Indonesia Black Cumin (*Nigella sativa* L.) Seeds Extract as Ameliorant Reproductive Function in Type-2 Diabetes Mellitus

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Received 4 January 2020; Received in revised form 29 April 2020; Accepted 17 May 2020; Available online 30 June 2020

**ABSTRACT**

Diabetes initiates augmented damage in levels of nuclear and mitochondrial DNA in males. As a result, the sperm DNA is affected, leading to lower levels of fecundity and influencing reproductive health. This study aims to improve male reproductive function and oxidative stress status in diabetic rats. Combination High Fat Diet (HFD) and Streptozotocin (STZ) injection 30 mg/kg intraperitoneally are the initiations of DM-2 for 14 weeks. Treat therapy using 80% ethanol extract of black cumin seeds takes five weeks. Based on parametric test on ANOVA test results followed by Duncan Multiple Range Tests on the concentration, abnormalities of spermatozoa and Super Oxide Dismutase (SOD) effects and the Kruskal-Wallis test followed by the Mann-Whitney test on non-parametric data of the spermatozoa motility and Malondialdehyde (MDA) levels, showed that obtaining black cumin dose 48 is an effective dose in improving sperm quality and stress oxidation level. It has the same effect with metformin, even has MDA level less than normal rats. This study finds out Ns-48 is an effective dose of ethanol extract of black cumin seeds to improve spermatozoa quality and stress oxidation level, so that it becomes valuable information for research related to the improvement of reproductive function in diabetes mellitus disease.

Keywords: diabetes; MDA; reproduction; spermatozoa; SOD

**INTRODUCTION**

Diabetes mellitus type 2 (DM-2) is one category of metabolic disorder characterized by increased blood glucose levels, and the body's cells are insulin impervious. Globally, the total of people with DM-2 continues to grow, 382 million people in 2013, 425 million in 2017, so that the assessed number of people travail from DM-2 is 592 million in 2035, about 629 million in 2045 (Saeedi *et al.*, 2019). Type 1 and type 2 diabetes are the two main types, with type 2 diabetes widely held (> 85%) of total diabetes prevalence. Prevalence will surge in all countries, but the highest decay will be experienced in low-income countries (Forouhi & Wareham, 2014; Cho *et al.*, 2018).

DM-2 sufferers are easy to experience oxidation stress and hypogonadism (Asmat *et al.*, 2016; Dhindsa *et al.*, 2018). The interaction of glucose with proteins leads to an amadori product and then advanced glycation end-products (AGEs). These AGEs, via their receptors (RAGEs), activate enzymes and alterations in their structures and functions. Moreover, AGEs cause diabetes hitches (Singh *et al.*, 2014). Radical Oxygen Species (ROS) and AGEs are mediators of diabetes worries (Matough *et al.*, 2014; Omolaoye & Du Plessis, 2018), such as a coronary arterial disorder (Fishman *et al.*, 2018), diabetic neuropathy and gloominess (Réus *et al.*, 2019), diabetic retinopathy (Solomon *et al.*, 2017), and interference of the structure-function of reproductive organs both women and men, thus it becomes diabetic barrenness (Bansal & Bilaspuri, 2011; Morrison & Brannigan, 2015). Diabetes mellitus causes increased damage in levels of nuclear DNA (sperm nuclear) and mitochondrial DNA in males (Basmatzou & Hatziveis, 2016; Pourmasumi *et al.*, 2017; Laleethambika *et al.*, 2019).

DM-2 generally performs aged 30 years, but currently, the disease also outbreaks someone aged 20 years. This age is an early
stage of reproduction, a period where the eminence of spermatozoa and ovum that are good is needed to produce offspring. Thus, efforts are needed to overcome reproductive failure caused by DM-2.

Distinctly, *N. sativa* has been studied as an antidiabetic, maintainer of reproductive wellbeing, and a source of antioxidants. Ethanol extracts *N. sativa* at dose 200 and 400 mg/kg body weight could decrease MDA levels in STZ-induced hippocampus mice (Abbasnezhad *et al.*, 2015). As an antidiabetic, inquiry shows the usage of *N. sativa* in patients and investigational animals. Feeding of *N. sativa* at a dose of 2 g/day set off a significant lessening in FBG, 2hPG, and HbA1c without generous changes in body weight, reduced insulin resistance, and developed pancreatic beta-cell function (Bamosa *et al.*, 2010).

