

Steroidogenesis and VEGF Production Doesn't Alter in Leydig Cells within the Homeostatic Range of Testicular Temperature

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ABSTRACT

Context: Physiologically defined homeostatic temperature range for human testis falls within 32°C–37°C and any deviation outside that range can result in impaired steroidogenesis. However, whether temperature deviation within the homeostatic range can affect the steroidogenesis, is still a question of the investigation.

Aim: In the present study, we investigated the production of progesterone, testosterone, and vascular endothelial growth factor (VEGF) within the homeostatic temperature range of testis, i.e., 32°C–37°C. **Setting and Design:** We used mouse Leydig tumor cells-1 (MLTC-1) cell line as a model in the present study. **Materials and Methods:** Progesterone and testosterone production by MLTC-1 cells was measured by radioimmunoassay and VEGF production was measured using ELISA. **Statistical Analysis Used:** Data were analyzed using GraphPad Prism software version 7.04. Student's *t*-test and ANOVA were used to calculate the *P* value. *P* < 0.05 was considered statistically significant. **Results and Conclusions:** Results of our study indicate that there is no significant difference in production of progesterone, testosterone, and VEGF within the homeostatic range of the testicular temperature. Thus, we concluded that testicular cells are able to regulate the steroidogenesis and VEGF production under the homeostatic range of the testicular temperature.

KEYWORDS: Heat, Leydig cells, progesterone, testosterone, VEGF

INTRODUCTION

Physiologically, the homeostatic temperature range for human testicular cells falls within the 32°C–37°C. This temperature homeostasis is necessary for normal testicular function and any deviation outside that conditions can have a profound effect on fertility.^[1] The homeostatic conditions of the testicular temperature is maintained by the countercurrent heat exchange system between incoming arterial blood and outgoing venous blood and consequent heat loss through the skin of the scrotum.^[2] A mild increase in the testicular temperature could be the potential contraceptive for men which was reported in the clinical conditions (cryptorchidism and varicocele) and certain occupations (bakers, welders, and professional drivers).^[1] Although it is well established that the deviation in temperature outside the homeostatic range can affect the testicular functions,^[1] whether the temperature deviation within the homeostatic

range can have any effect, is still a question of the investigation. It is important to note that the minor changes in temperature, even in Fahrenheit, can disturb the physiology of body,^[3] but in the testis, there occurs a temperature range (32°C–37°C) with the difference of 5°C which could possibly affect the basic functions such as steroidogenesis. However, no study has shown any proof till date with respect to such concept; therefore, in the present study, we investigated the steroidogenesis within the homeostatic range of testicular temperature.

The process of steroidogenesis starts with the release of gonadotropin-releasing hormone from hypothalamus

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which acts on the anterior pituitary and stimulates it for releasing of luteinizing hormone (LH). LH acts on the Leydig cells by binding to the cell surface G protein-coupled receptors and activate adenylate cyclase, leading to the production of cyclic adenomonophosphate (cAMP), which in turn activates the protein kinase A (PKA).^[4] Activated PKA leads to the steroidogenesis and vascular endothelial growth factor (VEGF) production. Because both steroidogenesis and VEGF production appear to be dependent on the gonadotropin-stimulated cAMP formation, it is possible to postulate that the increased steroid formation by Leydig cells in response to the gonadotropic stimulation may be the trigger for the induction of VEGF which was reported as one of the key factors responsible for the fine regulation of cross-talk among the endocrine, endothelial, and gametogenic compartments of the testes. However, it is not known till date whether the deviation in testicular temperature can have any effect on the VEGF production, and therefore, we investigated the VEGF production in Leydig cells along with the steroidogenesis in the present study.

In our study, we hypothesise that steroidogenesis and VEGF production should show the significant difference at two extreme points of the homeostatic temperature range. Therefore, we study the difference in the progesterone, testosterone, and VEGF production at two different temperatures, i.e., 32°C and 37°C in the mouse Leydig tumor cells-1 (MLTC-1) in the basal conditions as well as by stimulating the cells through 8-bromoadenosine 3',5' (8-Br-cAMP).

MATERIALS AND METHODS

Study design

MLTC-1 cells were used in the present study. The whole study was divided into two groups, i.e., basal and cAMP groups. In a basal group, cells were incubated separately at 32°C and 37°C for 6 h in the basal cell culture medium. In a cAMP group, 8-Br-cAMP was added in basal cell culture medium and then cells were incubated separately at 32°C and 37°C for 6 h. Temperature conditions of 32°C and 37°C were provided by maintaining respective temperatures in humidified Incubator.

