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The state of thermal stress on oxidative reaction exposure and its effect on DNA damages of sertoli cells *in vitro*

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ABSTRACT

Testis is an organ that its natural function depends on temperature. It was determined that heat stress in male reproductive system followed by at leasttwo negative results. One of them is the exposure of oxidative reaction and the other one is impairment in DNA structure. Thus the present study was conducted aiming at evaluating oxidative reaction indicators and the exposure of DNA damages by using TUNEL method in Sertoli cells under heat stress at 42°C in order to determine how heat stress can affect Sertoli cells and study the sort of resulted damages in these cells under these conditions, for this testes of 9 male lambs of 3-10 months were provided from slaughter house and the separation and culture of Sertoli cells were performed on these samples. To evaluate heat stress effect, Petry Dishes containing Sertoli cells were divided into 3 categories; first category was control group and was incubated at 32°C. Second category which were under mild thermal stress at 39 centigrade for 6 hours and the third category were under severe thermal stress at 42 centigrade for 6 hours. In order to account cells which are involved in planned death, colored TUNEL was done on three positive, negative control group and test. Four parameters related to oxidative reaction in control group and affected groups under thermal stress were conducted in per-oxidation-lipids test via measuring MDA concentration (TBARS),measuring the activity of super-oxide-dismothase enzyme, measuring nitric oxide quantity and FRAP test to study total value of oxidation compounds in the culture medium of cell. Finally DNA damages and oxidative reaction occurrence in control groups, under mild heat stress and under severe heat stress, were studied by experimental design (Random design) and statistical test (One way ANOVA) in statistical software (SPSS). The results of present study showed that by increasing temperature the rate of living cells decreases and the difference between living cells percent in control group 32 centigrade and the groups of 39 and 42 centigrade is significant ($P<0.05$). TBARS rate increases from control group to higher temperature and there is a significant difference between control group and the group of 42 centigrade ($P<0.05$). by increasing the temperature the amount of nitric oxide increases. More specifically, there is a significant difference between control group and the group of 42 centigrade ($P<0.05$). TUNEL dying evaluating showed that applying thermal stress causes an increase in the number of damaged cells from DNA and this increase in the group of 42 centigrade is more significant than control group ($P<0.05$).

Key Words: thermal stress, oxidative reaction exposure, DNA damages, Sertoli cells.

INTRODUCTION

Testicle is an organ that its natural function depends on temperature. So that testes inside the scrotum of many mammals or outside their abdomen makes to be in a lower temperature of 2 to8 degrees than body temperature [1]. Body temperature regulating is increasingly important because a slight increasing of temperature can impair sperm-producing and lend to some fertility problems. For example, in mice thermal stress exposure leads to loss of re-productivity cells in testicle, lower quality of sperm along whit sperms that are involved in DNA damages and

brings about early death of embryos resulted from fertilization whit such sperms [2]. Other on mice have shown that a transient thermal stress exposure (usually over 40'c) and placing the testes and epididium inside body in a surgical manner which motivates testes to be at body temperature for a long time causes testes weight loss, increasing of cell death, loss of reproductive cells and reduction of fertility of sperm cells [3 and 4]. According to this sensitivity, there are different mechanism to keep a proper temperature for testes activities [5]. Results of this study and some other related studies show the importance of heat stress interfere whit male reproductive function.

Mentioned materials show the importance of negative effects of heat stress on male reproductive function. As it is clear at least two negative consequences occurs for the thermal stress in the male reproductive system; first oxidative reactions and one another is interference of DNA structure. Each in its turn has proven negative effects on male reproductive performance.

One of the main male reproductive cells which are the most important supporting cells in the epithelium of sperm-producing tubules is sertoli cells. These cells have an underlying role in the nutritional and structure support of the sperm generating cells in the sperm-producing tubules. So they play an important role in spermatogenesis [6]. It is well known that Glycoprotein of sertoli cells are of various roles in the testicles sperm-producing procedure. Given the crucial role of sertoli cells in the testicle sperm-producing: Establishing the conditions that may damage cells or impair their performance can strongly affect the process of sperm production and fertility is males as well. One of these destructive conditions is heat stress. By now a few studies have examined how heat stress affects on sertoli cells .In these case clinical findings about sheep indicate destructive effect of heat stress. On male fertility parameters in this species and show negative effect of heat stress on reproductive efficiency in rams. But the cause of such a shift is not known in species. This study intends to determine how thermal stress affects sertoli cells in sheep and investigate the varieties of resulted damages in these sells under these conditions.