Utilization of seeds *N. sativa* in prosperous the reproductive status of male animals has also been carried out. Compared to the healthy reproductive organs of rats, the ethanol extract *N. sativa* at a dose of 400 mg/kg does not affect to the weight of male reproductive organs and reproductive cells but can significantly increase testicles and epididymis weight (Parandin *et al.*, 2012).

Based on previous research, it was shown that the study and utilization of *N. sativa* both in the form of seed powder and oil were more as antidiabetic and as maintainers of reproductive health than compared to diabetic infertility. This study aims to evaluate the effect of extract *N. sativa* seeds on sperm content, viability, motility, sperm morphology abnormalities, and their impact on SOD and MDA levels in DM-2.

**MATERIALS AND METHODS**

*Nigella sativa* Seeds Extract Preparation. Indonesian *N. sativa* seeds samples were 120 g obtained from Balitro, Bogor. *N. sativa* seeds dried handling an oven that is ±40°C for 2x24 hours, so it becomes dry, then ground until smooth and sifted with 60 mesh sieves. Extraction commences with a maceration process with 80% ethanol. Every 60 g of *N. sativa* seeds powder soaked using 300 ml of 80% ethanol solvent for 24 hours and stirred using a shaker for 3 hours. Then it was filtered. The filtrate extract obtained was concentrated with a rotary evaporator at 40°C, 70 rpm to become concentrated extract (Bahirin *et al.*, 2018), obtained are 23.57 g (39.28%).

**DM-2 Animal Induction.** Twenty-four rats were divided erratically into one regular group (non-diabetic) and five diabetic groups. Each group has four replication. All rats acclimatize for two weeks. After passing through the adaptation period, each rat except in the standard a control group was given a high-fat diet and taken ad-libitum for nine weeks. Test animals excluding negative controls were injected with STZ intraperitoneally with a low dose of 30 mg/kg bw on the first day of the 10th and 11th week when administering a high-fat diet. After five days of STZ administrations, a blood glucose tolerance test was conducted to prove the success of making entries animals in the DM-2 models. The animals were alleged to have DM-2 if blood glucose was > 200 mg/dl (Qinna & Badwan, 2015).

**Grouping and Treatment.** Into the bargain to the normal non-diabetic group (Normal), 20 diabetic rats were separated into 5 groups, 4 replication: dose 0 mg/kg bw, 24 mg/kg bw, 48 mg/kg bw, 72 mg/kg bw, and metformin as positive controls, successively coded as Ns-0, Ns-24, Ns-48, Ns-72, Metf. The remedy was given orally using gavage needles with a volume of 2.5 ml; treatment gave for four weeks. At the end of the examinations, rats were anesthetized and dissected for their reproductive organs. The cauda epididymis and sperm quality were observed, including concentration, viability, sperm abnormalities, and motility. The liver isolated for lysate preparation for measurement of SOD and MDA levels. The liver lysate was prepared from 0.1 g of the liver with diluent 1 ml 0.9% NaCl and centrifuged at 8000 rpm for 20 minutes (Aulanni’am *et al.*, 2011).