Culture of MLTC-1 cells

MLTC-1 cells purchased from ATCC (Manassas, USA) were cultured in a T-75 flask in Waymouth MB 752/1 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) at pH 7.3 at 37°C in 5% CO₂ in a humidified incubator. All experiments for this study were conducted on MLTC-1 cells between

passage numbers 10 and 30. The number of cells to be used in experiments was standardized and was seeded at a density of 2×10^5 cells/ml/well for estimating steroid and VEGF production.

MTT assay for cell viability test

Five milligrams of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) was dissolved in 5 ml of phosphate-buffered saline. Fifty microliters of the solution was added to each well containing 450 μ L of the fresh serum-free medium (SFM). The plate was incubated at 37°C in 5% CO₂ for 4 h. The reaction was terminated by adding dimethyl sulfoxide. The supernatant was transferred to a 96-well plates. The plate was read at a wavelength of 570 nm using multidetector microplate reader.

cAMP incubation

2×10^5 cells/ml per well were plated in a serum-containing medium culture plate for 24 h. Thereafter, the medium was replaced with fresh SFM supplemented with 0.1% bovine serum albumin. Cells were then incubated with 1 mM 8-Br-cAMP (8-Bromoadenosine 3',5'-cyclic monophosphate) for 6 h. After that, the medium was removed and stored at -20°C till assayed for VEGF.

Radioimmunoassay

Progesterone and testosterone secretion was measured in cell supernatant by radioimmunoassay (RIA). Tritium-labeled radioactive progesterone and testosterone were obtained from Perkin Elmer (Waltham, MA, USA). Mouse monoclonal testosterone antibody (clone 4E1G2) and rabbit polyclonal progesterone antibody were procured from Bio-Rad (Hercules, CA, USA) and used at a dilution of 1:500.

VEGF ELISA

VEGF secretion was measured in the cell supernatant using Mouse Quantikine VEGF ELISA kit from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's instructions. This kit specifically measures mouse VEGF₁₆₄ protein.

Statistical analysis

All the experiments were carried out at least in two replicates to ensure accuracy, and each experiment was performed at least twice to check the reproducibility. Data were analyzed using GraphPad Prism software version 7.04. Student's *t*-test and ANOVA were used to calculate the *P* value. *P* < 0.05 was considered statistically significant.

Ethical permission

Present study does not contain any studies with human participants or animals, performed by any of the authors.

We used commercially available established cell line which does not involve ethical concerns.^[5]

Language, grammar, and plagiarism

The references were inserted using EndNote software version 7.4. Language and grammar were checked by Grammarly software version 6.6. Plagiarism is corrected with the help of Plagiarism X software version 5.1. Present work was also edited by the professional proofreading and editing service (doc navigator, Chandigarh).

RESULTS

Cell number standardization

The number of cells to be used during the experiments was standardized by initially taking the 0.5, 1, 2, and 4 lakhs cells and measuring the respective progesterone/testosterone produced by them. In the basal conditions, the cells did not show any significant difference in progesterone production, but in the cAMP group, the cells showed a major significant difference between 1 and 2 lakh cells and showed a minor difference between 2 and 4 lakh cells [Table 1 and Figure 1]. In case of testosterone, a significant difference was observed between 1 and 2 lakhs in both basal and cAMP group [Table 1 and Figure 2]. Based on these results, 2 lakh cells were taken as final cell number for further experiments as progesterone/testosterone production show minor change beyond this number.

Cell viability analysis

The cell viability analyzed by MTT assay at two temperature conditions (32°C and 37°C) does not show any significant difference in the basal group as well as in cAMP group [Table 2 and Figure 3]. The mean difference in cell viability in the basal group at 32°C and 37°C was 0.01 ± 0.1675 which is nonsignificant ($P < 0.6645$). Similarly, the mean difference in cell viability in the cAMP group was 0.025 ± 0.1491 which is also nonsignificant ($P < 0.6502$).

Progesterone production

Progesterone production does not show any difference in two temperature conditions (32°C and 37°C) in basal as well as in a cAMP group [Table 3 and Figure 4]. The mean difference in progesterone production in the basal group at 32°C and 37°C was 0 ± 0.1155 which is nonsignificant ($P < 0.5340$). Similarly, the mean difference in the cAMP group was 0.375 ± 1.978 which was also nonsignificant ($P < 0.4154$).

