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Evaluation of thermal stress on Sertoli cells and its effect on nitric oxide production *in vitro*

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ABSTRACT

In order to identify and evaluate the thermal stress on Sertoli cells and its effect on nitric oxide production in vitro an assay was performed in the Institute of Embryo Technology by using of TUNEL method in Kord city. So testicles of 9 male lamb from3 to 10month were obtained through slaughter and the isolation and culture of Sertoli cells were performed these samples. In order to evaluate thermal stress, Petri dishes containing Sertoli cells were divided into three categories: the first group was the control group which was at 32 centigrade the second category was exposed in mild thermal stress for 6 hours which is equivalent with 39 centigrade the third category was under severe thermal stress of 42 centigrade for 6 hours. Measuring the nitric oxide in order to determine the total amount of oxidation compounds was done in cell culture. The result indicated that increasing the temperature makes the level of more specifically there is a significant difference between the control group and the group of 42 centigrade temperature ($P<0.05$). But despite a particular increasing of nitric oxide in the group with the temperature of 39 centigrade in comparison with control group there was seen no significant difference between these two groups.

Key words: Thermal stress, Nitric Oxide, Sertoli Cells.

INTRODUCTION

One of the important cells of male reproductive organ which is the most important supporting cells in sperm-making epithelium tubules are Sertoli cells. These cells play a basic role in supporting the nutritional and structure of sperm-generating cells in sperm-making tubules. So they play an important role in spermatogenesis [1]. It also has been found that Sertoli cell glycoprotein have different roles in the spermatogenesis testes.

Mammalian testes should be in a lower temperature than body temperature to function properly. Anatomical features of the testes and scrotum testicular enables the testicular thermoregulation. Thermal receptors in the skin of the scrotum can make reactions that cause body temperature to be dropped and breathing and sweating to be increased [2]. Sertoli cells are somatic cells that are from base to tip of the spermatogenic tubules epithelium and are in direct contact with developing sex cells in the process of spermatogenesis, several studies have shown that these cells play a crucial role in the evolution of the sex cells [3]. Numerous animal models have been developed to study the effects of heat on testes. These models include subjecting the testicles under a high temperature or (above 40 centigrade) placing them in the abdomen in a surgical procedure and making a cryptorchidism state, and causes testicles to be exposed in the temperature of body core for a long time (37°C). both methods had led to some changes such as testicular weight loss, increasing the apoptosis, loss of sex cells, changing of fertilizability of sperm [4].

Nitric oxide is a simple molecule with a single electron that is also known as free radicals, and has been affected by oxidation in the presence of oxygen, and creates secondary material. However, oxidation will occur if the levels of nitric oxide are high. Oxidation products of nitric oxide containing nitrite (NO2) in a solution of liquid and nitrate

(NO3) in the presence of oxy-hemoglobin (in the blood). Nitric oxide and its oxidation products are named as NOX altogether [5].

MATERIALS AND METHODS

This study was conducted in order to study and evaluate the thermal stress in Sertoli cells and its effects on production of nitric acid in vitro in the Institute of Embryo Technology in the university of Kord city. So, testicles of 9male lambs from 3 to10 months were provided through slaughter of Kord city located in Jongan area and were transferred to laboratory in the ice pack.

A. After moving the samples, testicles were washed and disinfected after 3 to 4 hours, by 70% ethanol for several times and then the layer of tunica Albojyna was cut by using a sterile scalpel blade.

B. After removal of sufficient required tissue, the testicular tissue was chopped by using two other scalpel and put into a sterile container. Then the fragments were moved into a conical tube. If the amount of sperm is high in the sample, it can be reduced by twice wash , in a way that a little (5mL) PBS is added to the pipe, then the centrifuge is cut once and by this sperm is removed from the tissue of the bottom of the tube and is suspended in phosphate buffer saline.

C. The PBS containing sperm was discarded. This time, the contents of the tubes were centrifuged for 4 minutes at 400g round. By second centrifuge the residues of PBS within sample were discarded.

D. A two-step enzymatic digestion:
During this stage, firstly the collagenase 0.1% enzyme was added to the sample, and was placed in incubator at 37 centigrade for half an hour. In order to do the digestion as the best way, the sample of testicular tissue was shaken with associated enzyme every 10 minutes. After this time, sample was removed of incubator and was centrifuged for 4 minutes at 400g round. After completing centrifuge, the supernatant containing Leydig cells was digested by collagen ease and discarded.

After completing this step, the second enzymatic digestion began. In this step, a next enzyme solution containing trypsin0.25 % and enzyme DNAse 20$\mu l/ml$ was added. The sample containing these two enzymes was incubated for 20 minutes, and as before, for the better digestion of enzyme, it was shaken every ten minutes. After enzymatic digestion, the sample was shortly centrifuged, to deposit the large pieces of tissue. At this stage, the supernatant was used for cultivation. The second enzymes are responsible for separating the spermatogenic tubules. It should be noted that in order to inactivate the second group of enzymes, we must use a culture medium containing 10%fetal bovine serum, because calcium neutralizes the trypsin.

E. Sample was centrifuged for 4 minutes at 400g round. The supernatant was discarded and the disposed cells on the floor were used. The culture medium DMEM[1] 4.5 mL and serum 0.5 mL were added to it to be quite uniform.

F. The resulting mixture was screened through a filter whose holes were of 70mm diameter. Then it was centrifuged for a minute at 60g round. Again it was centrifuged for 4min at 400g. The supernatant was discarded and the cells on the floor were mixed with DMEM medium and serum 10% for final cultivation and were moved to culture plates, and was qualitatively examined by a microscope, then placed in an incubator at 32 centigrade.

