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2nd International Symposium on Global Physiology 2018

"Enhancing Worldwide Collaboration in Basic and Applied Physiological Science"

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(2nd ISGP 2018)

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Grand Aston Yogyakarta, 13-12 oktober 2018

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WELCOMING SPEECH

Good Morning, Ladies and Gentleman…

Assamulaikum warohmatullahi Wabarokatuh.

Distinguished Guests, Speakers, Indonesian Physiological Society (IPS) members, Friends, and Families, All Welcome to 2nd ISGP 2018!

I am so pleased to see so many colleagues, professors, friends and families from several countries, all in one place! Thank you for participating in this symposium and Welcome to Yogyakarta.

The 2nd ISGP 2018 provides a very unique opportunity for all of us. Because this Symposium, covers a wide range of topics, namely medical physiology, exercise physiology, ergonomy, work physiology, oral physiology, veterinary physiology, livestock physiology, plant physiology, and environmental physiology, as well as other related topics, such as health education, biology, agriculture, forestry, pharmacy, conservancy, and biophysics. In this symposium I hope to be a media of sharing information, knowledge and research in the field of physiology. Our hope for the future can also develop research collaboration among all of us.

This symposium was carried out by a collaboration between Indonesian Physiological Society (IPS) jogjakarta region, Faculty of Medicine, Muhammadiyah University of Yogyakarta, Faculty of Veterinary Medicine, Faculty of Medicine and Faculty of Biology, Gadjah Mada University. The 2nd ISGP 2018 Organizing Committee members have worked extremely hard for the last year to prepare an outstanding symposium. For that, on behalf of the Committee I would like to give thanks to all committee members, sponsors and partners who have supported this symposium.

Enjoy your participation in the 2nd ISGP 2018 and memorable time visiting the Yogyakarta area. We hope you return next symposium with even more colleagues for 3nd ISGP 2020!

Thank you. Enjoy the symposium and welcome to the student city of Yogyakarta.

Wassalamu'alaikum warohmatullahi wabarokatuh

Yogyakarta, 12th October 2018
Dr. Slamet Widiyanto, S.Si., M.Sc.

PROGRAM OUTLINE OF 2ND ISGP 2018

Day 1, Friday, 12th October 2018
Grand Aston Hotel, Yogyakarta

Time	Programme	Venue
08.00 - 08.10	**Registration & Opening Remarks**	**Aston Hotel**
	Menyanyikan Lagu Indonesia Raya	
08.10 - 08.20	Dr. Slamet Widiyanto, S.Si, M.Sc	
08.20 - 08.30	Dr. ErmitaIsfandiary Ibrahim Ilyas, MS, AIFO	
08.30 - 08.40	Prof. Dr. drh. Isrina Oktavia Salasia	
08.40 - 09.00	**Coffee break + Poster Presentation**	
09.00-09.30	**Dr. Chatchote Thitaram, DVM** Vet Faculty of Chiang Mai University-Thailand Reproductive Physiology for Elephant Conservation	
09.30-10.00	**Prof. (Dr.) Prasanta Mitra Kumar** Head of Medical Biotech Department Sikkim Mannipal University	
10.00-10.30	**Prof. Datin Dr. Noriah Mohd Noor, MBBS., Ph.D** Kulliyyah of Pharmacy International Islamic University Malaysia	
10.30-11.00	**Prof. Jung Sok, Oak, Ph.D** Departmen of Kinesiology of Medical Science Dankook University	
11.00 - 11.30	Photo Session Day 1	
11.30 - 13.00	**Lunch Break + Friday Prayer**	
13.00 - 14.30	Paralel Session 1 Day 1	
14.30 - 15.00	**Coffee break**	
15.00 - 16.30	Paralel Session 2 Day 1	
16.30 - till end	**Coffee break + Closing Day 1**	

PROGRAM OUTLINE OF 2ND ISGP 2018

Day 2, Friday, 13th October 2018
Grand Aston Hotel, Yogyakarta

Time	Programme	Venue
08.30-08.45	**Registration + Opening Remarks**	**Aston Hotel**
08.45-09.00	**Coffee Break + Poster Presentation**	
09.00-09.30	**Dr. Suk Ho Lee**	
	Texas A & M San Antonio Exercise Physiology	
	Dr. dr. Denny Agustiningsih	
09.30-10.00	Department of Physiolgy Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada	
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12.30 - till end	**Coffee break + Closing Day 2**	

