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Effect of Mulberry (*Morus alba* L.) Leaves Infusion on the Reproductive Status of Chronic Diabetic Models

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ABSTRACT

Leaves of mulberry (*Morus alba* L.) is one of the medicinal plants that contains antihyperglycemic and antioxidant compounds. These compounds are known as agent that could decrease the high blood glucose levels and improve organ damage caused by free radicals in hyperglycemic conditions. This study aimed to determine the effect of mulberry leaves infusion on reproduction status of chronic diabetes model. This research was an experimental study using completely randomized design (CRD) with 6 treatments and 4 replications. The treatments were C+ (positive control), C- (negative control), T1 (400 mg/kgbw), P2 (600 mg/kgbw), P3 (800 mg/kgbw) and P4 (1000 mg/kgbw). 24 of wistar rats, male, 1 month old, and 70-100 grams of body weight were used as animal models. Data observed in this study include the number of germ cells (spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa), sertoli cells, seminiferous tubules diameter, leidig cells, testes weight and testosteron levels. Data were analyzed with One Way Anova, and then followed by DMRT (1%) analysis if there is a significant differences between treatments. The results showed that the infusion of mulberry leaves increased the number of germinal cells, sertoli cells, diameter of seminiferous tubules, leidig cell, testis weight, and testosteron levels of chronic diabetic model. The optimum infusion dose of mulberry leaves was T4 (1000 mg/kgbw).

KEYWORDS— Chronic Diabetic Model, Histology Preview, Mulberry Leaves (*Morus alba* L.), Rat, Testosterone

I. INTRODUCTION

Diabetes mellitus is a chronic disease caused by a complex metabolic disorder due to the reduced insulin either absolute or relative, which is characterized by hyperglycemia and glycosuria and the development of macrovascular and microvascular complications [1]. One impact of the complications of diabetes mellitus in chronic phase is the male reproductive organ dysfunction.

A complication of chronic Diabetes mellitus causes the decreasing of LH, FSH and testosterone levels [2]. FSH, LH and testosterone has an important role in spermatogenesis process [3]. If the amount of these hormones reduces, it will disturb the process of spermatogenesis, and the final consequency will be followed by the decreasing of germ cell numbers as well as testicular weight [4].

Recently, the utilization of syntetic antidiabetic medicine have side effects for the body and relatively expensive. One alternative to overcome this problem is to do research on plants that have antidiabetic effects ie mulberry (*Morus alba* L.). This plant is rich in chemical constituents such as alkaloids, flavonoids, and polyphenols [5]. Approximately fifteen polyhydroxylated alkaloids have been isolated from the leaves of mulberry, one of which is 1-Deoxynojirimycin (DNJ), which has potency to decrease blood glucose by inhibiting alpha-glucosidase [3]. This enzyme catalyzes the hydrolysis of bonds in maltose to produce two molecules of glucose [1]. Leaves and roots extract of mulberry contain 0.24% DNJ compounds [6]. Mulberry leaves also contain several chemical compounds such as ecdysterone [7] and scopoletin [6] which also contribute for the decreasing of blood glucose. In addition, mulberry leaves also contain folic acid and zinc [7] that are able to increase the number of sperm cells in men with infertility experience.

II. MATERIALS AND METHODS

A. Preparation for animal model

Before used for experimental study, model animals were acclimatized for 2 weeks, fed and watered ad libitum. Alloxan, a dose of 100 mg/kg or as much as 0,125 ml of intravenous induction, was administrated to make the condition of diabetes mellitus in mice and given 1 time and had fasted for 24 hours. Chronic complications caused by hyperglycemic conditions within a period of 5-10 years, if converted into a rat about 1 month, thus causing the interruption of blood flow, which can cause complications to various organs including the reproductive organs. Therefore rats after alloxan induced, left for 4 weeks and then treated with infusion of new mulberry leaves.

B. Measurement of blood glucose

Measurement of blood glucose levels was performed before and after treatment with alloxan as well as after the administration of mulberry leaves infusion. Before taking the blood, the rats' model were fasted for 16 hours. Measurement of blood glucose levels was performed by using glukotest, where the measured blood is from the blood veins of rat tail.

