrifuged for 4 minutes at 400g again. The supernatant was discarded and the cells on the floor were mixed with DMEM medium and 10 % serum for final culture and were removed to the culture plate and were qualitatively examined under microscope and were incubated at 32^{o}C.

Measuring of parameters related to oxidative reaction:
Four parameters associated with oxidative reaction were done in control group and under thermal stress: measurement of lipid per-oxidation is done by measuring the concentration of malondialehyde (TBARS) whish is done by spectrophotometric method [6]. Measurement of superoxide dismutase enzyme activity was performed in the cells by colorimetric-spectrophotometric method and based on measuring the reduction of nitro blue tetrazolium (NBT) AND IN THE PRESENCE OF zantyn oxide [7]. At last, the amount of DNA damage and oxidative reactions exposure were compared by means of mean comparison tests.

Statistical Calculations:
The amount of DNA damage and exposure of oxidative reactions in control groups, under mild heat stress and severe heat stress by using experimental design (Random design) and statistical test (one way ANOVA) were studied in statistical software (SPSS).

RESULTS AND DISCUSSION

TBARS test to study per-oxidation:
This test, as a selection method for screening lipid per-oxidation and a basic indicator has been placed to determine the oxidative reaction. This test provides a very useful data about production of free radicals and measurement of anti-oxidation activities of compounds. Results from this test are shown in chart 1 and table 1. As seen in the chart

amount of TBARS has increased from control group to higher temperature. More specifically, statistically there is a significant difference between control group and the group of 42°C (P<0.05). But statistically there is no significant difference between control group and the group of 39°C although amount of TBARS increases in the group compared to control group.

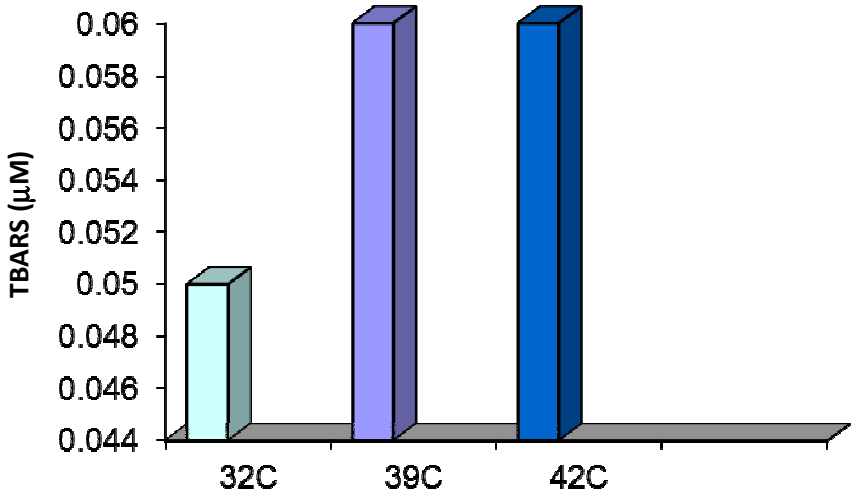

Chart 1: amount of measuring TBARS in the sample of Sertoli cells culture in different thermal groups. Different letters show a significant difference than the group of 32°C (P<0.05)
TBARS= Thiobarbituric Acid Reactive Substances

Accounting cells involved in planned death in TUNEL method:
Evaluating Sertoli cells related to different temperature groups by TUNEL dying showed that applying thermal stress makes a notable increase in the number of damaged cells from DNA and these cells gradually have been increased by increasing the temperature (9.9% in the group of 32°C , 17.24% in the group of 39°C and 37.39% in the group of 42°C). (Refer to figures 4-1, 4-2, 4-3, and 4-4).

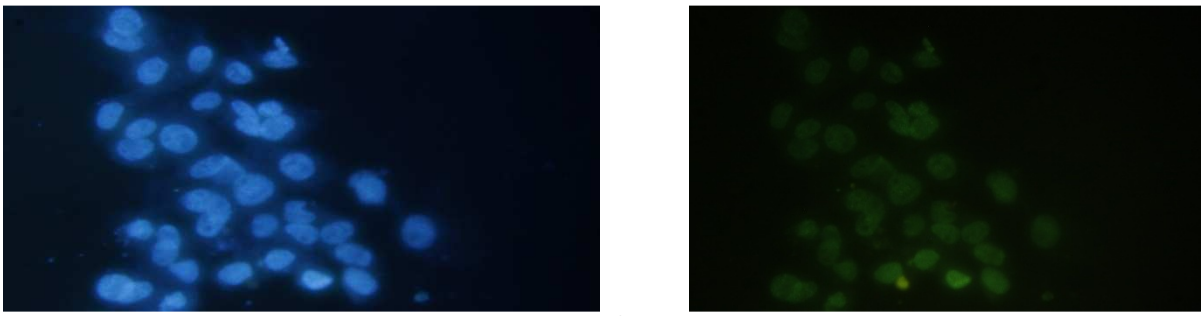

Figure 1: positive dyed control sample in TUNEL method (right figure). This sample was exposed under DNAse enzyme. All cells were involved in DNA damage under enzyme effect and became green. Colored left figure in Hoechst method for observe cell core