Measuring test of nitric oxide in Griess method:
Deproteinzation of serum:
A. 250mL zinc sulfate (75mM) was added to 300mL of sample.

B. and 350mL of NaOH (55mM) was added to the above solution and then vortexes and then were centrifuged for 3 minutes at 10000g round.

C. The supernatant was discarded.

D.750mL of supernatant with 350 mL of buffer-glycine (pH=9/7,4.5 g/l) was added to the prepared solution in the third step and then was vortexed.

Activating cadmium:
A. Two cadmium granules were taken and washed three times with distilled water.

B. Cadmium is shaken in copper (5mM) sulfate solution with buffer-glycine (pH=9.7; 15 g/L) for 5 minutes.

Measurement of nitrite:
A. 550mL of the product of first stage and two active cadmiums are placed in a micro-tube and are shaken for 10 minutes.

B. 350mL of above sample was added to 150mL of 1% sulfanilamide and was placed in darkness for 5 minutes.

C. 150mL of NED[1] (0.1%)is added to above solution and the absorbance is called in spectrophotometer in wavelength of 450nm for 10 minutes.

Blank preparation:
A. 750ml of distilled water is added to a 300ml glycine buffer and is briefly vortexed.

B. 350ml of above solution is removed and 150ml of sulfanilamide is added to it and it is placed in a dark place for 5 minute.

C. 150ml of NED is added to the above solution and then the spectrophotometer is set on zero.
This research was studied using random design and Statistical Presumption (one way ANOVA) in a Statistical Software (SPSS).

RESULTS AND DISCUSSION

Griess test to measure nitric oxide production:
Table 1 and figure1 show the rate of nitric oxide production in different temperatures. As seen in figure1 and table 1 as the thermal temperature increases levels of nitric oxide increases. More specifically, statistically there is a significant difference between control group and the group of 42 centigrade ($p<0.05$). However despite specific increasing of nitric oxide in the group of39 centigrade compared to control group there was no significant difference between these two groups, statistically. Chart 4-4 and table 1-4 represent the rate of change in SOD production in different temperature groups. As it can be seen by thermal stress exposure the production of the enzyme increases but rate of this change in two groups of thermal stress compared to control group is not significant.

It was determined that mammalian testes in order to function properly should be of lower temperature than body temperature. Anatomical features of the testis and scrotum enables adjusting the temperature. Meanwhile, major constituent cells of the testis tissues are Sertoli cells which are directly in contact with evolutionary sex cells in the process of spermatogenesis. So far several studies have shown that these cells play a crucial role in the evolution of the sex cells [3].

In an adult mouse, Sertoli cells constitute 17 to 19 percent of total volume of epithelium in spermatogenic tubules [6]. Several roles have been reported for Sertloi cells that represent their efficient roles in the evolution of sex cells. Regarding the influential role of Sertoli cells in the testes to produce sperm, damaging of this cell certainly impair male fertility. One of the destructive damagesis the effects of thermal stress which present study was conducted to investigate the effects of the stress on Sertoli cells. A very good criterion of measuring the destructive effects of thermal stress on cells is an assay test about survival of cells. In the present study Trypan Blue dye was used to evaluate the effect of thermal stress on the survival of Sertloi cells. It was found that the percentage of viable Sertloi cells can be significantly reduced by applying thermal stress which suggests the damaging effect of this kind of stress on Sertloi cells. In this case few clinical studies have been conducted on a ram that confirm this finding. In another study by [7], it was identified that by increasing scrotal temperature of rams from 1.5 to 2.2 centigrade 16 hours a day and for 21 days in every other day, the viable of embryo between 17to 65 days after inoculation compared with the control group significantly decreases.

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
Nitrite Rate (µm)	23.92 ± 2.84 a	27.4 ± 3.84 a, b	34.56 ± 3.7 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).

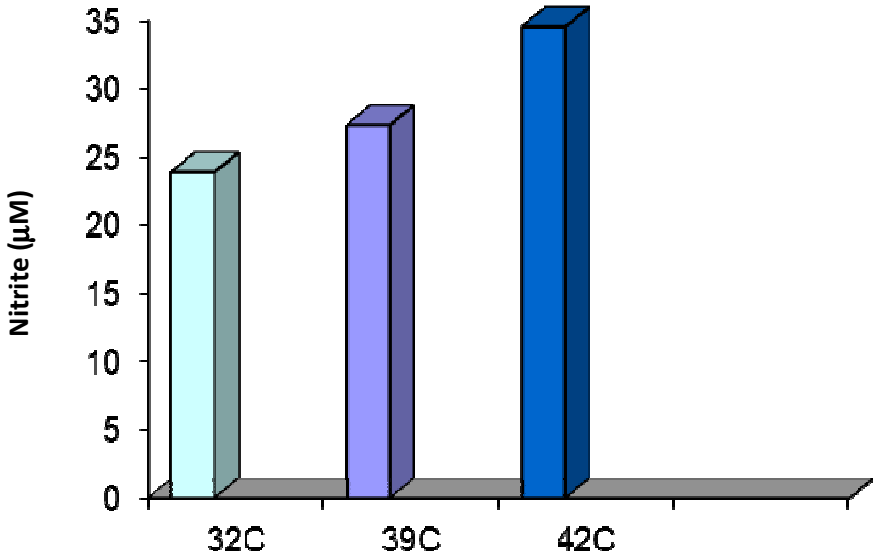

Chart 1: the amount of nitric oxide production in culture sample of Sertoli cells in different thermal groups. Different letters indicate significant differences compared to the 32 centigrade group (p<0.05).

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