**Data Retrieval.** 1) The measurement of sperm concentration was carried out using a Neubauer improved cell counting chamber. Sperm is gained from the epididymis duct and diluted with 0.9% NaCl solution, with a dilution factor of 200X. Preparations were noted and
calculated using a microscope with 400X magnification. Sperm concentration data state the number of sperm cells/ml; 2) Sperm motility was calculated using the Neubauer improved cell counting chamber. Sperm is acquired from the epididymis duct and diluted with 0.9% NaCl solution with a dilution factor of 200X. The progressive motility of sperm (frontal motion) was perceived and calculated using a microscope with 400X magnification. Sperm motility data states the percentage of sperm cells with a progressive movement from 100 sperm cells found; 3) The viability of sperm is assessed using eosin-nigrosin staining. Examine used microscope at a magnification of 400X. The dead sperm is indicated by sperm head stained, while the live sperm head is not colored. Viability data stated live-cell percentages of several sperms observed (100 sperm cells); 4) A sperm abnormality noticed by calculating sperm morphological abnormalities (compared to typical sperm forms) using eosin negrosin staining. Examinations were carried out using a microscope with 400X magnification. Inconsistencies of data state the percentage of sperm cells that experience morphological abnormalities of several sperms observed (100 sperm cells); 5) The liver method's measurement was provided by the MDA determination kit from Sigma Aldrich in which the wavelength setting was λ 532nm. As much as 100 µL of liver lysate comprised in the new microtube, added 550 µL of aquadest, 100 µl of 10% TCA, 250 µl of HCl 1 N, and 100 µl Na-Thio 1%. After that, it was separate out and centrifuged at 500 rpm for 10 minutes. The supernatant was transported to a microtube and soaked in a water bath at 100°C for 30 minutes. The supernatant cooled at room temperature 26-27°C, then measured the absorbance using a spectrophotometer. MDA levels were obtained by calculating the absorbance value into a standard curve linear equation; 6) The SOD activity measurement was carried out using nitroblue tetrazolium (NBT) under the SOD determination kit (from Merck) procedure in which the wavelength setting was λ 440 nm. A total of 400 µl of cold ethanol solution 37.5/62.5 (v/v) was added to the 150 µl of liver lysate. Then vortex splitting is constantly for 3 seconds and centrifuged at a speed of 4400 rpm at 4°C for 10 minutes. 2.9 ml of solution A (mixtures of xanthine solution and cytochrome c solution) was added 50 µl of ordinary solution (control) or sample and followed by the vortex. Then add 50 mL of solution B (xanthine oxidase) and then slowly vortex. SOD levels were experiential using the spectrophotometer absorbance. The SOD level is achieved by calculating the absorbance value into the standard curve linear equation.

**Data Analysis.** Data on concentration, viability, sperm abnormalities, and MDA levels followed a normal distribution with homogeneous variants. Testing the effect of treatment was analyzed by one way ANOVA. The treatment was carried out using the Duncan Multiple Range Test (DMRT) test. Motility and SOD data have homogeneous variants but are not normally distributed, analyzed by non-parametric using the Kruskal-Wallis test followed by the Mann-Whitney test.

### RESULT AND DISCUSSION

The statistical test results using ANOVA with a significance level of 5% showed a significant effect of 80% ethanol extract of *N. sativa* seeds on sperm concentration, abnormality, viability, and SOD. The Kruskal-Wallis test results on motility sperm and MDA also showed the same results as the ANOVA test (Table 1).

**Table 1. The analysis results of sperm quality and stress oxidation status in the liver of experimental rats**

| Parameters | Treatments | ANOVA |
|------------|------------|-------|
|            | Normal | Ns-0 | Ns-24 | Ns-48 | Ns-72 | Metf |       |
| Sperm quality |       |       |       |       |       |       |       |
| Concentration (10⁷/ml) | 20.00 ± 3.92d | 7.75 ± 1.26a | 13.50 ± 3.11bc | 18.25 ± 4.65cd | 11.25 ± 3.78ab | 15.25 ± 3.10bc | P <0.05 |
| Viability (%) | 71.25 ± 2.22c | 47.25 ± 2.22a | 67.75 ± 3.86bc | 68.50 ± 2.65bc | 63.75 ± 2.99b | 66.50 ± 4.04bc | P <0.05 |
The findings of Parandin et al. (2012) indicate that the integrity in the sperm nucleus, which raised to a normal level. The best treatment of dose 48 mg/kg, the motility equal to metformin, 72 mg/kg can increase the sperm motility, the extract of N. sativa could build the sperm viability and motility of rats. The results of this study. As a stimulate on increasing motility, the extract of N. sativa at the dose 24 and 72 mg/kg can increase the sperm motility of diabetic rats and the standard drug effects of metformin. The best treatment of dose 48 mg/kg, the motility equal to metformin, was raised to a normal level. This study's results generated better results than those of Parandin et al. (2012) who stated that 50% N. sativa ethanol extract did not significantly upsurge sperm viability and motility of rats. The findings of Desai et al. (2015) support the findings of this study. Verify that giving seeds of N. sativa powder dose of 300mg/kg bw for 45 days and the results of Parandin et al. (2012) which used ethanol extract and the two researchers succeeded increased testosterone to significant levels, increased luteinizing hormone (LH), and fertility indexes spermatozoa (Bashandy, 2007; Marbat et al., 2013; Desai et al., 2015). The hormones testosterone is the primary hormone accountable for signaling pathways in the process of spermatogenesis (Walker, 2011). LH/testosterone, and follicle-stimulating hormone (FSH) are important endocrine factors that can control testicular function (Ramaswamy & Weinbauer, 2014).