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Hyperglycemic condition occurs in rats when the blood glucose levels are more than 200 mg/dl in non-fasting or 126 mg/dl in fasting condition [8].

C. Preparation of mulberry leaf infusion

Preparation of mulberry leaf infusion was performed by washing mulberry leaves with water. After drying up at room temperature for 5 days, the pulverized of mulberry leaves was then cooked for 15 minutes a temperature of 90 °C and administrated as 0, 400, 600, 800 and 1000 mg /kgbw/day of 2.5 ml for 30 days.

D. Group of treatment

The group was divided as follows: Control (-): rat that not infused by both alloxan and mulberry leaves infusion, Control (+): rats induced by alloxan without mulberry leaves infusion, T1: rats induced by alloxan + mulberry leaf infusion of 400 mg/kgbw, T2: rats induced by alloxan + mulberry leaf infusion of 600 mg/kgbw, T3: rats induced by alloxan + mulberry leaf infusion of 800 mg/kgbw, T4: a rats induced by alloxan + mulberry leaf infusion 1000 mg /kgbw. Infusion leaves of mulberry was given for 30 days orally using a stomach sonde. At the end of the study, rat was sacrified and testis was dissected, weighed and used for preparations.

E. Determination of testis weight

Rat testes was removed and cleaned from fat, weighed and then soaked in formalin for histological preparations. The number of leydig, sertoli, spermatogonia, primary spermatocyte, secondary spermatocyte and spermatid cells were counted from five fields of view. For diameter of seminiferous tubules measurement, three seminiferous tubules were conducted on almost equal sized by measuring the distance of the longest and the shortest distance from the seminiferous tubules which are round or spherical considered then averaged. The histological observation was performed by using Olympus CX31 microscope with 400 x magnification.

F. Determination of testosterone level

Step of testosterone measurement was as follows: Prepare a wash solution by means of diluting the wash solution with deionized water. Preparation of standard, standard 5 (80 pg/ml) = 75 ml of wash solution + 75 ml stock wash solution; standard 4 (40 pg/ml) = 67.5 ml of wash solution + 75 ml standard 5; standard 3 (20 pg/ml) = 75 ml wash solution + 75 ml standard 4; standard 2 (10 pg/ml) = 150 ml wash solution + 75 mL standard 3; standard 1 (5 pg/ml) = 150 ml wash solution + 75 ml standard 2, 40 mL and 10 ml sample dilution of serum included in eppendorf, and mixed. 50 mL standard and the sample were then introduced into the well (except blank well), covered and incubated for 30 min at 37 °C, the membrane plate was opened, the liquid was absorbed by using a tissue, then washed with 100 mL wash solution that was created in point 1, shaken, the fluid was absorbed by the tissue discarded, washed 3 times, incubated for 30 min at 37 °C, the mixture was added 50 ml chromogen A and B into the well, incubated for 15 min at 37 °C, added 50 ml stock solution, the absorbance read at 450 nm in an ELISA reader.

G. Data Analysis

Data analysis for all treatment was performed by One Way ANOVA, and followed by Duncan test if F count> F table.

III. RESULTS AND DISCUSSION

Statistical analysis revealed that the number of spermatogenic cells, sertoli cells, leidig cells, seminiferous tubule diameter and testes weight of chronic diabetic mellitus rat model in T1 (400 mg/kg bw) are not significantly different with positive control treatment (C+). However, in T2, T3 and T4, the number of these cell are significantly different from (C+) and close to normal condition (Table 1).