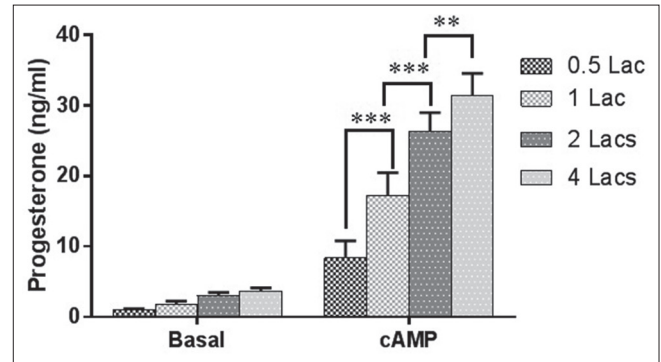


Figure 1: Mean progesterone (ng/ml) production with respect to cell numbers in basal and cAMP group (** indicates $P < 0.0095$ and *** indicates $P < 0.0001$)

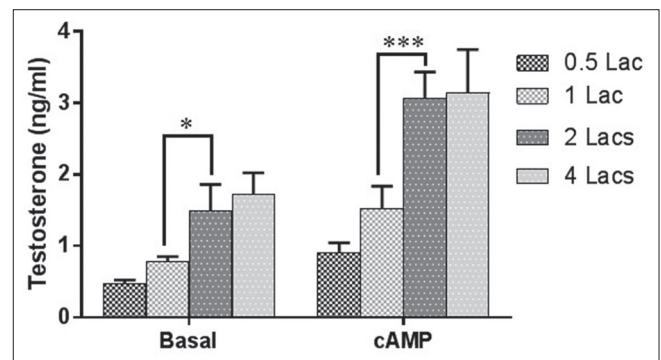


Figure 2: Mean testosterone (ng/ml) production with respect to cell numbers in basal and cyclic adenomophosphate group (* indicates $P < 0.0327$ and *** indicates $P < 0.0001$)

Table 1: Mean progesterone (ng/ml) and testosterone (ng/ml) production with respect to cell number in basal and cAMP group

Group	Cell number	Mean progesterone (ng/ml)±SD	Mean testosterone (ng/ml)±SD
Basal	0.5 lakh	1.05±0.11	0.48±0.04
	1 lakh	1.88±0.33	0.79±0.05
	2 lakhs	3.08±0.35	1.49±0.32
	4 lakhs	3.75±0.34	1.73±0.26
cAMP	0.5 lakh	8.38±2.1	0.91±0.12
	1 lakh	17.25±2.8	1.53±0.27
	2 lakhs	26.38±2.27	3.08±0.31
	4 lakhs	31.5±2.67	3.15±0.52

SD=Standard deviation, cAMP=Cyclic adenomophosphate

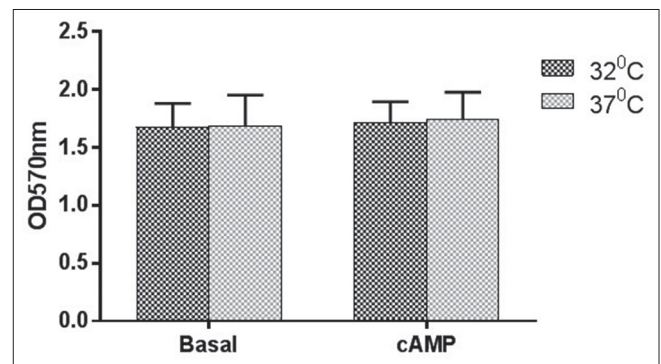


Figure 3: Mean optical density taken at 570 nm for the basal and cAMP group at 32°C and 37°C. Optical density gives the indirect measure of cell viability

Testosterone production

Testosterone production does not show any difference in two temperature conditions (32°C and 37°C) in basal as well as in a cAMP group [Table 4 and Figure 5]. The mean difference in progesterone production in the basal group at 32°C and 37°C was 0.01 ± 0.01904 which is nonsignificant (*P* < 0.1976). Similarly, the mean difference in the cAMP group was -0.3 ± 0.3182 which was also nonsignificant (*P* < 0.9581).

Measurement of VEGF

VEGF production also does not show any difference at two temperature conditions (32°C and 37°C) in basal as well as in a cAMP group [Table 5 and Figure 6]. The mean difference in VEGF production in the basal group at 32°C and 37°C was 13.37 ± 16.57 which is nonsignificant (*P* < 0.6227). Similarly, the mean difference in the cAMP group was 12.03 ± 14.69, which was also nonsignificant (*P* < 0.7100).

