Decrease Expression of Tumor Necrosis Factor - Alpha (TNF-α) and Sperm Count Increase in Type 1 Diabetes Mellitus Rat (Rattus norvegicus) Model with Turmeric Rhizome (Curcuma longa L) Extract

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Abstract. Diabetes mellitus type 1 (DM type 1) is a type of diabetes caused by pancreatic β cells damaged which producing insulin. Animal DM Type 1 caused inflammation of the pancreas and its impact on the male reproductive organs may decrease the quality and quantity of sperm. This research was aimed to determine the effect of turmeric rhizome ethanol extract (Curcuma longa L.) based on sperm count and expression of tumor necrosis factor-alpha (TNF-α) in the pancreas organ. This experiment was designed by Completely Randomized Design. Induction of DM Type 1 in rats was conducted by injecting Multiple Low Dosage - Streptozotocin (MLD-STZ) intraperitoneally (i.p) at the dosage of 20 mg/Kg BW for 5 days. This study used male rats (Rattus norvegicus) aged 3 months Wistar strain which divided into 5 groups: negative control (A), DM Type 1 (B), DM Type 1 treated with turmeric rhizome Extract 1.2 g/kg BW (C), 1.8 g/kg BW (D) and 2.7 g/kg BW (E) for 42 days. Each group consisted of 4 rats. spermatozoa counted with hemocytometer and measurement of TNF-α expression used immunohistochemical methods.

The result of the analysis on one way ANOVA (α = 0.05) data showed that spermatozoa count was significantly increased and TNF-α expression was decreased in rats which treated with turmeric rhizome ethanol extract 2.7 g/kg BW. This result indicates that turmeric rhizome extract may increase the number of spermatozoa and decrease the expression of TNF-α of DM Type 1 rats.

1. Introduction
Diabetes Mellitus (DM) is a disorder of carbohydrate metabolism which symptoms are generally hyperglycemia. Diabetes mellitus Type 1 (DM type 1) or Insulin-dependent diabetes mellitus (IDDM) is a type of diabetes that depends on the production of Insulin or occurs due to the presence of damage to the β-cells of the pancreas producing insulin [1]. Factors that can trigger the occurrence of diabetes in animals, such as age, gender, race, and Environment [2].

The prevalence of Diabetes mellitus in humans in 2002 according to the International Diabetes Federation is 8.39% [3]. Diabetes mellitus in animals occurs in pet animals such as dogs and cats, this is due to the feeding of high-fat feed as well as feeding exceeds the needs of the body. According to Fall et al. there were 13 cases of DM per 10,000 dogs annually in aged more than five years and the races are Australian Terriers, Samoyeds, Swedish elkhounds, and Swedish Lapphunds [2]. In cats the prevalence of DM incidence is 0.5%, the influencing factor is obesity, age, sex, and sterilization [4].

Diabetes mellitus type 1 is a disease caused by the presence of Inflammation in the pancreas or commonly called insulitis. Genetic and environmental factors in Type 1 DM triggers an immune
response that is mediated by the T cell lymphocytes and reacts to the pancreas cells as *self-antigen*. Cytotoxic macrophages and T cells produce cytokines, *tumor necrosis factor-α* (TNF-α) and interferon-γ (IFN-γ) that causes damage to the β-cells of the pancreas [5]. In patients with DM type 1, it will increase levels of expression of TNF-α. Patients with type 1 DM have low insulin levels that can affect the decline in hormone secretion-other hormones including reproductive hormones such as, LH and FSH. The Descent of Follicle Stimulating Hormone (FSH) levels affects the production of spermatozoa so that the concentration of spermatozoa decreases [6].

Turmeric (*Curcuma longa L.*) that has been used as the seasoning has several active ingredients, among others, essential oils, *curcumin*, *Demetoksikurkium*, *Bisdemetoksicurcumin*, *saponin*, *flavonoids*, and *polyphenols*. The bioactive in turmeric used for therapy is *curcumin*, as it is an antioxidant and anti-inflammatory substance. The administering of Turmeric (*Curcuma longa L.*) was expected to decrease the expression of TNF-α pancreas and increase the spermatozoa number in a rat model of diabetes mellitus.