TABLE 1. NUMBER OF SPERMATOGENIC, SERTOLI AND LEIDIG CELLS, SEMINIFEROUS TUBULE DIAMETER AND TESTES WEIGHT OF CHRONIC DIABETIC MELLITUS RAT MODEL

Dose mg/kg bw	Averages ± standard deviation							
	Spg	Spt 1	Spt 2	Sptd	Sptz	Srt	Ø Tbs (µm)	Testis weight (mg)
C+ (0)	69.00±3.37 a	63.50±2.52 a	53.75±5.50 a	5.08±0.82 a	14.75±0.50 a	7.50±1.73 a	297.75±6.99 a	3.20±0.72 a
TI (400)	79.75±1.36 a	83.00±1.07 ab	70.00±9.42 ab	59.67±5.60 ab	21.75±4.11 a	18.25±2.06 a	301.5±1.19 a	3.32± 0.20 a
T2 (600)	85.25±5.12 a	90.75±5.68 ab	79.75±2.63 ab	67.08±3.37 ab	36.50±4.65 a	35.25±2.36 b	317.25±1.22 a	6.00±1.98 ab
T3(800)	94.25±7.97 a	101.58±2.52 b	93.83±3.83 b	77.42±1.73 b	67.75±5.50 b	42.00±2.83 b	438.5±1.51 b	7.87±1.06 b
T4(1000)	148.5±2.72 b	132.25±1.95 c	115.75±3.15 c	118.83±1.69 c	87.00±1.01 b	54.50±5.92 c	467.5± 3.06 bc	9.2± 3.06 bc
C-Normal	168.25±1.68 b	174.67±2.09 d	175.00±26.79 d	142.25±2.17 d	147.25±2.4 c	7.,75±1.15 d	486.75±2.67 c	11.32±2.66 c

Spg = spermatogonia, spt 1 = primary spermatocyte, spt 2 = secondary spermatocyte, sptd = spermatide, sptz = spermatozoa, srt = sertoli, Ø Tbs = diameter of the seminiferous tubule.

After mulberry leaves infusion treatment, the histology of spermatogenic cells improved. The layout of spermatogenic and sertoli cells on the seminiferous tubules is gradually dense in C +, T1, T2, T3, T4. It is presumably due to the cell regeneration during the treatment and the number of cells increase in T4 treatment and almost close to normal condition (Fig.1). T4 (1000 mg/kgbw) is the optimal dose in increasing the number of spermatogenic cells and testicular sertoli cells.

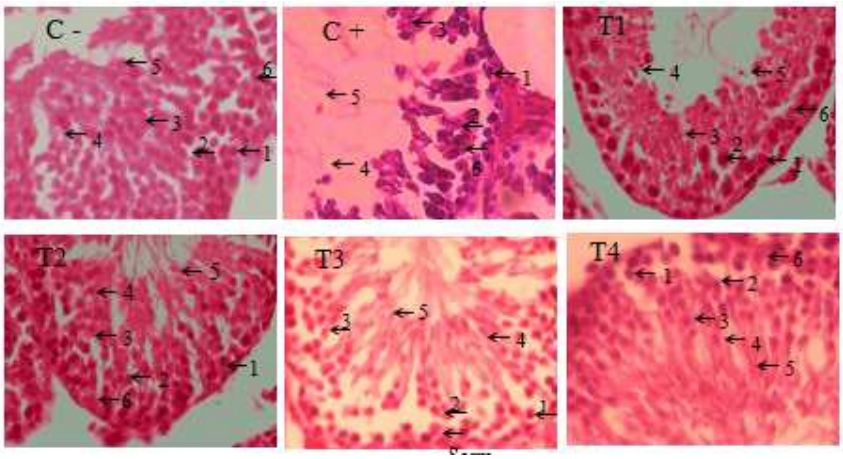

FIGURE 1. Histological picture of the testes on chronic diabetes model after dministration of murbei leave infusion. M: 400x. 1. Spermatogonia cell, 2.primary spermatocyte cell, 3. Secondary spermatocyte cell, 4.spermatid cell, 5.spermatozoa cell, 6. Sertoli cell

Free radicals in chronic diabetes mellitus rats increase due to the hyperglycemic conditions. Hyperglycemic conditions cause auto-oxidation of glucose, glycation of proteins, and activation of the polyol metabolic pathway which further accelerates the formation of reactive oxygen species. The formation of reactive oxygen species increase lipids, DNA, and proteins modification in various tissues [8].