DISCUSSION

Recent studies on the effects of heat on the testis in humans, either applied directly, by insulating

the scrotum or by making the testis cryptorchid have provided information on harmful effects of temperature deviation on testicular functions.^[6] Apart from humans, several rodent models have been used to study the impact of heat stress on the testis including transient exposure of the testes to elevated temperatures (typically >40°C), surgical induction of cryptorchidism resulting in long-term exposure of the testes to core body temperature (37°C), or housing of males at elevated temperatures (e.g., 35°C–36°C) for several hours^[1] and all of them suggested the similar results. Although the link between increases in

Table 2: Mean optical density taken at 570 nm for the basal and cAMPe group at 32°C and 37°C. Optical density gives the indirect measure of cell viability

Group	Temperature	Mean OD (570 nm)±SD, n	P
Basal	32°C	1.678±0.1014, 4	0.6645 (NS)
	37°C	1.688±0.1333, 4	
cAMP	32°C	1.715±0.0895, 4	0.6502 (NS)
	37°C	1.74±0.11920, 4	

OD= Optical density, SD=Standard deviation, NS=Not significant, cAMP=Cyclic adenomonophosphate

Table 3: Mean progesterone (ng/ml) produced by MLTC-1 cells in basal and cAMP group at 32°C and 37°C

Group	Temperature	Mean progesterone (ng/ml)±SD, n	P
Basal	32°C	2.15±0.09574, 4	0.5340 (NS)
	37°C	2.15±0.06455, 4	
cAMP	32°C	24.13±1.7, 4	0.4154 (NS)
	37°C	23.75±1.01, 4	

SD= Standard deviation, NS=Not significant, cAMP=Cyclic adenomonophosphate

Table 4: Mean progesterone (ng/ml) produced by MLTC-1 in basal and cAMP group at 32°C and 37°C

Group	Temperature	Mean testosterone (ng/ml)±SD, n	P
Basal	32°C	0.8175±0.0175, 4	0.1976 (NS)
	37°C	0.8075±0.0075, 4	
cAMP	32°C	2.025±0.2287, 4	0.9581 (NS)
	37°C	2.325±0.2213, 4	

SD= Standard deviation, NS=Not significant, cAMP=Cyclic adenomonophosphate

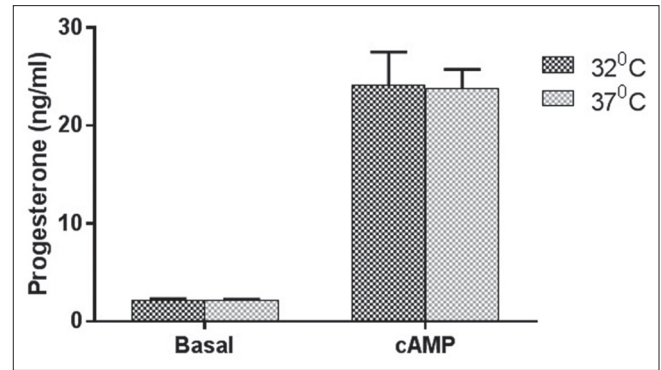


Figure 4: Mean progesterone (ng/ml) production by MLTC-1 cells in basal and cAMP group at 32°C and 37°C

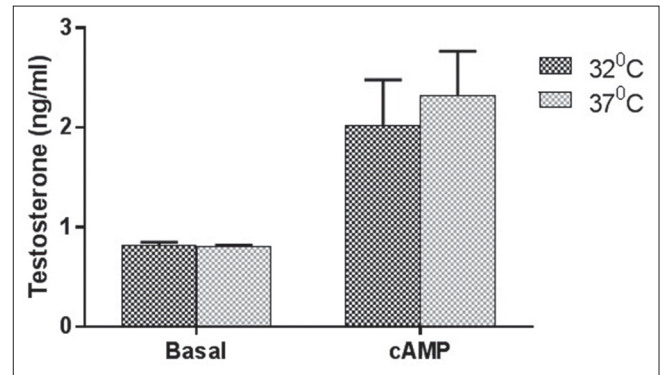


Figure 5: Mean testosterone (ng/ml) production by MLTC-1 cells in basal and cAMP group at 32°C and 37°C

