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Research Article
Effect of *Annona muricata* Leaf Extract Towards the Sertoli Cells on Alloxan-Induced Mice

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Abstract

Background and Objective: Prolonged and uncontrolled hyperglycemia in diabetes mellitus can increase the production of reactive oxygen and enhance the risk of male infertility by reducing the number of Sertoli cells. This study aimed to investigate the potential effect of Ethanol Extract of *Annona muricata* leaf (EEAL) on the amount of Sertoli cells in alloxan-induced mice antioxidant to prevent reducing the number of Sertoli cells. **Materials and Methods:** The samples used for this study are 30 alloxan-induced Swiss Webster mice divided into a negative control group, a positive control group (glibenclamide 0.65 mg kg^{-1}) and three plant extract groups (EEAL 150, 300 and 600 mg kg^{-1}). Every solution was given every day for 14 days. Histological examination using HE-stained preparations was performed on 40x magnification to evaluate many Sertoli cells counted using Image J software. **Results:** three EEAL groups of 150, 300 and 600 mg kg^{-1} have significant effects ($p<0.05$) to increase the amounts of Sertoli cells compared to a negative control group. In contrast, it does not significantly affect the amounts of Sertoli cells than the positive control group. **Conclusion:** The administration of *A. muricata* leaf extract during 14 days significantly reduced the number of Sertoli cells on alloxan-induced mice.

Key words: Alloxan, *Annona muricata* leaf extract, sertoli cell, alloxan-induced mice, FSH, LH, testosterone, spermatozoa

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes Mellitus (DM) is a chronic progressive metabolic syndrome that represents one of the greatest threats to global health. Its incidence is rising rapidly[1]. In 2013, Basic Health Research (Riskesdas) in Indonesia reported that 6.9% of the population experienced DM. It rose to 8.5% of the total population of Indonesia in 2018[1]. Factor such as older age is thought to be largely responsible in DM and it is known that DM is increasing in men of reproductive ages[2].

Prolonged and uncontrolled hyperglycemia on DM may affect male reproductive at multiple levels, such as pre-testicular, testicular and post-testicular[3]. It disrupts the hypothalamic-pituitary-gonadal axis at the pre-testicular level, resulting in an altered concentration of important spermatogenesis hormones such as FSH, LH and testosterone[4]. At the testicular level, DM causes decreasing lactate production by Sertoli cells[3]. It affects the lowering level of ATP in the testis and alters the spermatogenesis process. Thus, it negatively affects sperm quality, quantity and function as well as fertility[3]. The implication of anti-oxidant/oxidant imbalance resulting from overproducing reactive oxygen in hyperglycemia is also responsible for altering reproductive function at testicular level[5]. At the post testicular level, DM affects sexual behaviour and fertility outcome due to poor penile erection function[3]. These decreased functions are linked to the reduced Leydig cell secretion of testosterone[3].

Hyperglycemia in people with DM also causes reproductive disorders by changing testicular tissue. A recent study showed that induction of diabetes caused a significant decrease in the number of Sertoli cells in diabetic rats compared with the control group[6]. Sertoli cells have a critical function for male fertility, supporting the cell lineage of the male germline[7]. Sertoli cells are responsible for providing germ cells with a specialized environment to promote their survival and differentiation throughout life[7]. Reducing the number of these cells may affect the spermatogenesis process and decrease the quality and quantity of spermatozoa and fertility.

The use of natural ingredients as antidiabetic has been studied extensively. However, much attention is not given to the effects of DM on reproductive function, such as reducing the number of Sertoli cells. Until recently, most of the therapeutic interventions are targeted at eliminating the causes of hyperglycemia. Adeyemi *et al.*[8] succeeded in testing the effectiveness of *A. muricata* leaf extract as an antidiabetic agent. *A. muricata* leaf extract also has active compounds from the consisted flavonoids as an antioxidant, thus proving that it can be a candidate for prevent reducing the number of Sertoli cells[9]. Therefore, *A. muricata* leaf extract is expected could prevent reducing the number of Sertoli cells in alloxan-induced swiss webster mice.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Anatomical Pathology, Histopathology Lab, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia from August-October, 2019.