2. Methods

2.1 Animal Models

Animals models used were rats (*Rattus norvegicus*) male, Wistar strain, obtained from the Experimental animal development Unit UGM Yogyakarta with the age of 8-12 Week and weight 150-250 gram. The rat was divided into five groups, the negative control group (KN), the positive control group of diabetes mellitus type 1 (KP group), the DM 1 group which is treated with turmeric rhizome extract 1.2 g/kg BW (P1 group), the DM 1 group treated with Turmeric rhizome extract 1.8 g/kg BW (P2 group), and DM 1 group treated with turmeric rhizome extract 2.7 g/kg BW (P3 group). Each group consists of four rats. The cage used as a group cage with four-tailed mice per cage (one experimental group). Rats to be adapted to the environment for seven days and feed with the adult chicken feed from Wonokoyo Jaya Corporindo (maximum water content 12%; Protein 18-20%; Maximum fat 5-8%; Maximum fiber 5%; maximum Abu 7.5%; Calcium minimum 0.9-1.2%; phosphor minimum 0.6-0.8%) and drank *ad libitum* in all rat.

2.2 Animal models of DM type 1

One week after the adaptation, the blood glucose measured by GlucoDr®. The streptozotocin 20 mg/kg BW per day was injected intraperitoneal in each rat of the KP group, P1 group, P2 group and P3 group for five days. The measurement of blood glucose was continuously done every seven days. The blood glucose in a normal rat is ≤ 126 mg/dL [7]. The blood glucose results in the 14th days after the injection of the streptozotocin were > 400 mg/dL, it was shown that the rat has been diabetic.

2.3 Therapy of Diabetes mellitus Animal Model

The turmeric rhizome extract ethanol made with a maceration method [8], the stages begin with a clean wash of turmeric and cut thin, then inserted into the oven with a temperature of 40-60 °C to dried the turmeric. The next step is the extraction process, turmeric that has been dried with a blender until smooth, weighed as much as 100 grams and inserted into the Erlenmeyer 1 liter size of the glass. The dried turmeric was added with ethanol of 96% to 1 liter and shaken until thoroughly blended. A mixture of ethanol and turmeric’s active substances is then evaporated using a water bath with a temperature of 80 °C until the extract becomes viscous and weighed in an extract weight, then evaporated again using an oven to remove remaining ethanol. The evaporation used an oven with temperature 70 °C, every 15 minutes of extract weighed up to three times in the same weight extract (14.8 grams). The evaporated turmeric extract was diluted with a solution of Na₂CO₃ 0.1 N for intracardiac given by sonde through the mouth.
2.4 Therapy of Diabetes mellitus Animal Model

The determination of therapy medications was from dose assuming the content of Curkuminoids as much as 3% in the extract then converted with three doses of curcuminoid in the research Firmawati and Widjiati (2017) so that obtained three doses turmeric rhizome extract was 1, 2g/kg BW (P1 group) [9], 1.8 g/Kg BW (P2 group) and 2.7 g/Kg BW (P3 group). The therapeutic treatment of turmeric rhizome extract begins on the 15th day after the injection of STZ. Administration of therapy conducted on an oral basis through gastric sonde 2 mL per tail. The therapy was performed once a day for 42 days. During therapy, there is a measurement of blood sugar levels every seven days once in all treatment groups.

2.5 Sperm count

The rat was euthanized by cervical dislocation. The abdominal area was opened to take the rat testes. The calculation of spermatozoa was done by taking the semen from the end of the cauda epididymis (from the testicles that have been taken), it was cut with scissors and sorted for the sperm to come out and be loaded to the petri dish. The sperm fluid in the petri dish was taken 50 μL and placed on the glass object to count the sperm per field of view. In this research, an estimated number of spermatozoa per view was > 40 using a comparison of sperm and a solution phosphate buffer saline (PBS) for dilution is 1:20. One drop of sperm fluid taken and homogenized with a 1000 μL solution of PBS PH 7.4. The homogeneous sperm and PBS were taken using a pipette and were put in the room count improved Neubauer and covered with a glass cover, and make sure if no air bubbles. Spermatozoa are observed and were calculated under the light microscope [10].