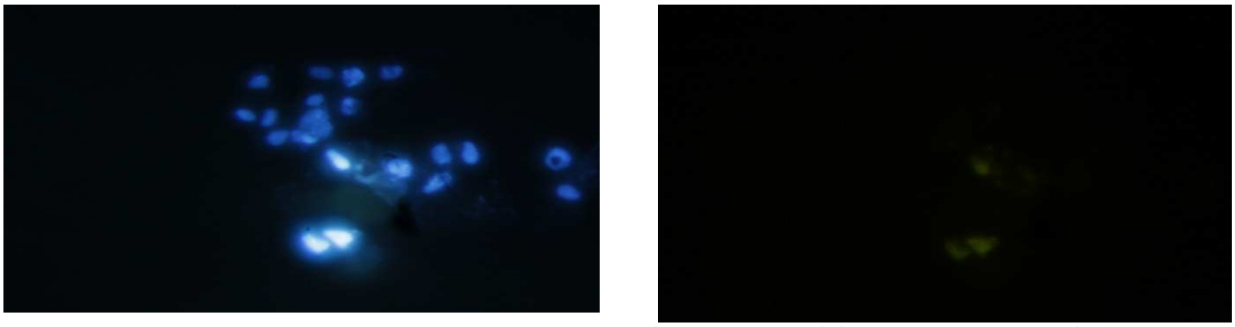

Figure2: a colored control sample (at 32°C) in TUNEL method (right figure). Colored left figure in Hoechst method for observe cell core.

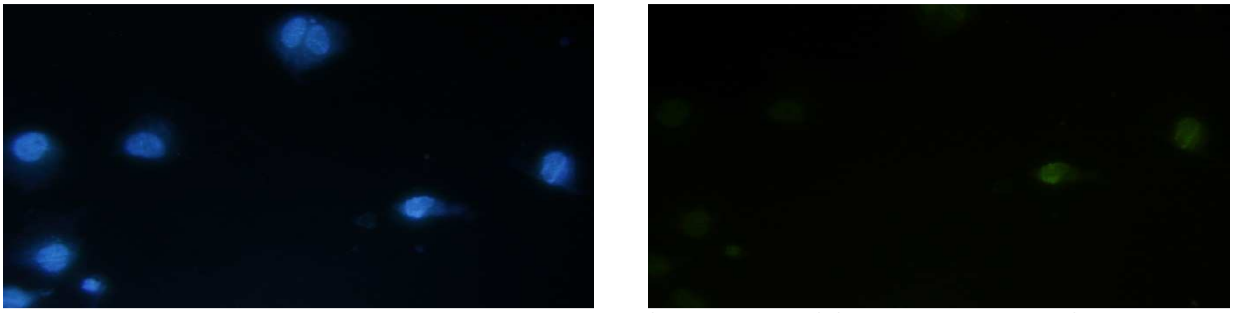

Figure3: a colored control sample (at 39°C) in TUNEL method (right figure). Colored left figure in Hoechst method for observe cell core

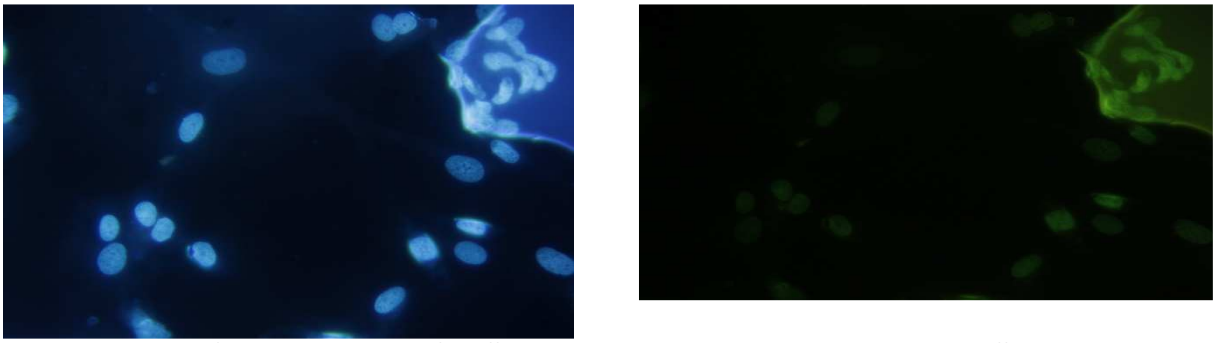

Figure4: amount of standard error I mean for different measures test in Sertoli cells culture sample in different thermal groups

