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Effect of AGE and Gamma-mangostin on Luteinizing Hormone Receptor (LHR) Levels in Leydig Cell Culture of Male Aging Rats: Preliminary study

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ABSTRACT

Products of Advanced Glycation End Products (AGE) are proteins that can cause cell destruction by increasing oxidative stress and inflammation. This product resulted from a series of chemical reactions after an initial glycation reaction. A Leydig cell is one of the types of cells affected by AGE. This cell is located in the interstitial of the testes and stimulated by the luteinizing hormone. This study aimed to compare the luteinizing hormone receptor levels in Leydig cell culture of Sprague-Dawley rat induced by AGE only and the one that administered gamma-mangosteen. We conducted an experimental laboratory study on luteinizing hormone receptor levels in Leydig cell culture of Sprague-Dawley rats induced by advanced glycation end products (200 µg/mL) and given gamma-mangostin (5 µM) compared to the one that was not treated with gamma-mangostin. The highest mean of LHR level was in group 3 given gamma-mangostin 5 µM (8.06 pg/ cells/24h), and the lowest mean was in group 1 (control) (7.78 pg/ cells/24h). The LHR levels in the rats' Leydig cell culture given gamma-mangostin (5µM) were higher than the other groups indicate the inhibition capacity on the oxidation process caused by AGE in aging rats Leydig cells culture.
Keywords: Luteinizing hormone receptor; Leydig cell culture; Aging rats; Advanced glycation end products; Gamma-mangostin

INTRODUCTION

Infertility, as a major health problem, occurs in an average of 15% –20% of couples. Both male and female factors can be the cause of infertility. The incidence of male infertility increased due to various factors, such as genetic, infection, and environmental factors. Sperm production depends on adequate testosterone levels maintained by Leydig cells in response to Luteinizing Hormone which is secreted by the anterior pituitary. This hormone acts on the surface of testicular Leydig cells by binding with luteinizing hormone receptors (LHR)(Goluža *et al.,* 2014; Zirkin & Papadopoulos, 2018).

Advanced glycation end-products (AGE) are complex and heterogeneous compounds that are usually formed endogenously like in diabetes conditions or exogenous conditions caused by tobacco, cigarettes, and food (Singh *et al.,* 2001). AGE will cause cell destruction and subsequent damage of tissue organs by the oxidative stress process (Bierhaus *et al.,* 1997). Binding to AGE receptors (RAGE) will induce NF-kB expression that is linked to the inflammatory response (Li & Schmidt, 1997). A previous study had localized the distribution of AGEs and RAGE in the male reproductive tract (Mallidis *et al.,* 2007).

Gamma-mangostin is one of the substances contained in the mangosteen pericarp, is the main substance that is classified as a xanthone derivative, and has anti-inflammatory and anti-oxidant properties (Chang & Yang, 2012). Extract of mangosteen pericarp showed antibacterial activity against Bacillus cereus ATCC10876 (Sze Lim *et al.,* 2013). Mangosteen pericarp extract has been widely used in Indonesia because it is well known to have many health benefits. With the increase of male infertility cases caused by hormonal problems, the authors are interested in examining the luteinizing hormone receptors levels in Leydig cell cultures of Sprague-Dawley rats induced by AGE (200 µg/mL) and treated with gamma-mangostin (5 µM).

METHODOLOGY

Materials

Samples

This study was an in-vitro experimental laboratory study conducted from April 2014–May 2015 at Cell culture laboratory, Department of Physiology, Faculty of Medicine, Public Health and

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Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia. The sample used in this study were male Sprague-Dawley rats aged 90 days, weighing around 300–350 g. Ethical clearance was obtained from the Institutional Review Board of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia (KE/FK/342/EC/2015).

Methods

Testis retrieval

Common surgical procedures in rodents from were used as a guideline in this study(Foley, 2005). Ninety days aged Sprague Dawley (weight around 200 gram) were fasted for about 10 hours before removing the testicle. As much as 0.3 mL/100 grBW ketamine HCL was injected intramuscularly as an anesthetic procedure. After that, the rope was used to fix the four limbs of the rats on the surgery table. Abdominal hair was moistened using wet cotton and shaved until the skin in the area to be opened was visible. This area was then sterilized with alcohol and incised about 2 cm along the midline of the abdomen with a scalpel. A peritoneal incision was made with a length of about 1.5-2 cm.

