

## Original Research Article

# Effect of thymoquinone: the extract of *nigella sativa* in accelerating soft callus formation in fracture

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## ABSTRACT

**Background:** Excessive oxidative stress on fracture case can inhibit fracture healing and decrease time of bone healing. Thymoquinone, an active substance of *Nigella sativa*, the so-called black cummin in common, is a potent antioxidant and have been studied as an antiosteoporotic agent. Thymoquinone is expected to be the adjuvant alternative that enhance the recovery process of fracture cases by reducing oxidative stress and promotes osteoblast proliferation on callus formation.

**Methods:** Among 32 male mice Wistar Strain divided into 2 groups, conducted tibia fracture and casted. Group 1 was the control group without supplementation of *Nigella sativa* while black-cummin extract were given in group 2 orally at a dose of 800 mg/kg for 14 days. On the 14th day, group 1 and 2 were sacrificed, each bone tissue was taken to measure the levels of MDA by utilizing TBARS method and calculate the number of osteoblasts under the microscope. Data analysis were done using independent t-test.

**Results:** There are both decreased MDA levels and increased number of osteoblasts that are histologically significant to the groups administered by *Nigella sativa* extract containing Thymoquinone compared to the control groups ( $p < 0.05$ ) on day 14.

**Conclusions:** The administration of Thymoquinone from the extract of *Nigella sativa* reduced oxidative stress in fractures as well as increase the number of the osteoblast and its differentiation in callus formation.

**Keywords:** Malondialdehyde, *Nigella sativa* extract, Osteoblast proliferation, Oxidative stress, Thymoquinone

## INTRODUCTION

Fractures are part of daily occurrence in orthopedic field. Economic impact in the United States of this musculoskeletal condition reaches 127.4 billion USD.<sup>1</sup> Advance in the field of healing is highly required to provide variants of therapeutic alternatives and also better outcomes of patients.<sup>2</sup>

Healing process in fracture is a complex case.<sup>2,3</sup> Healing process starts when there is an injury causing fracture and destruction to soft tissues and vasculatures surrounding the fractured area, resulting in tissue damage. This damage induces the increase of oxidative stress in tissues because of the existence of lipid peroxidase, marked by the increase of Malondialdehyde.<sup>4</sup>

*Nigella sativa* is a herbal plant classified into family of Ranunculaceae. This commodity known to be black cummin or habatus sauda has been proven to beneficially act multi systemically as antidiabetic, anticonvulsant, anti-nociceptive, and also antioxidant.<sup>5</sup> Thymoquinone has the highest concentration in this *Nigella sativa* with the levels of 30-48% from total substances.<sup>5-7</sup>

Researches about benefits of Thymoquinone in medical practice has been widely accomplished, one of them is from Saril in 2014 about wound healing properties of this particular substance.<sup>8</sup> In dentistry, Thymoquinone is acquired in mouth ulcers treatment with acceptable results<sup>9-11</sup> in the field of oncology, Thymoquinone also has the role as preventive agent for cancers.<sup>12,13</sup> This substance also operates as potent antioxidant by digesting free radicals such as superoxide, and stimulates osteoblast differentiation simultaneously with BMP-2 activation.<sup>14</sup> However, effects of Thymoquinone as potent antioxidant has no widespread evidence, especially in traumatic fracture cases. This research was held to analyze the effects of *Nigella sativa* supplementation in the form of Thymoquinone substance in the healing process of fractures through observation of MDA biomarker and TBARS (Thiobarbituric Acid Reactive Substance assay) and also the increase of osteoblast through histopathological examination of white rat's tibia (*Rattus norvegicus*).<sup>4,5</sup>

## METHODS

### *Experimental animals*

The inclusion criteria were a healthy male white rat, 3-4 months old, 180-200 grams in weight. The exclusion criteria were deformity in extremity, infection, dead before experiment and broken cast while sample being taken. The study for observation of the experimental animal was carried for a period of 14 days. The study design was a randomized control trial posttest design only. 32 male white rats from Wistar strain (*Rattus norvegicus*) were divided into two treatment groups and tibial fractures with cast fixation were accomplished upon them. Group 1 was the control group without *Nigella sativa* extract administration, whereas Group 2 is the treatment group with administration of *Nigella sativa* extract with the doses of 800 mg/kgBW in 14 days.