Molecular modification in various tissues resulted in an imbalance between protective antioxidants and free radical production. It was the beginning of oxidative damage known as oxidative stress [9]. Oxidative stress will increase the amount of ROS that can damage cell membranes, lipid peroxide or malondialdehyde (MDA), the amount of excess ROS affects the hypothalamic-pituitary axis thus inhibiting the secretion of FSH and LH.

The decreasing in FSH levels will cause disruption of mitosis process and proliferation of spermatogonia [4]. While FSH and LH hormones have an important function in the process of spermatogenesis. LH stimulates the function of leydig cell to produce testosterone in testes, while FSH stimulate spermatogenesis process which undergo the change of spermatogonia into spermatids. In addition, FSH also serves to stimulate the formation of Sertoli cell androgen-binding protein (ABP) in which this protein contributes in transporting testosterone to the seminiferous tubules and epididymis [3].

Hypothalamus secretes GnRH (gonadotrophin releasing hormone) anterior pituitary to produce FSH and LH. These hormones play a major role in regulating male sexual function. FSH is carried through the blood to the testicles to initiate the proliferation process of spermatogenesis. LH will stimulate leidig cell to produce testosterone to complete the maturation process and the formation of spermatozoa. Disruption in FSH and LH secretion causes the process of cell division and maturation of spermatogenic becomes blocked, and finally the number of spermatogenic cells was also reduced [11].

The decreasing in testicular spermatids of rat's seminiferous tubules possibly occurs through several mechanisms such as disruption of sertoli cell function, which causes the decreasing of germ cells nutrients, lactate and pyruvate. Lactate and pyruvate are energy sources of the spermatids. The decreasing of these energy sources might interference the meiosis process, the other possibility is because in the early spermiosis already impaired. With the disruption of spermiogenesis early the next process will be impaired [3].

Administration of the mulberry leaf infusion for 30 consecutive days increased in spermatogenic cells and Sertoli cells. In general, an increase in the number of spermatogenic cells and Sertoli cells occurs because the mice were initially experiencing a hyperglycemic condition after being fed mulberry leaves infusion blood glucose levels to be down, so that the free radicals that had increased to participate reduced. Free radicals are decreased to reduce tissue damage in the body, including damage to the organ-forming germ cells. Chemical constituents of mulberry leaf that play a role in lowering high blood glucose levels is the DNJ. DNJ has the potential to inhibit alpha-glucosidase, an enzyme that catalyzes the hydrolysis of bonds in maltose to produce two molecules of glucose [12].

In addition, mulberry leaves are able to repair tissue damage due to their antioxidant content. One of antioxidant content of mulberry leaves is vitamin C [10]. The role of ascorbic acid (vitamin C) for diabetes is as aldose reductase enzyme inhibitor [1], thus reducing the use of equivalent reduced. The willingness of the reducing equivalent is useful for the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH). It can further prevent the buildup of sorbitol in tissues [12].

In extracellular, this antioxidant could decrease the superoxide radicals which is generated from the glucose auto-oxidation and nitric oxide synthesis. If the superoxide radicals are in excess condition, they will react with nitric oxide and

finally produce peroxynitrite radicals that are cytotoxic. Inhibition of the formation of peroxynitrite radicals will keep vasodilatation function played by nitric oxide. In endothelial cells, ascorbic acid affects the enzyme nitric oxide synthase that can suppress superoxide radicals as a result of nitric oxide formation. Meanwhile, folic acid and zinc contained in mulberry leaves are to be tough to be able to increase the number of sperm cells [13].

The increase in weight of the testes of treated mice compared to control mice given positive after infusion mulberry leaves for 30 days this happens because the number of spermatogenic cells in the testes also increased. This is consistent with the statement of Zhang et al [3], that the rich content of spermatogenic cells in the seminiferous tubules in the testes can also increase the weight of the testis itself although testicular weight was also influenced by other factors. Testis weight is not only influenced by the presence of germ cells and seminiferous tubule diameter, testis weight was also affected by connective tissue and smooth muscle cells [7]. This network serves to support the process of spermatogenesis is done by the testes. So not only gained weight testicular tubules but also supported by a network of connective tissue and blood vessels.