Increasing the concentration, viability, and motility of sperm in this study because of the increase in testosterone levels due to the administration of ethanol extract of N. sativa. This condition causes an increase in the hormone testosterone and LH.

Regarding oxidation stress, the results of this study indicate that the ethanol extract of N. sativa seeds at all doses can reduce the level of oxidative stress. The results show an increase in SOD enzymatic antioxidants' levels, which inhibited the lipid peroxidation process. These antioxidants are evidenced by the decrease in MDA levels in the liver of diabetic experimental animals in line with the increase in SOD levels. These consequences are in line with the inquiry of Hasan et al. (2018) which informed that the infusion of N. sativa seeds equivalent to 100 mg/kg body weight/day in rats for three consecutive days instigated a reduction in oxidative stress, increasing SOD and GSH-Px in erythrocytes and decreasing MDA levels in serum. MDA reduction due to the content of 3 significant components in N. sativa oil can work as antioxidants, namely thymoquinone 43.46%, p-cymene about

| Abnormality (%) | 11.00 ± 1.83a | 21.25 ± 2.22a | 18.00 ± 1.63b | 14.25 ± 1.71b | 19.00 ± 2.16b | 16.25 ± 1.50bc | P <0.05 |
|----------------|---------------|---------------|---------------|---------------|---------------|---------------|---------|
| Motility (%)   | 61.53 ± 2.83c | 42.36 ± 6.05c | 53.65 ± 3.46b | 57.95 ± 5.16b | 51.78 ± 3.55b | 54.10 ± 1.19b | P <0.05 |

Liver

| SOD (U/ml)    | 7.30 ± 0.62b | 3.57 ± 0.82a | 6.21 ± 0.80b | 6.69 ± 1.48b | 6.03 ± 1.46b | 7.12 ± 1.26b | P <0.05 |
|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------|
| MDA (ng/ml)   | 1266.50 ± 30.97b | 1389.63 ± 71.49b | 840.25 ± 321.23a | 773.38 ± 274.75a | 846.50 ± 326.42a | 832.75 ± 313.02a | P <0.05 |

Note: Normal: normal rats, non-diabetic dyslipidemia; N-0: diabetic rats without extract and medicine; Ns-24: Diabetic rats treated at extracts dose of 24 mg/kgbw; Ns-48: diabetic rats treated at extracts dose of 48 mg/kgbw; Ns-72: diabetic rats treated at dose extract of 72 mg/kgbw; Metf: diabetic rats treated at metformin dose 3.6 mg/kgbw; the means followed by the different alphabets in the same line shows the significant differences in DMRT test with a = 0.05; ǂ significance in Kruskal-Wallis; ǂ means which are followed by the different alphabet in the same line indicating the significant difference in the Mann-Whitney test.
12.79%, and carvacrol (8.53%). Through the DPPH test compared to quercetin, carvacrol has adequate antioxidant activity (Burits & Bucar, 2000).

The results of this study also displayed a decrease in morphological abnormalities of spermatozoa in DM-2 rats. The conduct of giving ethanol extract of seeds N. sativa dose 48 mg/kg bw in DM-2 rats best repressed the occurrence of sperm morphological abnormalities. This upshot is in line with the results of the study (Bashandy, 2007; Marbart et al., 2013). This diminution in sperm morphological abnormalities caused by the enhancement of the reproductive status of DM-2 rats with N. sativa extract as an antioxidant. These data supported by Assi et al. (2016) reported that N. sativa decreased spermatozoa abnormality caused by free radicals.

CONCLUSION

The administration of 80% ethanol extract of Indonesia N. sativa seeds has an effect of plummeting oxidative stress to enable the process of spermatogenesis to run well. The accomplishment of spermatogenesis is shown by augmented concentration and decreased sperm abnormality, as well as increased sperm viability to surge sperm motility. For the clinical study using N. sativa seed herbs to improve diabetes infertility can use the equivalent of an effective extract dose of 48 mg/kg bw. Conversely, for the improvement of standardized herbal medicines, clinical trials with adequate trials are still required.

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