2.6 Immunohychemical preparations

The pancreatic rat organ was inserted into the solution Paraformaldehyde (PFA) 4%. Histopathology preparation with the staining of IHC, including dehydration, clearing, embedding, sectioning, adding on glass objects and then staining with Immunohistochemical. A primary antibody used was an anti-TNF-α Rat with a secondary antibody labeled biotin used Goat anti-Rat biotin labeled. The pancreatic TNF-α expression was observed and photographed at 40x and 400x magnification with five fields of view for each group. Then the calculation of the TNF-α expression per field of view for each group used Axio Vision software. The data used in this study is quantitative data. Quantitative Data number of rat spermatozoa and Tumor necrosis Factor-alpha (TNF-α) expressed and analyzed with SPSS 16.0 Edition for Windows with variety analysis ANOVA with α = 0.05 and continued with the test Tukey or real difference honestly (BNJ) when it turns out significant.

3. Result and Discussion

The diabetes mellitus type 1 (DM type 1) in the rat model measured the blood sugar levels using a glucometer. The result of STZ induction for the animal models of diabetes mellitus type 1 showed an increase in blood sugar levels of > 400 mg/dL, compared with the negative control that showed an average of 121 mg/dL (Figure 1) in accordance with Baric et al. (2008) that the normal blood sugar levels of rats ≤ 126 mg/dL. This result suggests that animal models have suffered from DM type 1. The administration of turmeric rhizomes extracts therapy provides influence in the form of a decrease of the blood glucose in the P1 group, P2 group, and P3 group (Figure 1). The administration of turmeric rhizomes extracts therapy also provides influence in the form of decreased expression of TNF-α pancreas, with an average decrease in expression TNF-α group C, group D, and group E versus Group B in a succession of 25.21%, 37.72% and 56.16% (table 1).
The calculation of the average pancreatic rats TNF-α expression through SPSS 19.0 followed by the ANOVA test shows a noticeable difference in the KN group compared to the average expression TNF-α Rat KP group. This is due to streptozotocin induction on animal models Diabetes mellitus Type 1 as a diabetogenic agent causes the activation of the T lymphocytes especially CD4 + that produces interferon-γ (IFN-γ). The interferon-γ will activate macrophages that stimulate the production of proinflammatory cytokines such as IL-1 and TNF-α as well as produce free radicals such as superoxide (O2-), hydrogen peroxide (H2O2) and nitrite oxide (NO) [5]. The interferon-γ also activates T lymphocytes CD8 + cytotoxic. Proinflammatory cytokines produced due to the activation of macrophages to the cytotoxic into pancreatic β-cells by transmitting death signals to the β-cells so that it occurs necrosis in the pancreas B cell. The free radicals produced from the activation of macrophages also trigger the occurrence of pancreatic B-cell necrosis in the presence of a bond between the Superperoxide (O2-) and nitrite oxide (NO) being Peroxynitrite (ONOO-). The peroxynitrite caused DNA fragmentation in pancreatic B cells and induces the occurrence of cell necrosis (Nugroho, 2006). A pancreatic B cell that is experiencing necrosis will emit a protein known as B-protein. Furthermore, this B-protein will be recognized as an antigen (b-Ag) by the APC and presented by the MHC-2 at the B-Ag specific T cell receptors that re-activate CD4 + TH1 [5]. This condition will occur continuously and resulting in an increase of the expression of TNF-α in the cell β pancreas and some damage to the cell β pancreas. The mechanism causes the emergence of the TNF-α expression increased in the positive control group (KP) compared to the negative control group (KN).

Table 1. The average of the TNF-α expression in the immunochemical preparations of the rat pancreas in each group of treatment

| Group                  | Average of the % area TNF-α expression ± standard deviation |
|------------------------|-------------------------------------------------------------|
| Negatif control (KN)   | 3.92 ± 0.38a                                                |
| Positif control (KP)   | 17.54 ± 7.98c                                               |
| Treatmen dose 1 (P1)   | 13.12 ± 1.66bc                                              |
| Treatmen dose 2 (P2)   | 10.93 ± 0.31abc                                             |
| Treatmen dose 3 (P3)   | 7.69 ±0.38ab                                                |

Figure 1. Rat blood glucose levels.
Description: The notation a, b and c indicate a significant difference between the treatment ($P < 0.05$).

The statistical result shows that there was no significant between the positive control group (KP group) with the P1 group and the P2 group. The results indicate that the average of the TNF-α expression after administration of therapy Turmeric rhizome Ethanol Extract (Curcuma longa L.) at a dose of 1.2 g/KGBB and 1.8/kgBW was still the same as a positive control group (KN group), but the P2 was no significant to with the KN group. On the other hand, the P3 group has a significant difference with the KP group and no significant difference with the KN group. This result indicates the administration of therapeutic dose 2.7 g/kg BW (P3 group) effective to lower the expression of TNF-α pancreatic in rats model of diabetes mellitus type 1.