### *Nigella sativa extraction*

*Nigella sativa* seed was pulverized into firm powder and added with 95% ethanol to purify them. Liquid extract then was purified with Rotavapor (Rotavapor R-205 Buchi®, Switzerland). The result extract then was processed in air compressor (oven) to gain rough extract in the form of soluble paste which was then added with corn oil.

### *Tibial fractures*

Subjects fasted for 3 hours before surgical process, beginning with anesthesia, accomplished by

administration of ketamine hydroxyde 40mg/kgBW, and then continued by administration of prophylaxis antibiotic Cephazolin 5mg/kg intramuscularly. Field of operation was sterilized with savlon, 70% alcohol, and povidone iodine, covered by sterile surgical covering. Rats were then assessed for being totally under anesthetic substance (closures of eyes, slow movements), and then operation was accomplished with operators wearing sterile gloves and gown and performing aseptic method. Anterior incision was accomplished in cruris shaft regions of the rats, and then the cut was deepened per layers until skeletal levels. Fracture of tibia was then achieved in the 1/3 middle region of the bone with osteotomy using bone cutting forceps. Tibial fracture lines should be exactly the transversal complete fractures.

### *Fixation of fractures with plaster of paris*

Plaster of paris was fixed into tibia of rats classified into Group 1. Area of fractures were then irrigated and closed with wound dressing. Subjects were then treated inside their cages with regular food. Rats also obtained analgesic medication in the form of Paracetamol 100 mg/kgBW if there were signs of pain, such as lethargic activity, difficulty eating, and chills. All rats in Group 1 and Group 2 were sacrificed at the second week after surgery.

### *Histological evaluation*

Sample of lower extremities bones were obtained from knee joint until the levels of ankle joints of rats to provide simpler identification of fractures positions. Bone tissues were then examined in Pathology Anatomy Laboratory, Faculty of Medicine, Brawijaya University to create histopathological samples and osteoblast calculation. Tissues were soaked into 10% formaldehyde and later decalcified with 5% nitrate acid within 1 week of estimated time until the samples were soft and could be modified into smaller cuts. After the cutting process, tissues were dehydrated with alcohol and clearing process was achieved by mixing the dehydrated contents into xylol liquid twice, each period of mixing was estimated to be 30 minutes. Tissues were then embedded into paraffin block and cut into thin pieces in longitudinal directions with the results of 3-5  $\mu$  objects. Samples were then relocated into waterbathagar for further processing and were finally placed into object glassed and labelled. Hematoxylin & Eosin (HE) staining was accomplished to these samples and samples were later covered by cover glass an ready to be observed under microscope.

### *MDA measurement*

After bone samples were taken in the area of fracture, measurement of Malonidialdehyde (MDA) levels was achieved by pulverizing bones and measuring the result into 50-100 mg separate parts, putting bone samples into petri tube and added buffer phospat, 100% TCA, HCl 1 N, 15 Natrium Thiobarbiturate, each 1 mL of substances. Water batch then was heated into 100° Celsius

temperature in 25 minutes and samples were centrifuged with 2000-3000 rpm speed for 15 minutes. After centrifugation was completed, supernatant was taken from samples, added with aqua bides until each sample was of 3 mL volumes, and assessed under spectrophotometry with 532 nm wavelength.

**Statistical analysis**

Statistical analyses were achieved using SPSS version 20. Test for homogeneity of variation was accomplished by Levene’s test to examine residual plot, while normality test was achieved by One-Sample Kolmogorov-Smirnov test. Normality and/or homogeneity of variance assumptions for other variables were not satisfied and prior to statistical analysis these variables were normally scattered for variables of osteoblast and MDA. Therefore, T test could be continued with all assumptions satisfied. Osteoblast dan MDA statistical analysis of data was achieved by using independent T-test. Data are presented as mean±standard deviation formats. The level of significance was set at p <0.05. The relationship between the amount of fibroblast and capillaries and collagen production was tested with Pearson Correlation.