Animal sample preparation: In this study, 30 male Swiss Webster mice (12-14 weeks old; average body weight ±30 g) were prepared from the Animal Laboratory of Center for Health Research and Development, Ministry of Health, Jakarta. During the experiment, the temperature in the breeding environment was ±25°C. Moreover, mice were kept at a 12:12 hrs light-dark cycle while having free access to standard food and *ad libitum* water. Health Research Ethics Committee approved this animal experiment of Faculty of Medicine Universitas Indonesia (approval no. KET-507/UN2.F1/ETIK/PPM.00.02/2019).

Induction of diabetes: Diabetes was induced by intraperitoneal injection of 40 mg kg^{-1} b.wt. alloxan. Fasting blood glucose was measured using glucometer three days after injection. A blood glucose level of $\geq$200 mg dL^{-1} was considered as an indicator of diabetes.

Design of the experiment: Before undergoing the 14 days of treatment, hyperglycemia animals were randomly divided into five groups (n = 6): 1) Negative control (K-) administered by water *ad libitum*; 2) Positive control (K+) administered by glibenclamide 0.65 mg kg^{-1}; 3) Low dose (P_1) administered by 150 mg kg^{-1} Ethanol Extract of *Annona muricata* Leaf (EEAL); 4) Medium dose (P_2) administered by 300 mg kg^{-1} of EEAL; 5) High dose (P_3) administered by 600 mg kg^{-1} of EEAL.

Tissue preparation: Testicular tissues were fixed about 24-48 hrs with a 10% formalin buffer, dehydrated, cleared by stratified xylol solution and infiltrated in paraffin using an automatic tissue processor. After that, testicular tissues were embedded into a paraffin medium in a box cassette and sliced with a thickness of 3-5 µm using a microtome. The block slices were soaked in water (40-50°C), fixed to glass slides and dried for 1 hr at 40°C

Hematoxylin-eosin staining: First, all preparations were soaked by xylol I and II for each 5 min and followed by submersion in ethanol gradient series (70 and 90%) for 5 min. Then, the preparations were stained by soaking them into hematoxylin solution for about 5-10 min, followed by rinsing them underwater. After that, the samples were soaked into eosin solution for about 3 min, followed by dehydrating them with stratified concentration alcohol (70, 90 and 100%) each 3-4x dyeing. Then, all preparations were dipped in xylol I and xylol II solutions for about 5 min each. Lastly, the samples were dripped with one drop of ingelan and covered by a cover glass.

Histopathological analysis: Testicular tissue preparations were examined based on their histopathological appearance using a light microscope and Axiocam ERc 5s camera (Carl Zeiss, Jena, Germany) at 40x magnification. The examination was conducted in 10 randomized seminiferous tubules in each preparation. The amount of Sertoli cells in each tubule were calculated by image-J software manually.

Statistical analysis: The distributions of data were analyzed using the Shapiro-Wilk test. Relationships between groups were analyzed by one-way ANOVA and Bonferroni test for *post hoc* analysis using SPSS-20. In this study, $p<0.05$ was considered the significance level and values were expressed as mean±standard error.

RESULTS

Histopathological changes: After 14 days of treatments, the K (-) group showed the fewest amount of Sertoli cells than other groups. The K (+) group has the most in many Sertoli cells, followed by the P_2 group, P_3 group and P_1 group (Fig. 1). Histopathological images of seminiferous tubules in the Sertoli cell mice were depicted in Fig. 2a of a negative control group, Fig. 2b of a positive control group, Fig. 2c of low dose EEAL (150 mg kg^{-1}) group, Fig. 2d of medium-dose EEAL (300 mg kg^{-1}) group and Fig. 2e of high dose EEAL (600 mg kg^{-1}) group.

Post-hoc analysis showed a significant result between K (-) and every EEAL group (P_1, P_2, P_3). The number of Sertoli cells significantly different in P_1 ($p = 0.019$), P_2 ($p = 0.000$) and P_3 ($p = 0.019$) compared to K (-) group. In contrast, every EEAL group (P_1, P_2, P_3) showed no significant difference from K (+) group (Table 1).