Turmeric is a plant that can be utilized as an exogenous antioxidant because it contains high curcumin compounds. Sharma suggests that the curcumin contained in the turmeric rhizome can serve as an antioxidant and anti-inflammatory [11]. Curcumin as an antioxidant will stabilize free radicals manufactured due to macrophage activation. As an anti-inflammatory, curcumin suppresses the activation of the nuclear factor kappa B (NF-kB) which is a eukaryotic transcription factor that encompasses the regulation of inflammation, cellular proliferation, transformation, and tumorigenesis. The activation of NF-kB will induce the expression of an inflammation gene and increase the production of COX-2, cell adhesion and pro-inflammatory cytokines [12]. Activation of NF-kB suppressed by curcumin will inhibit the induction of transcription of the pro-inflammatory cytokine, resulting in decreased expression of TNF-α. Curcumin may also inhibit the induction of cyclooxygenase-2 (COX-2) and lipoxygenase [11]. The curcumin inhibits the direct enzymatic activity of COX-2 whereas as we KNOW that COX-2 plays a role in the production of prostaglandins during inflammatory processes, pain, and fever response [11]. The lipoxygenase line plays a role in the production of arachidonic acid that will become a prostaglandin as an inflammatory mediator.
Figure 2. shows that in the B figure has a lot of TNF-α expressions on the part of Langerhans Island characterized by the brown color spread over the Langerhans island of the pancreas and the presence of cavities or intercellular chambers. The brown color arises due to the binding of antigen (TNF-α) and antibodies (anti-TNF-α) labeled Biotin. The TNF-α expression on B figure higher than the A figure because in rats diabetes mellitus pro-inflammatory cytokine production such as TNF-α increases. In figure C, figure D, and figure E occurs a decrease of expression TNF-α marked with a decrease in brown color on the island of Langerhans and the occurrence of inhibition of damage to Langerhans island. The pancreas was characterized by a lack of intercellular cavity. This described that the therapeutic treatment of turmeric rhizome ethanol extract containing curcumin compounds able to lower the expression of TNF-α pancreatic rat model of diabetes mellitus type 1.

**Figure 2.** The expression of TNF-α in the Langerhans island of rat pancreas. A: KN group; B: KP group; C: P1 group; D: P2 group; E: P3 group; →: B cell that expresses TNF-α; : B cell that not express TNF-α.
The influence of the turmeric rhizome (Curcuma longa L.) extract on the number of spermatozoa in the rat model of diabetes mellitus type 1 checked with count the concentration of the rat spermatozoa in the cauda epididymis. The results of the number of spermatozoa indicate a noticeable difference ($P < 0.05$) of the five groups of treatment. This suggests that the administration of turmeric rhizomes (Curcuma longa L.) extract in each treatment group affected an increase in the number of spermatozoa rats in the model of diabetes mellitus type 1 (table 2). Calculation of the average number of spermatozoa rat negative control groups (KN group) showed a noticeable difference compared to the average number of rat spermatozoa in the positive control group. The type 1 diabetes mellitus resulting in a decrease in the amount of insulin in the body, so there can be complications in the reproductive organs one of them on the number of spermatozoa. The standard deviation in the control group was large because the positive control group was a group of rats induced by Streptozotocin to become diabetic. During the research, a rat group of positive controls remained maintained to the end so that one rat died due to the worsening condition of diabetes mellitus. While the surviving positive control rats were found to have changed the macro anatomy of the testis (Figure 3).

![Figure 3. Macro anatomy of rat testis (left: the positive control group and right: the negative control group).](image)