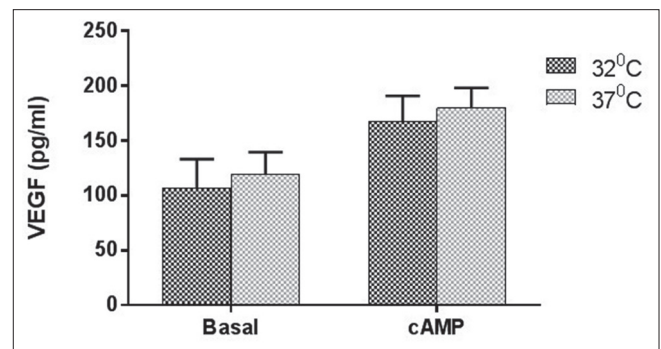


Figure 6: Mean VEGF (pg/ml) production by MLTC-1 cells in basal and cAMP group at 32°C and 37°C

Table 5: Mean VEGF (pg/ml) produced by MLTC-1 in basal and cAMP group at 32°C and 37°C

Group	Temperature	Mean VEGF (pg/ml)±SD, n	P
Basal	32°C	106.6±13.34, 4	0.6274 (NS)
	37°C	120±9.821, 4	
cAMP	32°C	167.9±11.52, 4	0.7100 (NS)
	37°C	179.9±9.116, 4	

VEGF= Vascular endothelial growth factor, SD=Standard deviation, NS=Not significant, cAMP=Cyclic adenomonophosphate

testicular temperature and impaired spermatogenesis is well established, it is not clearly established whether or not the steroidogenic function of the Leydig cells is affected by elevated temperature. The study of steroidogenesis in Leydig cells is requisite because the process of spermatogenesis ultimately depends on Leydig cells for an adequate supply of testosterone. Therefore, we conducted the present study which is exclusively focused around the steroidogenesis in Leydig cells.

In the previous study, it has been shown that, when gonadal cells subjected to heat shock, they faced the impaired steroidogenesis.^[7] The testosterone level in blood was found to be reduced in rats after exposure to a hot bath at 43°C for 10 min.^[8] Adult rats, when subjected to a single local testicular heat treatment of water at 43°C for 30 min, lead to insufficient testosterone biosynthesis.^[9] The mechanism behind the impaired steroidogenesis could be the disturbance in the cytosolic lipid pool reserve of Leydig cells. Cholesterol in the Leydig cells is stored as lipid droplets and a disruption of lipid droplet homeostasis can affect steroid hormone production by Leydig cells.^[10] Several large lipid drops were detected in Leydig cells of rats subjected to heat treatment when compared with nonexposed rats in which few small lipid droplets were detected.^[9] Disrupted lipid metabolism by raised testicular temperature further can lead to endocrine disturbance. However, our study suggests that Leydig cells are able to produce testosterone generally within homeostatic limits of testicular temperature.

Leydig cell viability following a heat shock is least studied area and most of the studies primarily focused on the germ cells and some of them on the Sertoli cells. Leydig cell viability was found to be not affected within the homeostatic range of testicular temperature in our study which is consistent with the available literature.^[11] Although VEGF plays several essential roles in the maintenance of testicular functioning such as it facilitates blood-borne hormones and nutritional elements transport,^[4] stimulates Leydig cell proliferation and testosterone secretion,^[12] and regulates spermatogenesis,^[13] but overexpression of VEGF in the testes can also lead to a massive disruption of

spermatogenesis.^[4] Till date, only a few studies are available establishing the relation of heat stress to the VEGF production. It has been documented that the VEGF pathway may play a role in neutralized the heat stress in the testicular cells.^[14] The detailed pathway by which VEGF act is not well understood, but still there is evidence suggesting that the scrotal heating may activate the testicular stress responses by increasing the expression of hypoxia-inducible factor 1 alpha which further induced the VEGF expression.^[14] In the present study, we also tried to investigate whether VEGF production alters within homeostatic range of the testicular temperature or not and found that it remains unaltered.

The aspect which makes our study unique is that we investigated the steroidogenesis and VEGF production within the homeostatic range of testicular temperature not by providing heat or cold shock. That was important because restricting the experiments under consideration of physiological limits is much relevant than to performing them outside the physiological limits of the body. To the best of our knowledge, the current study is the first study in which the effect of temperature on steroidogenesis and VEGF production was investigated within a homeostatic range. Although the results from our study is not same as our hypotheses, still, the present study gives important information about testis microenvironment.

CONCLUSIONS

Although several studies had been conducted to investigate the effect of temperature on the testicular functions, the main question still remains unanswered, is there any effect on the testicular function within the homeostatic range of testicular temperature. The present study put forward an evidence-based answer to the question by showing that the steroidogenesis and VEGF production does not alter within the homeostatic range of the testicular temperature.

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Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors contribution

Atul K Goyal designed and conducted the experiments and, generated the raw data. Jyoti Saini compiled the raw data, did the statistical analysis and drafted the manuscript.

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