The present study was conducted to:
1. evaluate oxidative reaction indicators in sertoli cells under thermal stress at 42'c.
2. evaluate DNA damages by TUNEL method in sertoli cells affected by thermal stress at 42'c.

MATERIALS AND DISCUSSION

All the procedures of sertoli cells culture were performed in vitro at Institute of Embryo technology of kurd university Advances in understanding the biology of sertoli cells was helpful in its intro is olation and culture.

Various studies were conducted to separate these cells. In these studies it was observed that sertoli cells have maintained their in vitro structure and biochemical properties as well as inside the body the most common method to isolate these cells include some enzymatic digestions of testicle [7].

Cultural procedures of sertoli cells:
Testes of 9 lambs of 3-10 months were provided from slaughter inside ice pack.

A)after moving the sample , they were washed and disinfected by 70%ethanol for several times after 3-4 hours and then Tunica Albojina was cut by sterile scalpel

B) After sufficient removal of required tissue , testicle tissue were sliced in another sterile by two scalpels. The slices were moved to Kunical tube. If sperm is high in the sample it can be reduced by twice wash. So that , some phosphate Buffer saline (5ml) is added to tubule, then centrifuge is short at once by which sperm is isolated from the tissue on the bottom of the tubules and is floated in PBS.

C) PBS containing sperm was removed and then tubules contents were centrifuged for 4 minutes at 400 ground. By second centrifuge the remaining PBS was removed.

D) two- step enzymatic digestion: over this step, first collagenase enzyme 1% was added to the sample and was incubated for half anhour at 37'c. the testicle tissue sample whit its enzyme in a best way. after this procedure, the sample was removed from oven and was centrifuged for 4 min at 400 g round. After centrifuge ,the supernatant containing leydig cells was digested by collagenase and was discarded. After this phase, the second enzymatic digestion was began . At this phase the next enzymatic solution containing Trypsin o.25% and DNA se enzyme 20ul\ml was added. the sample containing these two enzymes was incubated for 20 min and as before it was shaken every 10 min to do a better digestion. After second enzymatic digestion, the sample was centrifuged shortly to deposit the largetissue slices. At this phase, the supernatant was used for the rest of the culture. Enzymesof second

series functioned to isolate sperm- producing tubules. It is worthy of worthy of note that a medium containing 10% FCS is used inactivate the second enzymatic series because calcium neutralizes Trypsine.

E) The sample was centrifuged for 4 min at 400 grounds. The supernatant was discarded and deposited cells on floor were used.4.5ml of DMEM medium and 0.5 ml of serum were added to it to completely uniform.

F)the resulted mixture was screened through a filter whit holes of 70 um diameter, then was centrifuged for 1 minute at 60 ground. Then was centrifuged for 4 min at 400 ground again. The supernatant was discarded and the cells on the floor were mixed whit DMEM medium and serum10% for final culture. then were moved into the culture plate and were qualitatively examined under microscope and then were incubated at 32'c.

Evaluating parameters relating to oxidative reaction:
Four related relating to oxidative reaction in control parameters were group and groups of thermal stress were performed as following:

Measuring lipid-peroxidation via measurement of MDA concentration (TBARS) which is done by spectro-photometric method . Measuring the activity of superoxide Dismothuse enzyme of cells was done in colorimetric-spectro photometric method and based on Nitro Blue Tetrazolium reduction measure (NBT) in the presence of zantyn\ zantyn oxidase. At last DNA damages and oxidative reactions in control groups under mild heat stress and severe heat stress were compared by mean comparison tests.

C) 150ml NED was added to the above solution and then spectrophotometer was regulated at zero.

TBARS test:
Preparation of working solutions:
A) Normal Acid coloridric 0.25 was prepared

B) 0.375 gr of thiobarbiutric was added to acid coloridric

C)15gr of aced tri cloroacetic was added to the solution.

D)The final content is gradually gets to 100ml by normal acid coloridric 0.25

A)200ml of serum or sample whit 800ml distilled water was added to 2ml of working solution:

B) Blunk preparation: 1 . ml distilled water is added to 2 ml working solution.

C) Bone Mary is regulated at boiling temperature and samplewas put in bathroom at 100'C for 15 min .

D) thesample were let to be cold in the room temperature then it is centrifuged at 1000 rpm for 10 min.

E)the supernatant was removed and was poured into the coot and the light absorbance was read against Blunk in 535nm.

