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Research Article

The Effect of Thymoquinone Extract of Black Seeds Toward Socket New Blood Vessels Formation Process After Extraction in Diabetic-Induced Rats

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Abstract

Background: Hyperglycemia elevates the advanced glycation end product (AGEs) formation which induced complication after tooth extraction, such as endothelial dysfunction. Thymoquinone (TQ), the active compoud of black seeds (Nigella sativa L.), has been known to stimulates new blood vessels activity also for its anti-diabetic properties.

Objective: To determine the effect of Thymoquinone towards socket new blood vessels formation process (angiogenesis) after extraction on diabetic rats model.

Methods: 27 male rats were injected STZ intravenously and rats with blood glucose level ≥250 mg/dl were divided into three groups (K, P1, P2). Rats were given aquadest (K), Thymoquinone (P1) and Metformin (P1). On 7th day after treatment, its lower first left molar teeth was extracted. 3 samples from each group were euthanasized on 3rd, 7th, and 10th day after axtraction. HE and Immunostaining VEGF was used to observe the new blood vessels formation (angiogenesis).

Results and Conclusion: We found the lowest blood glucose in P1 group on 10th day after extraction. The new blood vessels formation in P1 showed the progressive formation compared to P2 and K group. We conclude that Thymoquinone treatment may improve the socket new blood vessels formation process and prevent the complication.

Keywords: *angiogenesis, diabetes, post extraction socket, thymoquinone*

Introduction

Diabetes mellitus (DM) occurs because the pancreas does not produce enough insulin or body cannot use insulin produced effectively there can be an increase in blood glucose concentration or hyperglycemia. Based on data WHO (World Health Organization), DM prevalence disease around the world is as much as 171 million on year 2000 and will increase twice become 366 million in 2030. The prevalence of DM in Indonesia reached 8.426 million in 2000 which is expected to increase by 2030 as much as 21,257 million people. This means that there has been a threefold increase in 30 years. Based on WHO statistics, from the top 10 countries that have diabetes, Indonesia is ranked 4th in the world.

Hyperglycemia in the long term triggers a chemical reaction called glycation and produce AGEs (advanced glycation end products). Product these AGEs can react with their receptors (RAGEs) or react directly with the body's cell-building proteins. This interaction will change the structure and properties of molecules so cell and network functions are also changing. In addition, the AGEs- RAGEs bond on the surface endothelial cells cause increased ROS (Reactive oxygen species). Increased ROS will be leading to increased iNOS regulation (Inducible nitric oxide synthase) and decrease regulation of eNOS (endothelial nitric oxide synthase) so the biovannance of NO (nitric oxide) decreases and cause endothelial dysfunction. On condition this is done if the extraction action of the tooth can be causing decreased vascularization of the wound post extraction of the tooth thus causing wound healing disorders. It can complicate post-extraction of teeth on people with DM, so that DM disease is now getting a lot of attention in prevention efforts and its management.

Preventive therapy has been done either by using Metformin (oral hypoglycemic medication). Metformin has the advantage of not raising the weight but has side effects on the gastrointestinal tract. In such a situation, the public begins glance at traditional and herbal medicine with utilizing a variety of plants.

One plant that can be used as a medicinal plant is black cumin (Nigella sativa L.). Thymoquinone content in black seeds has a hypoglycemic effect by increasing insulin sensitivity in body tissues and repairing damage to pancreatic β cells thus increasing insulin secretion. In addition, Thymoquinone also plays a role in accelerating the formation of new blood vessels (angiogenesis) in the wound healing process through increased protease activity and endothelial cell migration.

Research Method

27 rats were adapted for 1 week, fed and drank by ad-libitum. Before induced Streptozotocin (STZ) rats were fasted for 12 hours and continued to be fed with water. Prior to injection of STZ, blood glucose values were measured using glucometer by injuring the tip of the rat tail.

Diabetic induction was performed by injection of STZ doses of 50 mg / kg BW of rat performed intravenously in rat tails. The STZ

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powder was dissolved in 0.2 ml of a 0.1 M cittrate buffer solution pH 4.5. After 30 minutes, the rats were again fed with food and glucose solution.