Table 1: ± Mean standard error for different evaluated assays in sertoli cells culture sample in different thermal groups

	32°c temperature	39°c	42°c
Living cells number	95 ± 9.3 a	96 ± 8.6 a, b	78. ± 6.8 b
TBARS Rate (µW)	0.46 ± 0.05 a	0.64 ± 0.06 a, b	0.65 ± 0.06 b

TBARS: Thiobarbituric Acid reactive substances; FRAP: Ferric Reducing Ability of Plasma Assay; SOD: Super oxide dismutase
Different Letters in a row showing a significant differences (P<0.05).

The present study showed that by increasing temperature, living cells decreases and the difference between living cells in control group at 32°C With the groups of 39°C and 42°C is significant (P<0.05). The amount of TBARS has been increased from control group to higher temperature and there is a significant difference between control group and the group of 42°C (P<0.05). Also measuring Sertoli cells associated with different temperature groups of dyed TUNEL showed that applying thermal stress makes an increase in the number of damaged cells from DNA and the increase in the group of 42°C is significant than control group (P<0.05). Also the number of damaged cells in the group of 39°C is significantly more than control groups though the difference was not significant.

There are various functions for Sertoli cells showing their efficient role in the evolution of sex cells. According to the influential function of Sertoli cells in the procedure of sperm production in testes, damaging these cells will undoubtedly impair male fertility. One of the destructive damages is thermal stress effects which the present study was conducted to study the effects of the stress on Sertoli cells. A very good criterion for measuring destructive effects of thermal stress on the cells is the measurement test of viable cells. In the present study the Blue Trypan color was used for measuring the effects of thermal stress on the amount of viable Sertoli Cells. It was determined that the percentage of living Sertoli cells by applying thermal stress decreased significantly and indicates the destructive effect of this stress on Sertoli cells. A few clinical studies were conducted in this case confirm this finding. For example, Davis et al [8] showed that increasing the scrotum temperature to42°C in rams for 45min causes testes weight loss, average reduction of sperm-producing tubules' diameter, reduction the core diameter of Sertoli cells, reduction of reproductive cells after spermatogoniums A1and non-sperm of normal planning 20 days after applying thermal stress. Hydro-peroxide-lipidwas analyzed and Alkoxy and proxy radicals were formed at last different kinds of carbonyls were produced that are measurable via TBARS test. The applied lipid-peroxide on membrane is severely harmful for cell membrane and it can be the underlying cause of cell death [9]. The sensitivity of TBARS method has put this test as a selecting method of screening for per-oxidation lipid and a basic indicator in determining oxidative reaction. This test provides useful information about free radicals activities at hospitals and is an efficient method in measurement of anti-oxidation activity of different compounds. MDA which is released from breakage and degradation of unsaturated fatty acids is a suitable indicator for determining the amount of lipid-oxidation. DMA as a product from lipid-per-oxidation reacts with Thiobarbituric acid and makes a red spectrum and its absorbance in wavelength 535nm in the spectophotometric is measurable. At this study we concluded that by increasing the temperature the amount of lipid-per-oxidation increases which is in accordance with Papapetropolos

studies and collagenase in 1999. They showed that hydroxyl free radical which is resulted from metabolism activities and phagocytosis (respiratory burst in neutrophils) is severely reactionable and can react with unsaturated fatty acids and phospholipids of cell membrane and lipid-hydro-peroxide is formed. Lipid-hydro-peroxide is analyzed and forms Alkoxi and peroxy radicals and finally produces some different carbonyls such as MDA which is responsible for DNA degradation. Also they showed that anti-oxidants such as Alph-tocophrol reduces the MDA concentration. MDA production varies between different tissue samples and one of its reasons is different values of unsaturated fatty acid in different tissues. Since fatty acids containing at least 3 Methylene interrupted double bonds can ultimately form MDA. Variation of formed MDA is more reflecting the lipid compound to show their sensitivity to oxidation [10].

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