FRAR test:
In FRAPtest (ferric Reducing Ability of plasma Assay) deduct ability of antioxidants in considered sample was examined IN this method Fe3+ ions were used as raw material which in the presence of antioxidants is reduced to Fe2 to Fe2+can make colorful complex with Tris-2-pyridyl-s-triazine which the color intensity shows deductibility feature of sample.

Applied materials and FRAP process:
A)Prepation of FRAP reagent:
1-Buffer stat 300 mm and ph=316,311gr sodium stat- rehydrate with 16ml acid acetic was added to 11 water.

2-TPTZ solution: 10mm was solved in 40 mm acid cloridri3-20 mm colored iron.

Materials1. 2. And 3 during utilization mixed together in relation to 1:1:10,respectively.

B)Methodology :100 µ l of sample was mixed with 3 ml FRAP solution and ware length was read in light absorbance 59 nm.

First light absorbance was read at ominutes. Then the sample was put at 37' c for minutes and after this time the light absorbance was read again.

Blank Preparation:
100 µl distilled water was added to 3 ml FRAP solution and the machine was regulated.

C) Standardation by Asid Ascorbic:
100 µlStandard solution 1,25,50,75 and 100 µl was added to3 ml FRAP solution and light absorbance was read at o' and 4.

D) laying in formula for estimating FRAP sample value:

$$\frac{zerolig\ tabsorbance - 4t\ minutelig\ tabsorbance}{zerostandardlig\ tabsorbance - 4t\ standardlig\ tabsorbance} = \textbf{sample FRAP value}$$

Test of total Protein Measurement in Bradford method:
Bradford Prepared sigma solution was used.
A) 25 µ l of sample was added to500 µ l agent.
B) It was placed in medium temperature for 5 minutes.
C) Light absorbance was read in 595nm were length.

Blank preparation:
All above-mentioned stages were preformed exceptfor 25µl distilled water is making instead of sample.
Standard table was used for final measurement.
Later all mentioned parameters were measured based on raw protein.

Statistical calculations
DNA damages and oxidative reaction in the control groups, under mild heat stress and severe thermal stress were examined by using Experimental design (Random design) and statistical test (one way ANOVA) in statistical software (SPSS).

RESULTS AND DISCUSSION

TBARS test to examine per oxidation:
This test as a selection method was used for screening per oxidation lipids and has been as a basic indicator in determining oxidative reactions. This test provides useful data about free radical productions and measurement of antioxidant activities of compounds.

Results from this test are seen in Table 1-4 and chart 1. As seen in chart TBARS increases from control group to higher temperatures. More specifically, statistically there is a significant difference between control group and the group of 42°c ($P<0.05$). But there is no statistically significant difference between control group and the group of 39°c though TBARS in this group is increasing compared to control group.

FRAP assay to investigate antioxidant ability:
As said before antioxidants predictability is estimated by this test. Results of antioxidants reducing ability in examined sertoli culture samples, in chart 2 and table 1 are given. Attained values indicating antioxidant ability of materials available in culture medium has increased in relation to control group but the increase is not statistically significant.

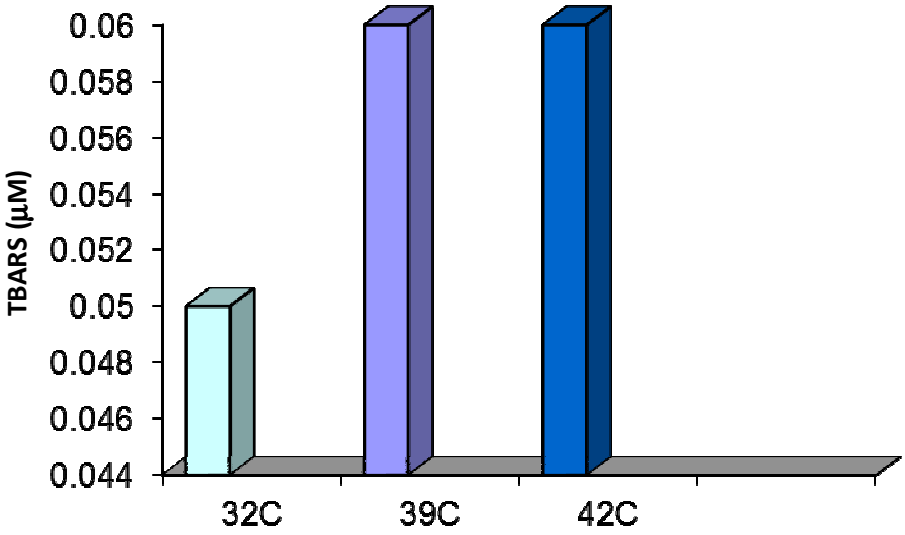

Chart 1: TBARS measured in sertoli cells culture sample in different thermal groups. Different letters showing a significant difference in relation to the group of 32°c (p<0.05)
TBARS= Thiobarbituric Acid Reactive Substances