On the 1st day (D-1), post-induction measurements of BGL values were performed. Rats with randomized BGL values ≥250 mg / dL were categorized positive DM and divided into groups of K, P1 and P2. Each group consisted of 9 rats subdivided into 3 subgroups based on observation days, i.e., the 3rd, 7th and 10th days of post-extraction (subgroups D-3, D-7 and D-10). Group K was given 1.5 ml of distilled water for 1x1/day [1-4]. The P1 group was treated with Thymoquinone extract (dose 80 mg / kg BW of rat) dissolved in 1.5 ml of olive oil for 1x1 / day. Group P2 was treated with metformin (dose of 100 mg/kg BW of rat) dissolved in 1.5 ml of distilled water for 3x1 / day [5-8]. All three solutions are given to the sample intragastrically. The treatment was started on day 1 after the mouse was positive DM. On the 7th day (D-7) post-treatment, the lower left molar molars of the mouse were extracted. Prior to dental extraction, a BGL value was measured.

The rats were anesthetized with 0.1 ml / 100 g BW with intramuscular ketamine. The rat is then placed on the rat dental chair and extraction of the left lower left molar is performed with a clamp, semicircle and excavator artery [9-12]. After the teeth are taken, the tooth socket is coated with a lowspeed diamond round bur contraangle number 1 with a speed of 5000 rpm for 2 seconds.

After extraction, treatment was continued until the mice were euthanized on the 3rd, 7th and 10th days of post-extraction (according to the subgroup observation day). Before rats were euthanized with ether inhalation method, BGL measurement was performed. The left lower jaw of the mouse was removed and fixed in 10% formalin for 24 hours. Subsequently the sample was dececcified in 10% formic acid for 7-10 days. Tissues were processed by paraffin embedding method. The tissue is cut vertically with a microtome blade with a thickness of 4 micrometers to produce apico-coronal socket pieces of the mesials [13-15]. Preparations are placed on a warmer slide for 24 hours and stained with Hematoxylin & Eosin (HE) and Immunohistochemical (IHC) staining. The preparations were observed with an optical microscope with magnification of 40x and 1000x on a 1/3 apical socket area of 20 square meters. The process of formation of new blood vessels is observed through the number of endothelial and monocyte cells that express the vascular endothelial growth factor (VEGF).

Result

The results of this study were random blood glucose level (BGL). The results showed that the value of BGL after treatment was decreased in the Thymoquinone (P1) group on the 10th day post extraction.

The randomized BGL measurements showed the highest average values found in the P2 group on the 10th day of post extraction. While the mean value of randomized BGL group P1 on the 10th day post extraction decreased and reached the lowest point (Table 1).

The results are also available in HPA images. The observed expression of postoperative VEGF socket extraction can be seen in Figures 1, 2 and 3.

Figures 1, 2 and 3 show a positive reaction of VEGF endothelial and monocyte cell expression on day 3, 7 and 10 post extraction with IHC staining. Furthermore, this positive reaction will be calculated and summed up. The results of VEGF post extraction socket expression calculations can be seen in Table 1 and Figure 4.

Table 1: Results of randomized BGL measurements on day 3, 7 and 10 post extraction.

Group	D-3 (X±SD)	D-7 (X±SD)	D-10 (X±SD)
Control	467±119	425±35	538±55
Treatment 1	512±48	521±10	343±9
Treatment 2	560±57	465±117	575±43