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REPRODUCTIVE PHYSIOLOGY FOR ASIAN ELEPHANT CONSERVATION

Chatchote Thitaram DVM, Ph.D, Dipl.TBT

Center of Excellence in Elephant and Wildlife Research,

Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100, Thailand

The world's populations of Asian elephants (*Elephas maximus*), both *in situ* and *ex situ*, are decreasing at an alarming rate due to the habitat destruction, human elephant conflict, poaching for ivory, meat and skin, diseases and injury with a low birth rate in captivity. As a result, it is crucial to breed elephants in captivity so that captive stocks do not need to be supplemented by removing animals from the wild. In contrast to the poor captive breeding, the human elephant conflict is one of the major problems in several elephant range countries due to over wild elephant population in limited area. Moreover, many captive bulls are aggression and harmful to human and other elephants; thus, reproductive control is an alternative way to solve these problems. Therefore, we need to improve our understanding of the reproductive physiology and, in particular, the estrous cycle and musth of the Asian elephant to strengthen captive management strategies

Female: puberty and ovarian cycle

Puberty (i.e., initiation of ovarian cycles) in young female elephants occurs between the ages of 4 and 10 years of age and depends on several factors; e.g. birth origin (e.g. wild or captive), nutrition and growth rate. Cyclical changes in ovarian activity and structures are accompanied by changes hormones of the hypothalamo-pituitary-gonadal (HPG) axis, including gonadotropin releasing hormone (GnRH) from hypothalamus, follicular stimulating hormone (FSH) and luteinizing hormone from anterior pituitary gland, estrogens during the follicular phase and progestagens (progesterone and related steroids) during the luteal phase from the ovary

TESTOSTERONE INCREASES THE EXPRESSION OF ENDOTHELIAL ER-B IN NORMOGLUCOSE BUT NOT IN HIGH GLUCOSE ENVIRONMENT

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ABSTRACT

Testosterone may have contributing role in endothelial dysfunction. A part of testosterone that diffuses to the interior of endothelial cells will be converted to estradiol by aromatase enzyme. It is known that endothelial cells contain estrogen receptors (ER) as well as androgen receptor (AR). In the development of cardiovascular diseases, ER has been regarded as a protective factor. To examine the influence of testosterone to the expression of endothelial ER-β, we have exposed testosterone in 4 doses: 0 nM, 1 nM, 10 nM, and 100 nM to the endothelial cells culture derived from human umbilical vein (HUVEC) in either normoglucose (NG) or high glucose (HG) medium. HG medium was used to mimic hyperglycemia, a condition that can trigger activation of endothelial cells. Immunocytochemistry was used to stain the endothelial ER-β. The results showed that proportion of endothelial cells positively stained with ER-β antibody was significantly higher in NG medium + testosterone than in NG medium only. However, proportion of endothelial cells positively stained with ER-β antibody in HG medium + testosterone did not significantly differ than in HG medium only. In conclusion, testosterone increases the expression of beta estrogen receptors in resting endothelial cells but not in activated endothelial cells.

Keywords : endothelial cells, beta estrogen receptors, testosterone, high glucose, immunocytochemistry.

INTRODUCTION

Cardiovascular disease is one of the leading causes of mortality that are suffered by male subjects more frequently than female ones, so that the male gender is known as one of the classic risk factors for cardiovascular diseases. The concentration of androgen hormones, particularly testosterone, in plasma which is much higher in male subjects compared to female subjects led to proposition of the role of testosterone in cardiovascular events [1-2].

Endothelium plays important roles in cardiovascular events. Endothelial cells have a capacity to inhibit platelet activation, through biosynthesis and release tromboregulators, such as endothelium-derived relaxing factor (EDRF) or nitric oxide (NO) and prostacyclin (PGI_2). PGI_2 is the most potential platelet antiagregator as well as a vasodilator. Endothelial PGI_2 will bind to PGI_2 receptor (IP_1) on the platelet membrane. IP_1 ligation-which is a GPCR-by PGI_2 causes the activation of the enzyme adenylyl cyclase, which catalyzes the conversion of ATP into cyclic adenosine monophosphate (cAMP), resulting in increased cAMP intratrombosit, activation of protein kinase A (PKA), decreased concentration of calcium ions, and finally inhibition of platelet aggregation [3-4].