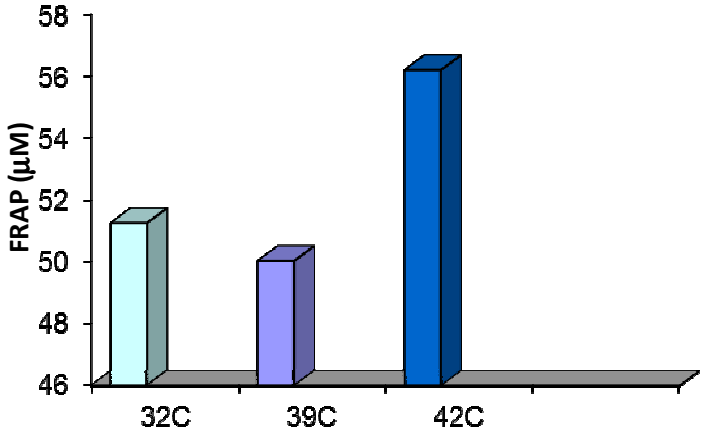

Chart 2: Estimated FRAP in sertoli cells culture sample in different thermal groups P>0.05 among 3 groups.
FRAP= Ferric Reducing Ability Of Plasmas Assay

Chart 3 and Table 1 show variation in SOD production in different temperature groups. As seen by thermal stress, enzyme production increases, but this change is not significant in two thermal stress groups in relation to control group.

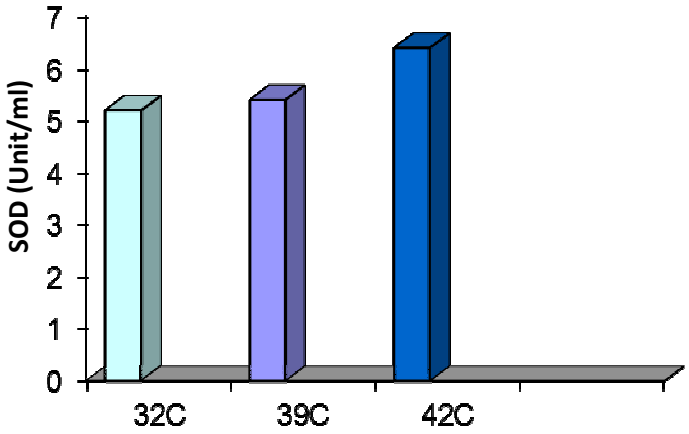

Chart 3: Estimated SOD in sertoli cells culture sample in different thermal groups. P>0.05, among 3 groups, SOD= Super Oxide Dismutase

Gris Assay for estimating Nitric Oxide Production:

In chart 1 and Nitric production is seen in different temperature groups. As seen in chart 5-4 and Table 1 by increasing temperature, Nitric Oxide rate rises. More specifically, there is a significant difference between control group and the group of 42°c, statistically (P<0.05). But despite distinct increase of Nitric Oxide in the group of 39°c there is no statically significant difference between these two groups in relation to control groups.

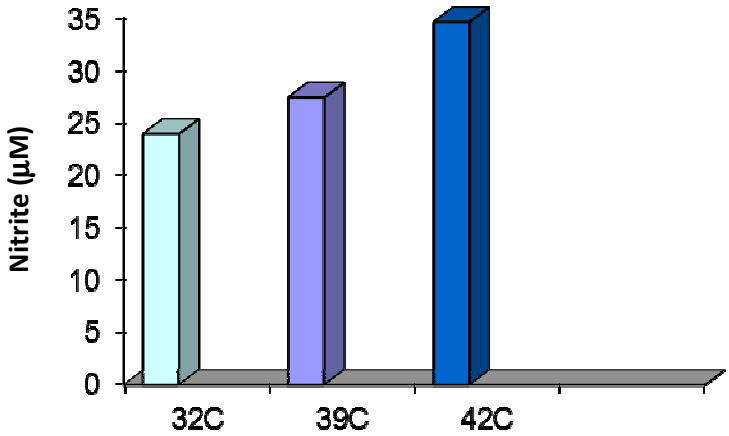

Chart 4: Nitric Oxide production in sertoli cells culture sample in different thermal groups. Different letters showing a significant difference in relation to 32°c (P<0.05)

Counting cells involved in planned death in TUNEL method:

Evaluating sertoli cells related to different temperature groups by dyed TUNEL showed that applying thermal stress caused a notable increase in the number of damaged cells from DNA and these cells are gradually increasing by rising 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). (Figures 1, 2, 3, and 4).