**RESULTS**

According to analytical descriptive results from Table 1, it can be concluded that Osteoblast and MDA can be assessed as follows:

- Osteoblast of Thymoquinone with 16 sampel has the estimated values of 16,625 with 3.074 standard deviation. Highest value of Osteoblast Thymoquinone is 22 and lowest value is 11.

- MDA of control with 16 samples has the estimated values of 0,1255with 0.024 standard deviation. Highest MDA control value is 0.1663 and lowest value is 0.09.
- MDA of Thymoquinone with 16 samples has estimated values of 0,0549 with 0.0066 standard deviation. Highest value of MDA Thymoquinone is 0,0648 and lowest value is 0.0445.

Independent t-test calculation is done by using SPSS software version 20.00 for osteoblast variable and show the result in Table 2.

Table 2 show sig. t is 0,000 (<α=5%), which means H0 is refused and H1 is accepted. Then, it can be concluded that there is significance difference which osteoblast count in treatment group is higher compare to control group

Independent T test calculation is done by using SPSS software ver 20.00 for MDA variable and show the result in Table 3.

Table 3 show sig. t is 0,000 (<α=5%), which also means H0 is refused and H1 is accepted. Then, it can be concluded that there is significance difference between control and treatment group which MDA value in control group is higher compare to treatment group From the results in Table 4, it can be observed that the coefficient correlation values are negative (-0.813) which means X variables (decrease of MDA levels increases Y variables, osteoblast) will increase. This value shows there is extremely strong correlation between MDA levels and osteoblast amount. Correlation of MDA levels variables with osteoblast amount has significant correlation with p-value 0,000 (<0.05) (5%).

**Table 1: Descriptive analysis.**

Variables	N	Minimum levels	Maximum levels	Mean	Standard deviation
Osteoblast control	16	2.000	9.0000	4.687500	2.0238165
MDA control	16	0.921	0.125506	0.125506	0.0241181
Osteoblast thymoquinone	16	11.0000	22.0000	16.625000	3.0748052
MDA thymoquinone	16	0.0445	0.0652	0.054931	0.0066055

**Table 2: Osteoblast T test.**

Osteoblast	Uji homogenitas		T	df	Sig. t	Mean difference
	F	Sig				
Equal variances assumed	3.854	0.059	-12.974	30	0.000	-11.938
Equal variances not assumed			-12.974	25.946	0.000	-11.938

**Table 3. MDA T test.**

MDA	Uji homogenitas		T	df	Sig. t	Mean difference
	F	Sig				
Equal variances assumed	19.520	0.000	11.289	30.000	0.000	0.071
Equal variances not assumed			11.289	17.238	0.000	0.071

**Table 4: Correlation of variables.**

Variable	Spearman correlation	Significance
MDA levels and osteoblast amount	-0,813	0.000