Recent studies showed that endothelial cells contain androgen receptor (AR). Endothelial cells have also been known to have 5α-reductase enzyme, which catalyzes conversion of testosterone into DHT (5-10% of bioavailable testosterone; BioT), as well as aromatase enzyme, which catalyzes conversion of testosterone into estradiol (0.1-0.2% of BioT). Endothelial cells are also known to have estrogen receptors (ER), both ER-α and ER-β. With the AR, ER, and enzymes to metabolize testosterone, endothelial cells emerges as target cells for testosterone action [5-6].

The protective role of endothelial cells to the cardiovascular system, such as antiplatelets aggregation, is disturbed in condition of endothelial dysfunction and/or endothelial activation. Endothelial dysfunction occurs when biosynthesis, secretion and/or activity of endothelial NO is decreased. This condition can be extended to the imbalance between NO, PGI_2 and/or EDHF in the vascular system. Endothelial activation is related to the changes in endothelial phenotypes, such as the increased expression of adhesion molecules, therefore endothelial cells become more pro platelets aggregation and pro thrombosis. Hyperglycemia is one of factors, as well as pro inflammatory cytokine, free radicals and shear stress, that induce endothelial dysfunction and/or activation [7-8].

As a part of our study, this paper will report our attempt to answer the question whether exposure testosterone to the endothelial cells will influence the presence of ER-β in normoglucose and high glucose medium.

METHODS

To answer the objective of the study we conducted an in vitro study using primary endothelial cell culture derived from human umbilical vein (HUVEC). We collected the delivered mothers who donated the umbilical cord. They were having no hypertension, diabetes mellitus, and pre-ecclampsia. Their babies were aterm at the time of delivery and having Apgar score >10. The delivered mothers were the person that signed the informed consent. The study had been approved by the Institutional Review Board of the Faculty of Medicine, Gadjah Mada University (FM-GMU), Yogyakarta.

Primary endothelial cells culture

We used Medium 199 (M199) as the culture medium that prepared from the powdered M199 (Gibco) added with 2 g of sodium bicarbonate and 2 g of HEPES sodium salts. We supplemented M199 with 10% fetal bovine serum (FBS) (Caisson), 100 IU/mL of penicillin and 100 µg/mL of streptomycin (Sigma), 0.5% fungizone (Gibco), and 2 mM of L-glutamine (Sigma). We prepared the umbilical cord buffer from 9.6 g of Dulbecco's phosphate buffered saline (PBS) powder that dissolved with distilled water into 1000 mL in total volume. We sterilized PBS solution with autoclave at 121^{0}C for 15 minutes. We enriched the PBS with 100 IU/mL of penicillin and 100 µg/mL of streptomycin that obtained from crystalline procaine penicillin-G powder (Meiji) and streptomycin sulfate powder (Meiji) dissolved with sterilized water for injection. We kept the buffer solution was kept at 4^{0}C.

Human umbilical vein endothelial cells (HUVEC) culture was established according to the technique describes by Jaffe et al. [9], Tanaka [10] and Baudin et al. [11]. We have reported our protocol in previous manuscript [12]. Briefly, it was divided into three phases: umbilical cord collection, endothelial cells collection and endothelial cells culture.

Umbilical cord collection

We had taken the umbilical cord from the placenta of the delivered mothers by sterile technique. We put the umbilical cord (10-20 cm length) in a sterile bottle containing phosphate buffer saline with the antibiotic penicillin streptomycin 1%. We carefully placed the bottle at 4^{0}C environment until we processed the sample in the Cell Culture Laboratory in the Department of Physiology FM-GMU for not more than 12 hours after sample collection.