Table 1: *Post hoc* analysis of comparisons of the number of sertoli cells between groups

		CI 95%**		
Groups	Mean difference	Minimum	Maximum	p-value
K (-) vs K (+)	-1.860	-3.282	-0.437	0.015*
K (-) vs P_1*	-1.130	-2.058	-0.201	0.019*
K (-) vs P_2*	-1.620	-2.193	-1.047	0.000*
K (-) vs P_3*	-1.080	-1.970	-0.189	0.019*
K (+) vs P_1	0.730	-0.651	-0.651	0.526
K (+) vs P_2	0.240	-1.321	-1.321	0.478
K (+) vs P_3	0.780	-0.597	-0.597	1.000

*p<0.05 showed statistically significance between groups, **CI 95%: Confidence interval 95%

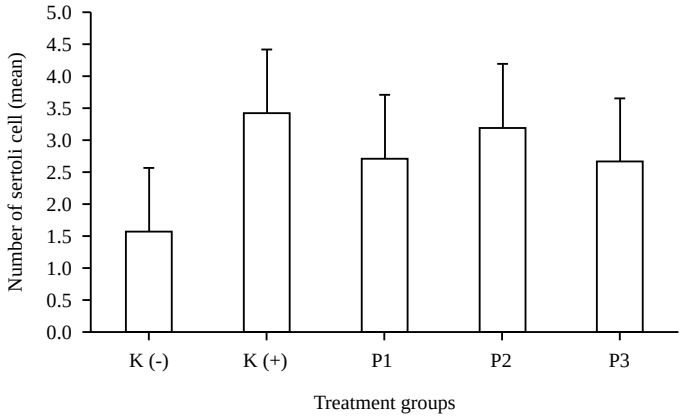

Fig. 1: Average number of the sertoli cells between groups

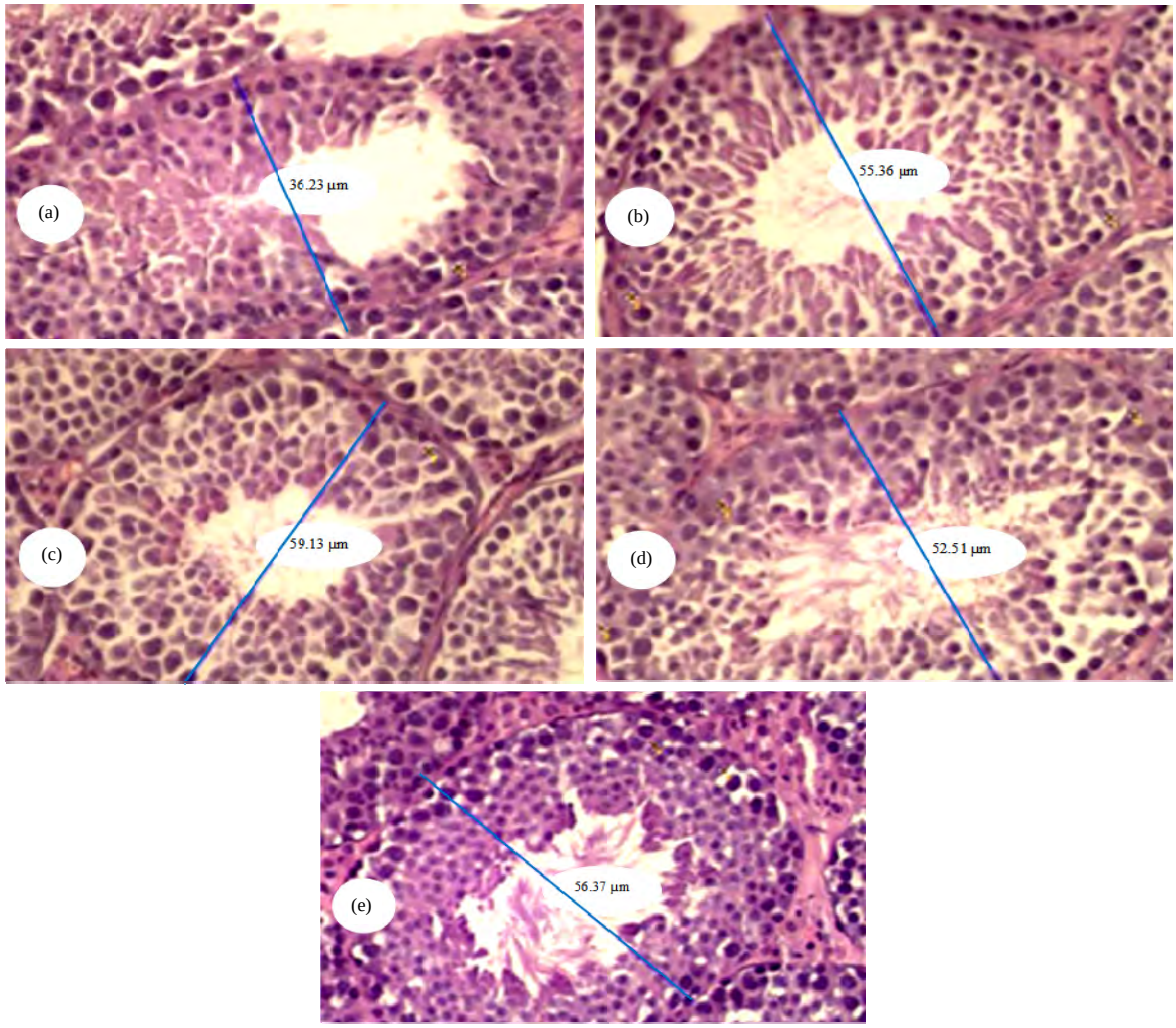

Fig. 2(a-e): Histopathological appearance of testicles in HE staining of Seminiferous tubules from one side to the other side of the basement membrane, in 40× magnification

(a) Negative control group, (b) Positive control group, (c) Low dose EEAL (150 mg kg^{-1}) group, (d) Medium dose EEAL (300 mg kg^{-1}) group and (e) High dose EEAL (600 mg kg^{-1}) group

DISCUSSION

This study used alloxan to induce hyperglycemia on Swiss Webster mice, resulting in Type 1 Diabetes Mellitus (T1DM) condition on those mice. The results of this study showed that the mean number of Sertoli cells in all test groups and the positive control group was higher than the average number of Sertoli cells in the negative control group. However, there was no statistically significant difference between the three test groups (150, 300 and 600 mg dL^{-1} EEDS) and the positive control group (glibenclamide). Thus, the administration of glibenclamide or EEDS extract, which both have antidiabetic effects, both have no statistically significant difference in effectiveness in preventing the decrease in the number of Sertoli cells.

Sertoli cells are greatly involved in testicular energy metabolism and form testis barrier[7]. Previous *in vivo* study using streptozotocin (STZ)-induced T1DM have reported that DM reduces testicular LDH activity, that is important to maintain steroidogenesis and spermatogenesis[10]. Another study showed that one of the important reasons for a reduced number of Sertoli cells and testicular damage in diabetic rats is decreased LH and FSH serum[11]. This reduction of important hormones happens along with impaired spermatogenesis and Sertoli cell destruction[12]. Thus, decreasing LH and FSH as the result of DM could alter the number of Sertoli cells. Other than that, a reduction in lactate production (one of the Sertoli cell functions) seemed to happen and reduce insulin level (diabetic condition). Thus, decreasing insulin serum is another reason for a reduced number of Sertoli cells in the diabetic