The increasing of the testes weight in diabetic rats after mulberry leaves infusion is due to folic acid and zinc in mulberry leaves that finally support the increasing the number of spermatogenic cells [4]. In addition, the antioxidant content owned mulberry leaves supposedly able to suppress the increase of free radicals that can interfere with spermatogenesis, so that when the number of cells increases, testicular spermatogenic will experience weight gain

Effect of mulberry leaves infusion on histological preview of leidig cell and testosteron level of chronic diabetic models

From Figure 2 (a) and (b) the number of leidig cells and testosterone levels significantly increased. Low level of leidig cell and testosterone was found in the C + and not significantly different from T1. While in T2 and T3, and T4, leidig cell number and testosterone levels increased up to normal condition.

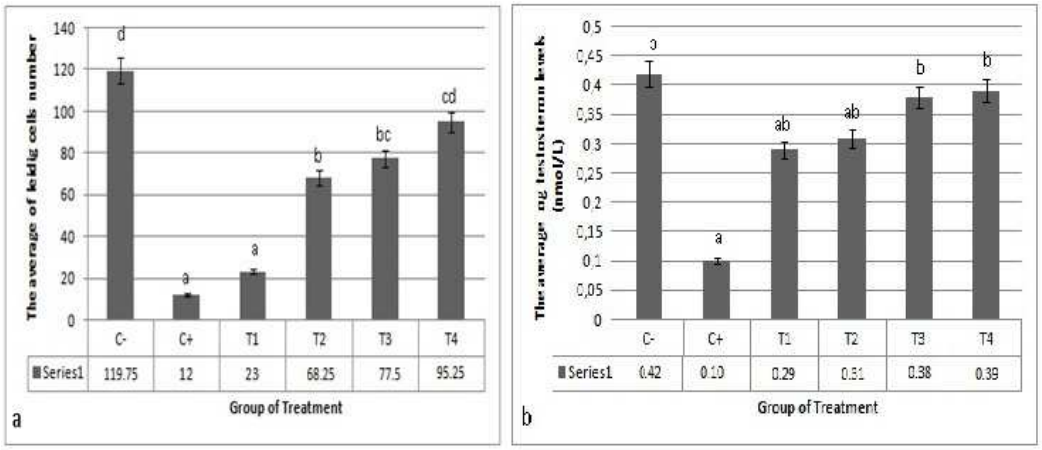

FIGURE 2. (a) Leidig cell number and (b) testosteron level on chronic diabetic models after treatment of mulbery leave infusion.

Oxidative stress due to hyperglycemia will increase the amount of ROS that can damage cell membranes, the lipid peroxide or Malondialdehyde (MDA), which ultimately affects the hypothalamic-pituitary axis work so that secretion of LH and FSH decreased. FSH levels will lead to disruption the mitosis process and spermatogonia proliferation. According Zhang [4] FSH and LH hormones have an important role in the process of spermatogenesis. LH stimulates the Leydig cell function to produce testosterone in testes. Furthermore, FSH could stimulate spermatogenesis process, the formation of spermatogonia into spermatids. In addition, FSH also serves to stimulate the formation of Sertoli cell androgen-binding protein (ABP) in which this protein plays a role in the transport of testosterone to the seminiferous tubules and epididymis.

The destruction of Leydig cells due to the inhibition of the luteinizing hormone (LH) secretion in rats with diabetes mellitus can be seen from the number of Leydig cells and testosteron level that decreased as shown in Fig. 3. Mulberry leaves infusion can improve the number of leydig cells, because mulberry leaves contain vitamin C as an antioxidant that can counteract on free radicals, in addition to other compounds that have antioxidant functions that moracetin, isoquersetin, eugenol, and carotene .

IV. **CONCLUSION**

Mulberry leaves infusion can improve the reproductive status of the rat model of chronic diabetes mellitus characterized by the increasing of spermatogenic and sertoli cells number, seminiferous tubule diameter, testes weight, leydig cells and testosterone levels, where the optimal dose is 1000 mg / kg. It is suggested to do further research on the effect of mulberry leaf infusion on sperm quality models of chronic diabetes mellitus.

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