Endothelial cells collection

This step was done in the laminar air flow in a sterile condition. We identified the inlet of umbilical vein of the cord and we cleaned the outside of the umbilical cord using povidone iodine. After that, we inserted a cannula into the vein and kept it tightly at its position with a sterile clamp. We then flushed the lumen of the umbilical vein using ringer lactate solution to wash away from blood clotting. To disaggregate the endothelial cells from its basal membrane, we injected 0.25% trypsin-EDTA (Gibco) into the lumen of the umbilical vein. We then clamped the other end of the lumen to prevent the enzyme solution dropped outside the lumen. We incubated the umbilical cord for 3-5 minutes in warm PBS. After that, we taken the clamp off and collected the enzyme solution that already contained the disaggregated endothelial cells into a sterile bottle filled with 1 cc of FBS.

Endothelial cells culture (HUVEC)

This step was also done in the laminar air flow in a sterile condition following endothelial cells collection. We centrifuged the cells effluent at 2000 rpm for 10 minutes. We then removed the supernatant and washed the pellet with M199. We resuspended the cells with growth medium and inoculated them on a gelatin-coated 60 mm-diameter tissue culture dish (Iwaki). We incubated the dish at 37^{0}C and 5% CO_2 for 24 hours. We changed the growth medium of the dish every 2 days until the HUVEC reached 80% sub confluence. We observed the confluent cells under an inverted microscope (Eclipse, Nikon, Japan). We examined the expression of von-Willebrand factor (vWF) using immunocytochemistry method to characterize the HUVEC.

HUVEC subculture

We took the TCD containing primary HUVEC out from the incubator and observed the cells with an inverted microscope. If the endothelial cells already reached the sub confluence (80%), we put the TCD into the hood. With the aseptic technique, we poured the culture medium out from the TCD and put the warmed PBS solution containing antibiotics penicillin-streptomycin 1%

into the TCD three times until the TCD was clear from the culture medium. After that, we put 1 cc of trypsin-EDTA solution into the TCD to dispatch the endothelial cells from the vessel. We incubated the TCD with Trypsin-EDTA 1% for 5 minutes and observed it with the inverted microscope. If the trypsination was success, the endothelial cells will be looked rounded and mobile. Then we put the culture medium into the TCD to stop the action of the trypsin. We pipetted the cell suspension out form the TCD into the conical tube 15 cc sterile and centrifuged it at 2000 rpm for 10 minutes. Then we poured carefully the supernatant and suspended the cell pellet in the bottom of the tube with 1 cc of the culture medium. We did endothelial cell counting with the improved Neuebauer counting chamber and calculated the endothelial cells needed for the subculture. We finally did subculture (1st passage) with $3x10^5$ endothelial cells for each well in 24 wells microplate (Iwaki) already contained gelatinized culture cover slip at the bottom of the wells.

Treatment

After 24 hours we replaced the growth medium of the secondary HUVEC with either normoglucose (NG) medium (5.6 nM of glucose) or high glucose (HG) medium (M199) (22.4nM of glucose) without or with testosterone (T) at 0, 1, 10, or 100 nM. The detailed of the treatment groups were as follows:

- G1: 0,45 cc complete M199 with NG + 0,05 cc DMSO 0,1%
- G2: 0,45 cc complete M199 with NG + 0,05 cc T 10 nM
- G3: 0,45 cc complete M199 with NG + 0,05 cc T 10^2 nM
- G4: 0,45 cc complete M199 with NG + 0,05 cc T 10^3 nM
- G5: 0,45 cc complete M199 with NG + 0,05 cc DMSO 0,1%
- G6: 0,45 cc complete M199 with NG + 0,05 cc T 10 nM
- G7: 0,45 cc complete M199 with NG + 0,05 cc T 10^2 nM
- G8: 0,45 cc complete M199 with NG + 0,05 cc T 10^3 nM

These treatment groups were arranged in 2x4 factorial designs for testosterone and glucose medium factors as follows:

Table 1. 2x4 Factorial designs

Testosterone (T)	Normoglucose medium (NG)	High glucose medium (HG)
T0	G1	G5
T1	G2	G6
T10	G3	G7
T100	G4	G8

Note: Normoglucose medium = 5.6 mM glucose. High glucose medium = 22.4 mM glucose. T0 = without testosterone. T1 = testosterone 1 nM. T10 = testosterone 10 nM T100 = testosterone 100 nM.

For the next 24 hours, we removed the treatment solution and did staining for the endothelial cells attached on the culture cover slip.