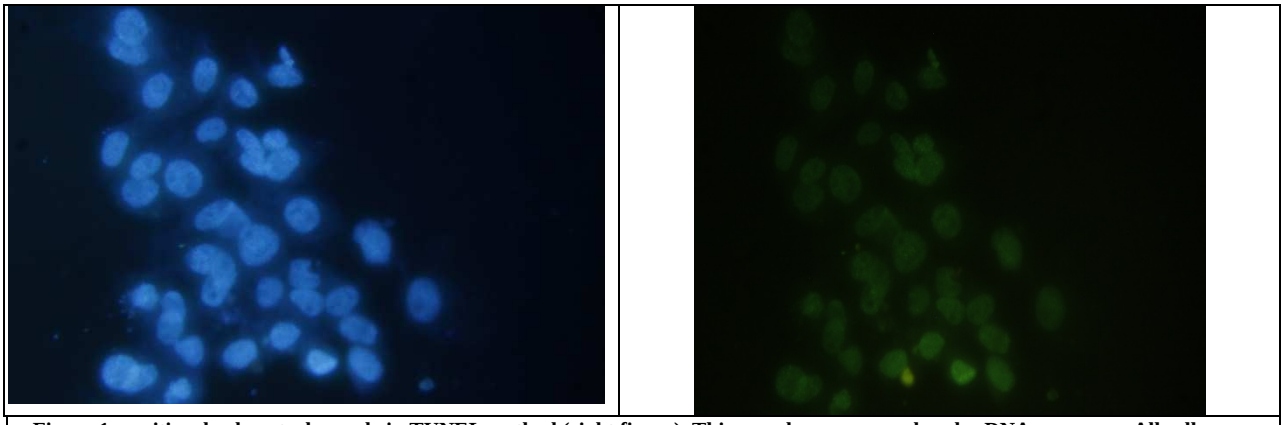

Figure 1: positive dyed control sample in TUNEL method (right figure). This sample was exposed under DNAse enzyme. All cells were involved in DNA damage under enzyme effect and became green. Colored left figure in Hoechst method for observe cell core.

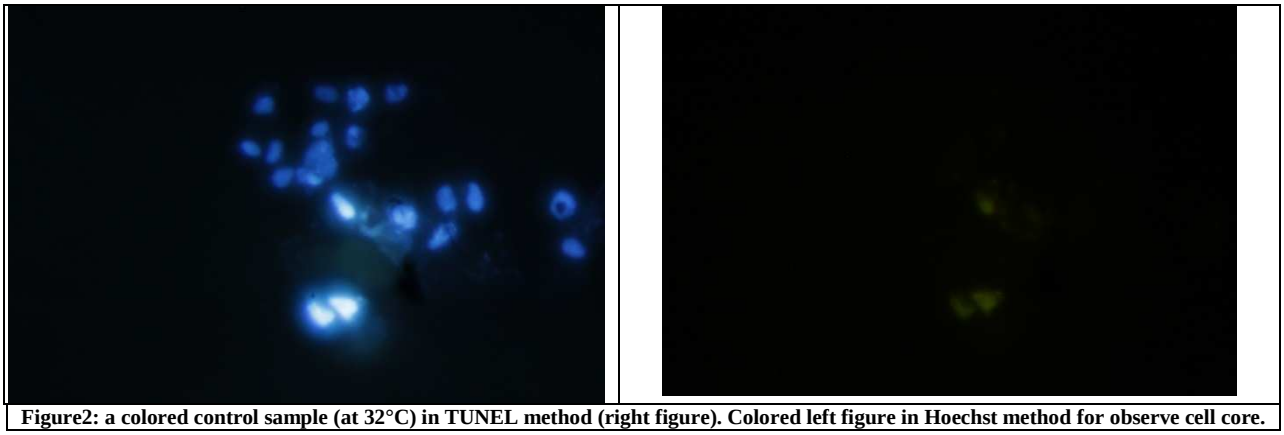

Figure2: a colored control sample (at 32°C) in TUNEL method (right figure). Colored left figure in Hoechst method for observe cell core.