group than the control[13,14]. Another study showed there was a reduction in testicular antioxidants such as Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxide (GPx) activities in alloxan-induced T1DM rats[15,16].

Previous research has found that *Annona muricata* leaf extract contains several enzymatic (catalase and superoxide dismutase) and non-enzymatic (Vitamin C and E) antioxidant[9]. Therefore, EEAL has the probability of preventing oxidative stress. In addition, Johnson *et al*. proved that vitamin E (-tocopherol) is a vital lipophilic antioxidant that can protect mammalian sperm from Sertoli cell apoptosis[17]. Other research showed that vitamin E could suppress lipid peroxide (one cause of oxidative stress) in testicles mitochondria cells[18]. *A. muricata* leaf also contains phenolics, catechin, epicatechin, rutin and other flavonoids as antioxidants to prevent an imbalance of oxidant/antioxidant levels of the cells[19].

Histopathological examination results showed the ability of *A. muricata* leaf extract to prevent reducing the number of Sertoli cells, possibly due to the antioxidant effects of flavonoids, catalase, superoxide dismutase, vitamin E and vitamin C. Those antioxidants can prevent apoptosis cells by overproducing reactive oxygen in hyperglycemia condition.

CONCLUSION

Administration of *Annona muricata* leaf extract in low dose (150 mg kg^{-1}), medium dose (300 mg kg^{-1}) and high dose (600 mg kg^{-1}) have the same ability in reducing the number of Sertoli cells in alloxan-induced mice. These changes may play an important role in male infertility observed in alloxan-induced subjects.

SIGNIFICANCE STATEMENT

This study revealed the potential action of the *Annona muricata* leaf extract as an antioxidant by reducing the Sertoli cell in the testis of alloxan-induced mice. This study will suggest the use *A. muricata* as plant remedies in managing diabetes mellitus (DM), particularly in male Sertoli cells. Thus, the application of this plant extract would be beneficial for human reproduction cells with DM.

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