Immunocytochemistry

We did staining with the procedure as follows. We did fixation for the cells with absolute methanol for 15 minutes. Then we poured the fixation solution out. Next we dripped 3% of H_2O_2 solution to the endothelial cells monolayer and incubated it for 20 minutes to inhibit the activity of endogenous peroxidase. We washed the monolayer with tapping water for 3 minutes followed with the aquadest. We then washed the monolayer with the PBS solution two times for 3-5 minutes. Next we incubated the monolayer with the blocking serum for 15 minutes. We then incubated it with mouse anti-human-ER-β (Abcam) as the primary antibody for ER-β, with 1:100 dilution for 1 hour. We washed it with the PBS solution for 2 times. We then incubated the monolayer with trekie universal link as the secondary antibody for 20 minutes. We washed it with the PBS solution for 2 times. Next we incubated it with trekie avidin horse reddish peroxidase (HRP) for 10 minutes. We washed it with the PBS solution for 2 times. We dripped chromogen DAB (1:50) into the monolayer and incubated it for 2 minutes. We then washed it with tapping water. We did counterstain with Mayer's hematoxylin for 2 minutes. We washed it with tapping water. Next we dripped graded alcohol: 70%, 96% and 100%. We then did mounting the monolayer with Canada balsam.

Cell counting

We observed the slide with an optic microscope with magnification 10 times. We observed the endothelial cells that positively and negatively ER-β

stained (browned). ER-β enzyme is localized in the nucleus. We then counted the proportion of the endothelial cells that positively and negatively ER-β stained in 5 fields of view for each slide. We calculated the mean and the standard of deviation for the counting results.

Statistical analysis

We used unpaired t test to analyze the influence of each doses of T to the presence of endothelial ER-β as compared to the negative control in either NG or HG medium. We used one way ANOVA to analyze the influence of T at graded doses to the presence of endothelial ER-β in either NG or HG medium. We used analysis of variance to analyze 2x4 factorial designs of the influence of testosterone and glucose medium to the presence of endothelial ER-β. We considered p value <0.05 as statistically significance.

RESULTS

Endothelial cells culture

We have reported the validity of our model of endothelial cells culture (HUVEC) in the previous report [12].

Endothelial ER-β

The proportion of endothelial cells that ER-β positively stained in our study is presented in the following table:

Table 2. Proportion of the ER-β positively stained endothelial cells following the exposure of testosterone in graded doses in normoglucose and high glucose medium

Testosterone (T)	Normoglucose medium (NG)		High glucose medium (HG)	
	Mean (%)	Standard of deviation	Mean (%)	Standard of deviation
T0	1.7	2.74	11.11	7.35
T1	14.09	4.40	4.14	4.0
T10	11.01	5.36	4.53	2.82
T100	23.21	10.52	6.22	2.85

Note: N = tripiclates of $3x10^5$ cells / group. Normoglucose medium = 5.6 mM glucose. High glucose medium = 22.4 mM glucose. T0 = without testosterone. T1 = testosterone 1 nM. T10 = testosterone 10 nM T100 = testosterone 100 nM.

Statistical analysis

Unpaired t-test:

a. Proportion of endothelial cells positively stained with ER-β antibody in normoglucose medium without testosterone (1.7 ± 2.74%) was significantly lower than proportion of endothelial cells positively stained with ER-β antibody in normoglucose medium with 1 nM testosterone (14.09 ±4.4%; $p = 0.0001$), 10 nM testosterone (11.01 ± 5.36%; $p = 0.014$), and 100 nM testosterone (23.21 ± 10.52%; $p = 0.009$).
b. Proportion of endothelial cells positively stained with ER-β antibody in high glucose medium without testosterone (11.11 ± 7.35%) did not significantly differ with proportion of endothelial cells positively stained with ER-β antibody in high glucose medium with 1 nM testosterone (4.14 ±4%; $p = 0.11$), 10 nM testosterone (4.53 ± 2.82%; $p = 0.119$), and 100 nM testosterone (6.22 ± 2.85%; $p = 0.222$).

One way Anova:

a. Exposure of testosterone in incremental doses in normoglucose medium significantly influenced proportion of endothelial cells positively stained with ER-β antibody ($p = 0.001$).
b. Exposure of testosterone in incremental doses in normoglucose medium did not significantly influence proportion of endothelial cells positively stained with ER-β antibody ($p = 0.107$).