Using a pair of curved tweezers and small scissors, an incision in the midline along the lower abdomen was made for about 1 cm long and 0.5 inches anterior towards the genital area. The rat skin was then opened to the left and right so that both testicles could be removed in one incision. Both vas deference will then be identified from one side of the testis. The left vas deferens then gently grasped using forceps and lifted partially so that the incision was observed. Vas deference then ligated and cut as explained above. After the testis retrieval, the incision area was then cleaned and observed for any bleeding. An absorbable thread was used to re-sewn the peritoneum and the skin, then povidone-iodine was applied to it until dried. The lethal dose (0.45 mg/kg) of ketamine was administered for euthanized the rats.

AGE-BSA preparation

AGE-BSA solution is Advanced Glycation End Products-Bovine Serum Albumin, an extraction product of BSA which reacted with glucose and going through incubation for several weeks. The powder form of AGE-BSA was used in this study, we then dissolved this powder into phosphate-buffered saline (PBS). The AGE-BSA used in this study is a Biovision product (catalog number 2221–10). This product has a specificity of 98% and shows an AGE content of 7000% compared to common BSA.

Leydig Cell Isolation and Cell Culture

This procedure was done by the guideline from the previous study by Sun *et al.* (2011). Microscissors were used to remove epididymis, visible blood vessels, fat, and other connective tissue from the testes. The tunica albuginea was then dissected out and incubated into a 50 ml centrifuge tube. Testes were then placed in a cooled mixture of Dulbecco's modified Eagle medium (DMEM)/Ham's F12 Media (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) 1:1, then mixed with 15 mM $NaHCO_3$, 20 mM HEPES, pH 7.4; 100 U/ml penicillin, 2.5 pg/ml amphotericin B and 0.1% BSA, with continuously maintained laboratory temperature. All solutions and procedures were processed in sterile conditions. Testicular tissue was then washed three times using media and finely chopped in a petri dish then placed in a 0.04% collagenase solution (type I, Sigma Chemical Co., 130 U/mg) and 1.0 μg/ml trypsin inhibitors in the culture media mentioned above, under constant agitation at 34 °C for 40 min.

Collagenase solution was then diluted four times with a culture medium, and a small piece of tissue was inserted and allowed sedimentation for 10 min. Centrifugation of the supernatant was done at room temperature for 3 min at 200 g, and the cell pellets were washed twice and stored in fresh tissue culture media. The remaining tissue pieces were administered both suspensions for 30 min with the same conditions. Cells then were collected and washed with the same procedure as described before. The two suspensions obtained from the collagenase treatments were mixed, and the sedimentation product from the 10 and 30-min treatments was taken to reduce the remaining tubular segments.

Percoll layer gradient used to obtain supernatant was 60, 40, 34, 26, and 21%. The gradient was centrifuged at 800 g for 30 minutes at room temperature. The layer formed between the concentrations of 40 and 60% was taken and washed to remove Percoll media and the viability was confirmed by Trypan blue assay. A number of $2\times105/cm^2$ cells were planted in the well with the DMEM/Ham's F12 media, and then added 15 mM NaHCO3, 20 mM HEPES, pH 7.4; 100 U/mL penicillin, 2.5 pg/mL amphotericin B, 10 μg/mL transferrin; 5μg/mL hydrocortisone and 2% Fetal Bovine Serum (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany).

Cell culture was done in an incubator at the temperature of 34°C and 5% CO_2 air pressure. Cells were estimated to coagulate in 24 hours after culture, then begin to grow to nearly confluent within 7 days. AGE-BSA 200μg/ml and AGE-BSA

Table I. The Mean Value of Research Subject Characteristic

Characteristic	Group 1	Group 2	Group 3	
	group 1 Control (n=3)	**AGE-BSA 200 µg/mL (n=3)**	**AGE-BSA 200 µg/mL + gamma-mangostin 5 µM (n=3)**	**p**
The Level of LH Receptor (pg/10^5/24h)	7.78 ± 0.00	7.79 ± 0.01	8.06 ± 0.26	0.10

Data were displayed in mean ± SD; p = 0.10 (p > 0.05 is considered not statistically significant); AGE-BSA: Advanced glycation end products-Bovine Serum Albumin; LH: luteinizing hormone.