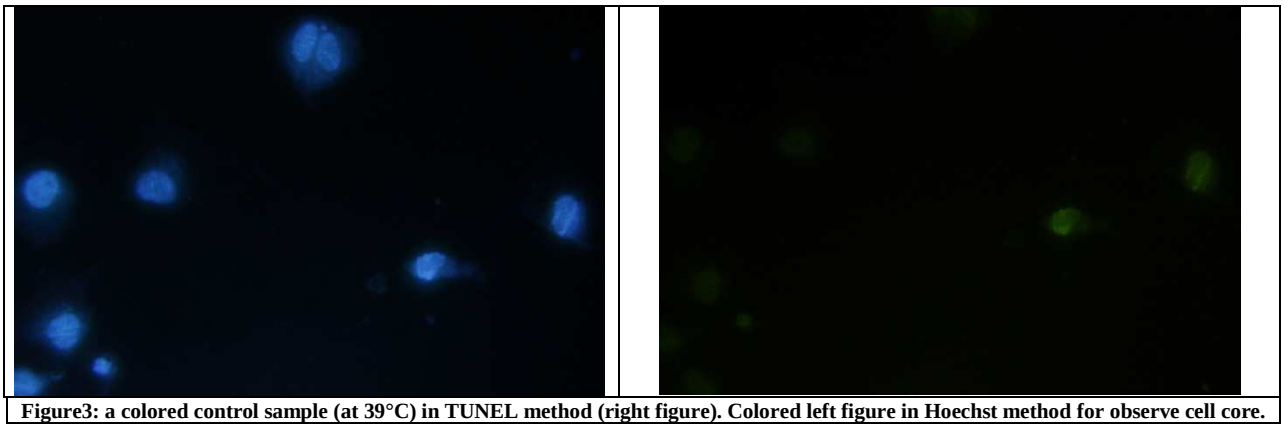

Figure3: a colored control sample (at 39°C) in TUNEL method (right figure). Colored left figure in Hoechst method for observe cell core.

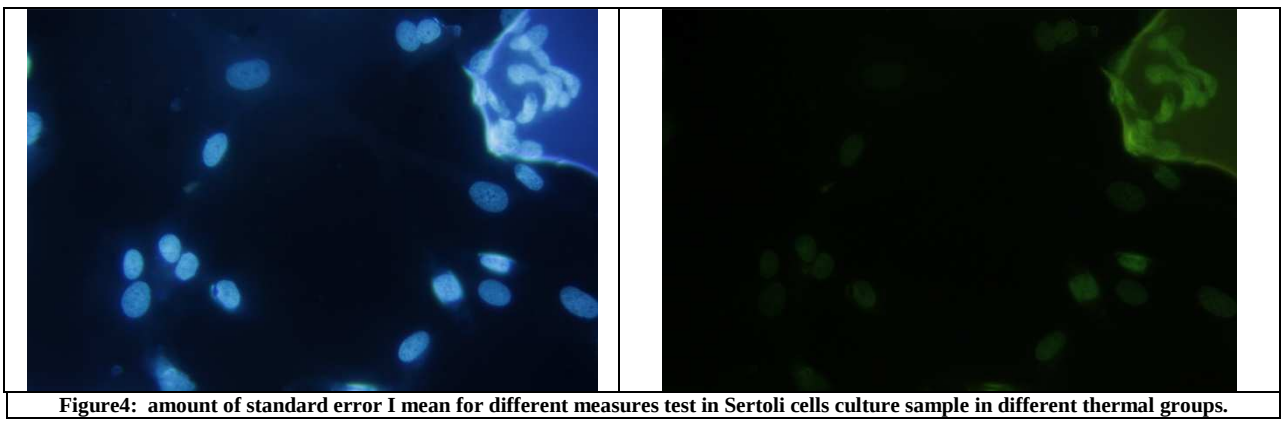

Figure4: amount of standard error I mean for different measures test in Sertoli cells culture sample in different thermal groups.