Analysis of variance for 2 x 4 factorial designs:

a. There was main effect of glucose medium to proportion of endothelial cells positively stained with ER-β antibody ($p = 0.002$).
b. There was main effect of testosterone to proportion of endothelial cells positively stained with ER-β antibody ($p = 0.012$).
c. There was interaction between testosterone and glucose medium to proportion of endothelial cells positively stained with ER-β antibody ($p = 0.0001$).

DISCUSSION

This study showed that exposure of testosterone to the endothelial cells increases expression of ER-β of endothelial cells in normoglucose but not in high glucose medium.

Our study supports previous studies, such as done by Schulze et al. [13] and Venkov et al. [14], which reported the presence of ER in the endothelial cells (HUVEC). These researchers showed that: 1) the nuclear extract from endothelial cells contains 67 kD protein that reacts to anti-ER monoclonal antibody, 2) the nucleus of endothelial cells are positively stained to ER specific antibody, 3) endothelial cells express protein that binds to estrogen receptor response elements (ERE), which in turn is inhibited by anti-ER antibody, 4) mRNA ER is detected in the endothelial cells. Moreover, they [13-14] reported that the ER density in HUVEC is 2-8 x 10^4 ER/endothelial cells with Ka ~ 5 nmol/L. The binding activated ER-β to ERE in the specific gene promoter shows the role of activated ER-β as a transcription factor.

Moreover, our study supports the presence of ER-β in the endothelial cells, as had been reported by Toth et al. [15] and Su et al. [16-17]. Based on the review from Villablance et al. [18], endothelial cells contains two ER isomers, namely ER-α and ER-β. ER-α is located either in the nucleus or cytoplasm, whereas ER-β lies in the nucleus only of the endothelial cells. Toth et al. [15] and Su et al. [16-17] proposed that ER-β expression dominates in the human umbilical vein and human placenta villous, respectively, as compared to ER-α expression.

Our study shows that exposure of testosterone to endothelial cells increases the expression of ER-β in normoglucose medium. It indicates that testosterone maybe metabolized into estradiol in the endothelial cell. This finding supports the results of previous studies. Meyer et al. [19] reported that exposure of 17β-estradiol increases mRNA expression and ER-β protein in endothelial cells (HUVEC). Villablanca et al. [18] reported that exposure of testosterone to the endothelial cells culture derived from mouse aorta increases expression of aromatase, ER density and biosynthesis of estrogen.

Gradient differences in glucose concentration between intracellular and extracellular compartment favor for glucose influx. It has been recognized that insulin is not necessary for glucose uptake by endothelial cells. The mechanism by which hyperglycemia induces endothelial dysfunction and/or endothelial activation is through free radicals builds up and NF-κB translocation. Free radicals is increased in the background of high glucose environment; it is related to the increased activity of electron transport chain in mitochondria. Hyperglycemia induces endothelial dysfunction directly by free radicals and indirectly by activation of PKC-β_2 and the development of advanced end glycation (AGE) products [20-22].

The opposite results in high glucose medium as compared to normoglucose medium in terms of expression of endothelial ER-β following testosterone exposure indicated that high glucose environment stimulates endothelial activation, which in turn changes the endothelial phenotypes, such as the expression of endothelial ER-β. The changes in the endothelial ER-β expression may inhibit the activity of vascular aromatase enzyme; therefore, the metabolism of testosterone to become estrogen in the endothelial cells is depressed in high glucose environment. This supposition is supported by Burul-Bozkurt et al. [23] who reported the decline in the activity of aromatase in diabetic mice. The depression of the expression of endothelial ER-β may in part explains the loss of endothelial contribution as a protective factor in type 2 diabetic mellitus patients from cardiovascular events.

CONCLUSION

Testosterone may influences the function of endothelial cells by modulates the expression of beta estrogen receptor (ER-β) in the endothelial cells, either in resting or activated state. In resting endothelial cells (normoglucose environment), exposure of testosterone increases ER-β expression. In activated endothelial cells (high glucose environment), exposure of testosterone tends to inhibit ER-β expression.

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