200µg/ml + Gamma-mangostin 5µM were added in the group 2 and 3 respectively, then homogenization was done in both groups. Samples were centrifuged and the supernatant was taken and incubate at -80°C for 2 weeks.

Luteinizing hormone receptor levels measurement

Luteinizing hormone receptors (LHR) levels in the media were examined by ELISA. Samples were centrifuged after the thawing process. An amount of 50µl was added as standard and samples per well. HRP-conjugate then was added to the wells (50µl/ well, except the blank well). A number of 50µl antibodies were added to each well and incubated for 1 hour at 37°C. All the liquid components in the well were aspirate and washed using Wash Buffer (200µl), this process was repeated until obtained three times washing process. Substrate A (50µl) and B (50µl) were added to each well, mixed, and incubated for 15 minutes at 37°C. After the incubation, 50µl of Stop Solution was added to each well then the optical density was determined within 10 minutes using a microplate reader set at 450 nm.

Gamma-mangostin preparation

The powder form of gamma-mangostin used in this study was a product from Sigma-Aldrich (MG 6824 catalog numbers) which then dissolved with dimethyl-sulfoxide (DMSO) to obtain a final solution concentration of 20 mM and that can be diluted as needed. The purity level of gamma-mangostin was 98%.

Statistical analysis

SPSS Statistics 22 program (SPSS Inc., Chicago, IL, USA) was used to analyzed all recorded data in a computer database. Statistical analysis results were displayed as mean values ± standard deviation (SD). One-way ANOVA was used to analyze the mean difference of each group.

RESULT AND DISCUSSION

Correlation between gamma-mangostin, AGE, and LHR level

As depicted in table 1, the mean value of LHR level in group 3 (8.06 ± 0.26 pg/10^5cells /24h) was the highest compare with group 1 (7.78 ± 0.00 pg/10^5cells /24h) and group 3 (7.79 ± 0.01pg/10^5cells /24h). Based on One-way ANOVA statistical analysis, there was p value = 0.10, which means that the LHR levels mean of each group has no statistically significant differences.

The result showed that there was a mean difference in LHR levels in each group, although not statistically significant. Data from table 1 showed that the highest mean of LHR level was in group 3 (8.06 pg/10^5 cells/24h), and the lowest mean was in group 1 (7.78 pg/10^5 cells/24h). This result may be indicating that gamma-mangostin can inhibit the oxidation process caused by AGE so that LHR remains expressed in Leydig cell culture.

Almost the similar level of LHR in group 1 (control) vs group 2 (7.78 ± 0.00 vs 7.79 ± 0.01) indicates that the administration of AGE-BSA 200 µg/mL has not shown an effect on reducing LHR in Leydig cell cultures.

This is in line with the results of the study by Rasheed *et al.* (2011) in different sample cells, which investigate whether AGEs induce the expression of IL-6 and IL-8 through RAGE-activated pathways in human OA chondrocytes. This study explained that AGE-BSA up to 200 µg/ml had no significant cytotoxic effects on OA chondrocytes compared with controls treated with 200 µg/ml native BSA (P > 0.05). LHR plays an important role in the process of testicular testosterone production via the hypothalamus-pituitary axis. GnRH which binds to the pituitary membrane receptors stimulates the secretion of LH then will be bind to LHR on the surface of Leydig cells resulting in the initiation of the intracellular

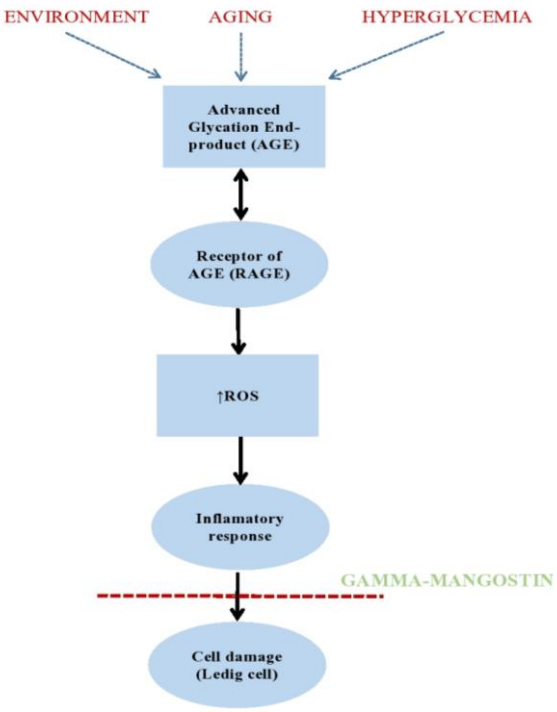

Figure 1. Conceptual Framework of Gamma-mangostin Effect on Leydig Cell Damage Inhibition

signaling cascade. This process resulting in cAMP production which plays a critical role in steroidogenesis (Zirkin & Papadopoulos, 2018).