X = mean of BGL (mg/dL)
SD = standart deviation

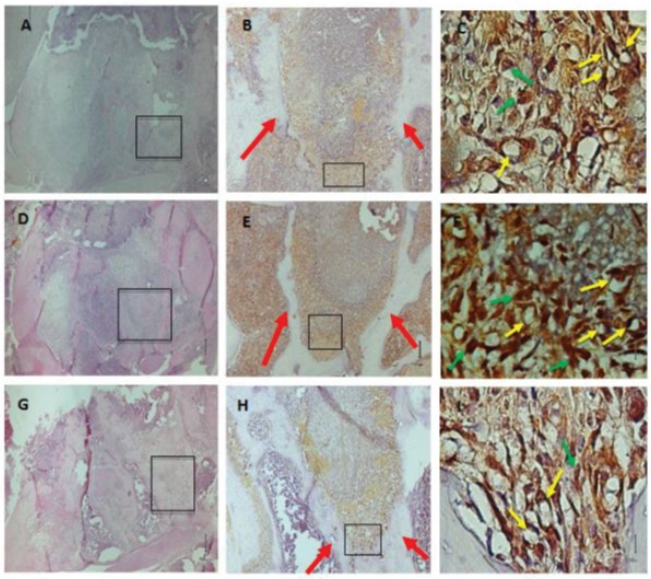

Figure 1: Histologic features of post-extraction 3-day socket gear with HE staining (left panel, 40x magnification) and IHC staining (center panel, 100x magnification and right panel, 1000x magnification). Group K (A-C); Group P1 (D-F); Group P2 (G-I). The arrow (↑) indicatesthe alveolar bone of the tooth socket. The arrow (↑) indicates a new blood vessel. The arrow (↑) is the cell that expresses VEGF. The black box is the area to be enlarged.

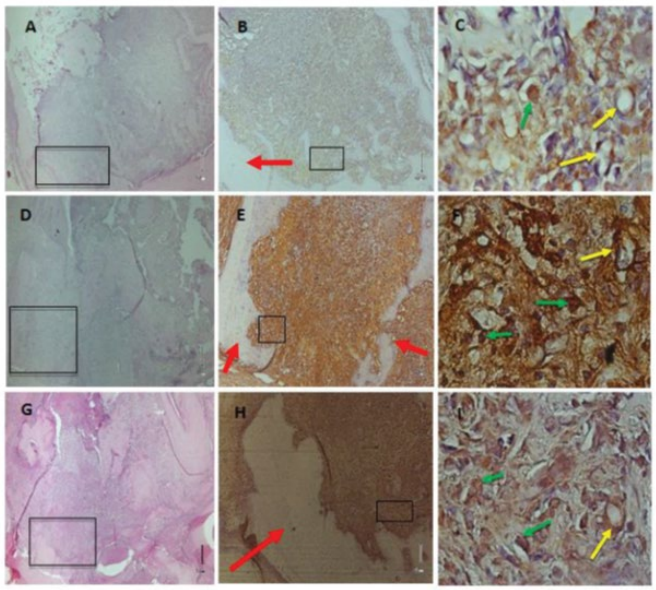

Figure 2: Histologic features of post-extraction 7-day socket gear with HE staining (left panel, 40x magnification) and IHC staining (center panel, 100x magnification and right panel, 1000x magnification). Group K (A-C); Group P1 (D-F); Group P2 (G-I). The arrow (↑) indicates the alveolar bone of the tooth socket. The arrow (↑) indicates a new blood vessel. The arrow (↑) is the cell that expresses VEGF. The black box is the area to be enlarged.

Table 2. shows the average number of VEGF post-extraction sockets most days 3rd extraction in the P2 group compared to P1 and K groups. While on the 7th and 10th days the average number of VEGF expression post extraction was highest on Group P1 with Thymoquinone extract compared with group K or group P2. The results can be seen in Figure.

Figure 4. shows the average number of VEGF post-extraction socket expression steadily increasing from day 3 to day 7 but

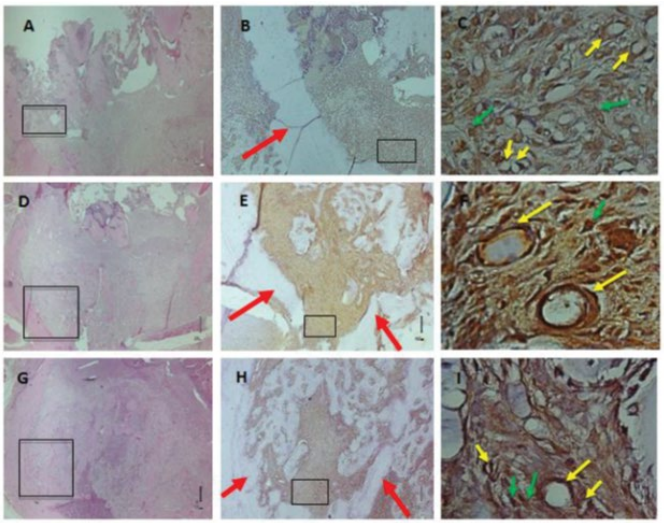

Figure 3: Histologic features of post-extraction 10-day socket gear with HE staining (left panel, 40x magnification) and IHC staining (center panel, 100x magnification and right panel, 1000x magnification). Group K (A-C); Group P1 (D-F); Group P2 (G-I). The arrow (↑) indicates the alveolar bone of the tooth socket. The arrow (↑) indicates a new blood vessel. The arrow (↑) is the cell that expresses VEGF. The black box is the area to be enlarged.