The present studies showed that by rising temperature, number of living cells has reduced and the difference between living cells percent in control group at 32°c and the groups of 39°c and 42°c is significant (P<0.05). TBARS scale has increased from control group to higher temperatures and there is a significant difference between control group and 42°c group (P<0.05). But there is no significant difference between control group and the group of 39°c. Resulted values indicate that anti oxidant ability of existing substances in culture medium has increased in relation to control group, but this increase is not statistically significant. In the other hand by thermal stress occurrence the amount of SOD enzyme production has been increased but this change in two thermal stress groups. In relation to control group is not significant. By rising temperature the amount of Nitric oxide increases. More specifically, there is a significant difference between control group and the group of 42°c (P<0.05). But despite distinct increasing of Nitric oxide in the group of 39°c there is no significant difference in relation to control group. Also, evaluating sertoli cells related to different temperature groups by dying TUNEL method showed that applying thermal stress causes an increase in the number of damaged cells from DNA and this increase in the group of 42°c was significant in relation to control group (P<0.05). Also number of damaged cells in the group of 39°c was notably higher than control group though the difference was not significant. It is identified that mammalian tests should be in a lower temperature than body temperature to function properly, which anatomical prosperities of testes and scrotum enable the temperature regulating. Mean while, one of the most important constituting cells of testicle tissue are sertoli cells that are in direct contact with developing sex cells in spermatogenesis process. By now a few studies have shown that these cells play a crucial and vital role in evaluation of sex cells [8]. In an adult mouse sertoli cells form 17 to 19 percent of epithelium capacity in sperm-producing tubules [9]. There care various roles for sertoli cells showing their influential role in evolution of sex cells. Regarding influential role sertoli cells in the process of sperm-producing in tests, damaging these cells definitely will impair male fertility. One of these destructive damages, are thermal stress effects which present study was conducted to examined effects of this stress on sertoIi cells. A really good scale standard to estimate destructive effects of thermal stress on cells is measuring assay of cells survival. In the present study Tripan Blue Dye was used to estimate thermal stress effect on viable of sertoli cells. It was determined that percentage of living sertoli cells is significantly decreasing by applying thermal stress indicating destructive effect of this on sertoli cells. A few clinical studies performed on ram in this relation confirming this finding. For example, Poyer et al [10], showed that raising scrotum temperature to 42°c in ram for 45min causes testicle weight loss, relative reduction of diameter of sperm-producing tubules, reduction diameter of sertoli cells core, reduction the number of reproductive cells since sperma togoniu A1 and non-occurrence of normal sperm since 20 days after applying thermal stress. I n an another study by Mieusset et al [11], it was determined that increasing scrotum temperature of ram from 1.5 to 2.2 °c for 16 hours a day and over 21 days in every other day caused notably reduction in the viable of embryo over 17 to 65 days after insemination in comparison with control group. In addition to these results the present study showed that by rising temperature Nitric oxide increases as well. More specifically, there is significant difference between control group and the group of 42°c (P<0.05). But despite distinct increasing of Nitric oxide in the group of 39°c in relation to control group there are no significant difference between these two groups. Similarly, Divis et al [12] showed that high concentration of Nitric oxide can react with molecule O2 and or super oxide ions, and results in solving cell skeleton proteins in Nitrogen, Nitration and oxidative damages and leads to cell function failure and even planned cell death [12]. They found that survival of systean Tobol in by oxide Nitrogen or its derivation including ONOO-ativated can be easily oxides. SoulPhidryl systean survival in oxides to Disoulphids that can inhibit dimeration α and β Tobol in units and lead to cytoskeleton damage of normal cells [13]. In 2008, Yung and his colleagues found that no low concentration maintains T-GSH and SOD activities and normally keeps the cell and Micro Tubules viability. While, No high concentrations reduce SOD,T-GSH and T-AOC activity and cell viability and destroy Micro Tubules .these researches suggested that NO can play a vital role. Using Tyrol polyptids or proteins for formation of S-Nitrosil series make anti-oxidative lose their activities. This disorder makes some problems in cleaning O2 free radicals and abnormal formation of the cytoskeleton. Damaging the frame of sentoli cells in body may cause a failure in Amenia-testicular ban which affects quality and quantity of stem cells. Also the researchers showed that No high concentration can inhibit transferring secration from sertoli cells [14]. In this study after measuring Nitric oxide we found increasing this material in the samples and this means that by increasing temperature leading to sertoli cells death, Nitric oxide amount increases as well, and this could help explaining the reasons of cell death which match with results of above-mentioned studies. It has been expressed that Nitric oxide can react with oxygen radicals, metals and other radicals. Nitric oxide also can produce proxy nitrite reacting with super oxide radical and Di-nitrogen tri-oxide reacting with oxygen. Of course it has been described that Nitric oxide direct effects occurs when Nitric oxide concentration is low and time is short. For example, Nitric oxide reaction with Heme proteins leads to activation of Goanylyl sicolase and sicloxinase. This interactive is very important in pro inflammatory process. Nitric oxide produces revival form of cyclotron Ksyzhnaz in low concentration which converts ferrous iron to active Ferric form, here Nitric oxide acts as a sweeper superoxide. The researchers showed that absence of Nitric oxide, H2O2 can lead to tissue damage and stopping the energy metabolism. So, it was shown that low concentration of nitric oxide can control free radicals production in addition to control role. And in the absence of this substance generated free radicals can destructively affect these tissues. It is worth noting nitric oxide is itself a harmful substance for cells