The previous study showed that infertility and cryptorchid conditions resulted from ablated LH pathway in the mutant mouse model system (Lei *et al.,* 2001; Zhang *et al.,* 2001). The significant decrease in LH binding site number related to age indicates the plasma membrane LHR reduction. In some conditions, approximately 10% of LHR is required to achieve a biological response. The current fact showed that Leydig cells from young rats, LH-suppressed condition, even with a low number of LHR were found to have the capacity for cAMP synthesis in response to LH compare with aged cells (Wang *et al.,* 2017).

Various factors cause the formation of AGE, including the amount of protein for the glycoxidation process, long-time hyperglycemic conditions, and the high level of environmental oxidant stress. AGE formation can also be caused by the physiological aging process and the presence of leukocytes in semen even within the normal range according to WHO criteria (Agarwal & Said, 2005; Sharma *et al.,* 2001).

This research was conducted on AGE-induced aging mice, given gamma-mangostin. As the conceptual framework depicted in figure 1, we assume that gamma-mangostin has an inhibitory activity on Leydig cell damage caused by AGE production.

AGE binding to AGE receptors will induce NF-kB expression that is linked to the inflammatory response (Li & Schmidt, 1997). Mallidis *et al.* (2007) found the distribution of AGE and RAGE in the male reproductive tract and there was a local production of AGE/RAGE in seminal plasma that possibly related to pathologic condition.

A previous study reported that AGEs showed an inhibitory effect on the production of testosterone by mouse Leydig cells by inducing oxidative and endoplasmic reticulum stress (Zao *et al.,* 2016). A preliminary study by Rizal et al (2019) showed that administration of AGE-BSA 200 µg/mL decreased testosterone levels in Sprague-Dawley rats Leydig cell cultures compared to the control group (untreated). A study *in-vitro* and *in-vivo* by Ohno *et al.* (2015) reported that hot water extracts of mangosteen pericarp showed an inhibitory effect on the pentosidine formation as one of the AGE biomarkers in the ribose-gelatin sample. Serum pentosidine contents were reduced in volunteer subjects that orally administered hot water extract or mangosteen pericarp at 100 mg/day for three months.

A previous study stated that the pericarp extract of mangosteen has a higher antioxidant

capacity than the pulp and seed extracts. The results of the study showed that all components of mangosteen extract could inhibit *Staphylococcus aureus* ATCC11632 bacteria, and only the pericarp extract has an inhibitory effect against *Bacillus cereus* ATCC10876 (Sze Lim *et al.,* 2013). Xanthones as the major bioactive compounds α-, β-, and γ-mangostin exhibited various biological activities including anti-inflammatory, anti-bacterial, and anti-cancer effects by reduced on prostaglandin E2 (PGE2) levels through inhibition of cyclooxygenase (COX-2) activity and NO production (Akao *et al.,* 2008).

Baek *et al.* (2021) investigate the apoptosis inhibition effect of γ-mangostin in mouse hippocampal HT22 cells. They used 5 mM of glutamate for apoptotic induction and it was found that γ-mangostin exhibits a strong protection capacity against glutamate-induced cell damage through the reduction of reactive oxygen species production.

Another study declares that water extract of mangosteen demonstrated an anti-apoptotic activity in H_2O_2-induced endothelial cell death through ROS formation inhibition and p38 MAPK suppression (Jittiporn *et al.,* 2019).

CONCLUSION

The LHR levels in the rats Leydig cell culture given 5μM gamma-mangostin were higher than the other groups, although not statistically significant. Administration of AGE-BSA 200 μg/mL did not indicate a decrease in LHR levels in the Leydig cell culture. Various concentrations of gamma-mangostin and larger samples are needed for future studies to confirm our findings.

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