## DISCUSSION

Healing process in fracture cases is a simultaneous, complex biological process. Several methods can be done to shorten healing time of fractures, both invasive procedures, such as mechanical and biophysical approaches, and biological interventions, for example bone grafting method, growth factors, and also natural substances from surrounding environment.<sup>15,16</sup> When traumatic fractures occur, reactive oxygen species (ROS) will increase in amount, causing an inclining number of osteoclast and stimulating bone resorption, which results in decreasing differentiation of osteoblasts. Increase of ROS will create disturbance in regeneration of fractures.<sup>17-19</sup> Data of this fourteen day research exhibits decreasing levels of MDA in groups which receives Thymoquinone of *Nigella sativa* extract by the fourteenth day, compared to control groups (control =  $0.126 \pm 0.024$  ng/100mg; groups with Thymoquinone administration:  $0.055 \pm 0.007$  ng/100mg). T test analysis shows significant decrease of MDA in groups receiving *Nigella sativa* extract compared to control groups (sig. t (0,000)  $< \alpha = 5\%$ ). In control groups, it can also be found that after 14 days of fractures, there is an increase of MDA levels, which marked regeneration process of ischemic injuries, in which oxidative stress resulting tissue injuries is induced at the same time. These results are also supported by other researches by Paskalev in 2011 about the increase of MDA in subjects of study in which fracturing process was accomplished within 12 hours into 2 weeks afterwards.<sup>20</sup> Turgut, et al, (2009) also provided evidence about increasing levels of MDA in rat subjects with fracturing process of seventh and fourteenth day of study.<sup>21</sup> In treatment groups with administration of Thymoquinone from *Nigella sativa* extract, Thymoquinone acts as antioxidant agent and decreases MDA levels, compared to MDA levels of control groups. These phenomena give evidence about the effects of Thymoquinone in *Nigella sativa* extract as potent and effective antioxidant agent in decreasing oxidative stress in traumatic fracture tissues.

Thymoquinone functions as antioxidant agent by reducing lipid peroxidase and increases the activity of antioxidant enzymes, and also neutralizing superoxide anion (O<sub>2</sub><sup>-</sup>), hydroxyl radicals, and single oxygen molecules. Further researches also prove administration of Thymoquinone increases the expression of natural antioxidant genes, such as SOD, catalase enzyme (CAT), and glutathione peroxidase in animal subjects, so that Thymoquinone is also capable in reducing oxidative stress directly through inhibition of endogenous antioxidant induction. In the healing process of fractures, the increase of free radicals can overcome the

natural immunity of host's body and causing disorders in skeletal regeneration process. Antioxidant supplementations of Thymoquinone in *Nigella sativa* extract is beneficial in increasing natural antioxidants of the body, so that the expression of oxidative stress can be suppressed and skeletal healing process can be held without hindrance.<sup>22-24</sup>

Continual data which were used as parameters of study is osteoblast amount, calculated from pathology anatomical samples and observed with microscope. Number of osteoblasts in rat samples with fracturing process of tibial bones exhibit more abundant number of cells in groups with administration of *Nigella sativa* extract compared to the amount of cells in control groups (groups with Thymoquinone administration =  $16.625 \pm 3.074$ , control groups =  $4.688 \pm 2.024$ ). Independent T test was then accomplished, and the results show that there is a significant increase of osteoblasts in groups receiving Thymoquinone compared to control groups (sig. t (0,000)  $< \alpha = 5\%$ ).

These results reveal Thymoquinone activity as potent antioxidant substance which has the effect of osteoblast differentiation. This conclusion is also supported by other studies of Wirris, et al, (2013) which discusses Thymoquinone effects in accelerating in vitro osteoblast differentiation and activation of BMP-2.<sup>14</sup> This research also shows there is correlation between decreasing MDA which is analyzed previously with the amount of osteoblast, which can be observed in decreasing expressions of oxidative stress in healing process of fractures, which is also marked by decreasing MDA levels which stimulates proliferation and differentiation of osteoblasts, so that the amount of osteoblasts will also increase. Correlation analysis of Pearson also shows the extremely strong correlation (r =  $0,813$ ) between decreasing MDA levels and increase amount of osteoblasts (p <  $0,05$ ). Several other studies about osteoporosis also supports the opinion of increasing ROS resulting in bone structural destruction, which is caused by the increase of osteoclast activities and disturbance to osteoblasts' functions, which in turn, reduce the number of osteoblasts from collagen synthesis.<sup>17,20,24</sup>

## CONCLUSIONS

As conclusions, Thymoquinone supplementation from *Nigella sativa* extract is proven to be potent antioxidant in decreasing oxidative stress in the context of tibial fractures. Rats subjects which underwent fracturing process and cast fixation benefits from antioxidant activities of Thymoquinone, which is proven by decreasing amount of osteoblasts in the callus formation process by the fourteenth day of study.

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