Insulin is produced and synthesized by the ß cells of the pancreas so that if there is damage to the ß-cells such as in the state of diabetes mellitus type 1 will occur a decrease in the amount of insulin. One of the roles of insulin in the body is to propagation protein synthesis in the liver. Insulin levels that decreased due to the DM type 1 condition will inhibit the work of organs affected by insulin. In the liver, organs decreased levels of insulin resulted in the loss of protein synthesizes by the liver, one of the synthesizes of IGF-1. Insulin-like Growth Factor – 1 (IGF–1) plays a role in enhancing the effects of gonadotropin in the Sertoli cells and Leydig cells [13]. Decreased IGF-1 results in a decrease in the effects of gonadotropin (LH and FSH) in the Sertoli and Leydig cells that play a role in spermatogenesis processes. The LH stimulates the Leydig cells to proliferate and produce testosterone, whereas the FSH directly affects the process of spermatogenesis and against the proliferation of Sertoli cells that produce ABP [14]. Androgen Binding Protein (ABP) produced by the Sertoli cells with FSH stimulation serves to transport the hormone testosterone. In the condition of diabetes mellitus, there is a decrease in the effect of LH and FSH in the Sertoli and Leydig cells. This resulted in decreased production of testosterone and the ABP used in the formation of Spermatozoa so that the spermatogenesis process would be obstructed. The obstructed spermatogenesis process will have an effect on the resulting amount of spermatozoa. Decreased number of spermatozoa in the state of diabetes mellitus caused by the destruction of the spermatogenesis process due to decreased testosterone production and Androgen Binding Protein. This caused a decrease in the number of spermatozoa in the positive control group (KP group) compared to the negative control group (KN group).

| Group             | The average number of rat spermatozoa ± standard deviation |
|-------------------|---------------------------------------------------------|
| Negatif control (KN) | 81.400 ± 4056,3 c                                        |
| Positif control (KP) | 26.775 ± 24596,8 a                                      |

Table 2. The average number of rat spermatozoa in each treatment group.

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| Treatment dose 1 (P1) | 26.913 ± 147.801 a |
|----------------------|-------------------|
| Treatment dose 2 (P2) | 43.713 ± 422.66 b |
| Treatment dose 3 (P3) | 61.450 ± 1033.755 bc |

Description: The notation a, b and c indicate a significant difference between the treatment (P < 0.05).

In rats the model of diabetes mellitus with induction of Streptozotocyn, where the rat became insulin-dependent diabetes so that this resulted in a decrease in the Leydig cell function and production of testosterone due to the absence of an insulin stimulation effect on Leydig cells and insulin-dependent conditions cause a decrease in the FSH, which eventually leads to a decrease in LH, so that this reduction of FSH and LH is a decrease in the quality of spermatozoa [15]. The administration of Turmeric rhizomes (Curcuma longa L.) extract in rat model diabetes mellitus type 1 (P1 and P2 group) was not different with the positive control (KP group). These results indicate that the average number of spermatozoa after administration of turmeric extract therapy (Curcuma longa L.) at dose 1.2 g/kg BW and 1.8 kg BW still had the same as the positive control spermatozoa number. This was due to the bioactive content of curcuminoids in the turmeric rhizome ethanol extract in this study which acts as a suspected antioxidant and anti-inflammatory is not enough to stabilize free radicals and suppress the number of proinflammatory cytokines [16].

Therapy in dose 2.7 g/kg BW (P3 group) did not differ real with the negative control group (KN group) and it differed with the positive control group (KP group). It showed that in the P3 group the number of spermatozoa becomes increase to normal. Turmeric rhizome (Curcuma longa L.) extract has high bioactive content of curcuminoids as antioxidant and anti-inflammatory [17]. Antioxidants are substances that are able to slow down or prevent oxidation processes. Curcumin as an antioxidant (AH) gives a hydrogen atom rapidly to free radicals (R *, ROO *) or converts it to a more stable form, while the antioxidant radical derivative (A *) has a more stable state than free radicals [18]. Curcumin also slows the rate of autooxidation by lowering the enzyme levels of xanthine oxidase which catalyzes the reaction of the formation of active superoxide anions. Curcuminoids as an anti-inflammatory works by suppressing the production of pro-inflammatory cytokines that trigger damage to the β-cells of the pancreas, thus able to reduce the level of cellular damage [18]. In this study we suggest that the free radical and pro-inflammatory cytokines decline to occur with the administration of turmeric rhizome extract, it is expected to reduce the rate of pancreatic cells damage and increase the production of insulin. The increase of insulin levels can trigger the synthesis of IGF-1 in the liver which enhances the effect of LH and FSH in Leydig cells and the Sertoli cells. Such improvements can trigger elevated levels of testosterone and ABP used in the process of spermatogenesis.

Conclusion

The administration of turmeric rhizome (Curcuma longa L) extracts at a dose 2.7 g/kgBB decreases the expression of Tumor Necrosis Factor-Alpha (TNF-α) on the pancreatic organs and increase the number of spermatozoa in rat model diabetes mellitus type 1.

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