[15]. In this study oxidative reaction which can result from thermal stress is examined. It has been proved that oxidative reaction can damage membranes, proteins, cell DNA and RNA. Naturally testes are full of various oxidants including enzymes such as superoxide Dismutase, glo tatumn peroxides, Heme oxen's and Glotatiun Transferees. Exposing some stressful conditions, will disturb the balance between free radicals production and testicles antioxidant capacity and generates oxidative reaction in testis. Results of present study showed that by increasing cell death, lipid per oxidation increase as well, especially this increase was notable in the highest thermal stress scale i.e.,42'c .It has been identified that initial thermal target of free radicals, is cell membrane. Free radicals are strongly reaction able can expose lipid- per oxidation by capturing an electron from structure of unsaturated fatty acids and phospholipids of cell membrane. Hydro peroxide lipid, is analyzing and from Alkali and proxy radicals and finally produces different carbonyls such as MDA which are measurable by TBARS assay. Applied lipid-peroxide can provide cell death [16]. Sensitivity of TBARS method has made this assay as a selection method for screening per oxidation lipids and as an under-lying indicator in determining oxidative reactions. The assay offer useful data about free radicals activities in disease is an efficient method in estimating antioxidant activities in disease and is an efficient method in estimating anti oxidant activity of different combinations. MDA which is released through breakdown and degradation of unsaturated fatty acids is a really good indicator for measuring oxidation lipids.MDA as a production from per oxidation lipids reacts with Thiubarbiutoric acid and generates a red range which its capture rate in wavelength 535nm and spec to photometric method. In this study we concluded that by rising temperature, the amount of lipid per oxidation increases which is in accordance with Papa Petropolis's studies and colleagues (1999). They showed that free radical Hydroxyl which itself is resulting from metabolism activities and phagocytosis (aspiration Burt in Neutrophils) is strongly reactive and can react with unsaturated fatty acids and phospholipids of cell membrane and form lipid Hydro peroxide. Lipid Hydro peroxide is analyzed and forms Alkoxy and Proxy radicals and finally forms different carbonyls such as MDA which functions to damage DNA. They also showed that anti oxidations such as Alpha-tophrol reduces MDA concentration. MDA production varies among different tissues samples and one of the reasons is various values of unsaturated fatty acids in different tissues. Since fatty acids containing at least three interrupted Methylene double bonds can at last form MDA.Difference in the amount of formed MDA more reflecting lipids combination to show their sensitivity to oxidation [17]. In this study FRAP assay was used to estimate the amount of change in total capacity of anti oxidation of sertoli cells. Also super oxide-dismutase activity as one of the testes anti oxidations was specially estimated. It was determined that although total capacity of anti oxidation and activity of super oxide-dismutase-enzyme increases by temperature rising, their amount didn't have significant change. In this study A notable increase occurred in MDA following by temperature rising show high production of free radicals in cell culture medium. Other conducted studies confirm this. For example, it was determined that placing spermatogenesis cell culture of mice under thermal tress between34 to 40c cause a significant increase in the amount of MDA showing high quantity of produced free radicals in medium [18].

These produced free radicals in medium can be origin of Cytoplasm a Mitochondria or Cytoplasm membrane [19] Destructive effects of these free radicals on DNA cells is a completely proved effect. As lipids, free radicals can invade DNA molecules too and damage it for several times [20]. Damage location can directly be alkalizes or DNA molecules structure. Damage such as point mutation, Poly morphs, deletion, breakdown in single-strand or both DNA strands [21]. Due to this content in this study DNA damages can be as a possible cause for the lack of a significant increase in the antioxidant system power of sertoli cells. This means that chance of Gene expression increase of antioxidant enzymes from sertoli cell may achieved after these damages expression. Generally the present study showed that thermal stress is a detrimental factor for the survival and function of sertoli cells and mechanism of this effect by increasing the production of free radicals was in the environment that can severely damage fatty acids in cytoplasm a membrane and affect antioxidant enzymes activities and cause cell death.

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
FRAP Rate (µW)	51.36 ± 4.67	50.03 ± 3.68	56.21 ± 5.58
SOD Rate (IU|M)	5.2 ± 0.9	5.4 ± 0.8	6.4 ± 0.7
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).

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