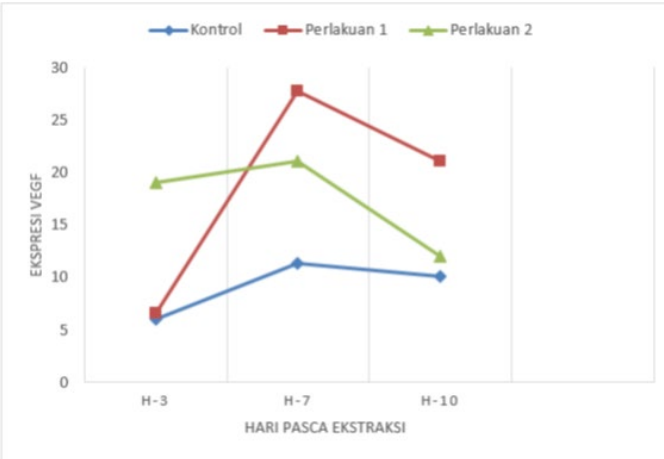

Figure 4: The average graph of the number of VEGF expression of groups K, P1, and P2 on the 3rd, 7th and 10th days showed an increase in the number of VEGF expression on the 3rd day to the 7th day and the decrease on 10th day. A significant increase occurred in the P1 group days 3 to 7 days.

Table 2: Average number of VEGF expressions of tooth socket post extraction group K, P1 and P2.

Group	D-3 (X±SD)	D-7 (X±SD)	D-10 (X±SD)
Control	6±2,06	11±3,24	10±4,32
Treatment 1	10±6,24	28±9,69	21±8,84
Treatment 2	19±7,56	12±5,06	12±6,20

X = mean of VEGF expressions
SD = standart deviation

decreasing on day 10. This occurs in all groups of groups K, P1 and P2.

Discussion

The formation of new blood vessels (angiogenesis) from the inflammatory process of wounds or lesions plays an important role in the wound healing process [16,17]. Wound healing after dental extraction is not much different from wound healing on other body parts. Healing sockets after dental extraction begins 24 hours after the formation of blood clots and mobilization of inflammatory cells (inflammation) to the wound area. Within the next 2-4 days, fibroblasts and endothelial cells proliferate into the bone marrow space and gradually enter into the blood clot [18]. On the 3rd day it is still the end of the inflammatory phase and the beginning of the proliferation phase and the angiogenesis process in normal wound healing begins. On day 3, neutrophils have largely been replaced by macrophages and the granulation tissue begins to invade the wound area [19]. The existing endothelial cells proliferate to form new capillaries [16]. According Rubin and Reisner (2008) on days 4-8 blood vessels proliferate in the process of healing wounds in humans. On days 10-14 the number of new blood vessels will decrease. This is in accordance with the results obtained in all groups, the number of new blood vessel formation increased from day 3 to day 7 but decreased on day-to-10. The increase in the formation of new blood vessels was seen to be quite drastic in the P1 groups on the 3rd day to the 7th day (Table 2, Figure 4).

According to Saad et al (2015), the condition of hyperglycemia in DM can cause disorders of the blood vessels including endothelial dysfunction that causes decreased circulation of blood vessels. Decreased circulation of blood vessels can lead to hypoxia. In a hypoxic state the endothelial progenitor cell (EPC) performs its vasculogenic function. According to Nababan et al (2007) EPCs are cells that have the ability to differentiate into endothelial cells. EPCs are part of more mature and unipotent stem cells. EPC can improve disease conditions that begin with damage to endothelial cells, both structurally and functionally through neovascularization mechanisms. One EPC source that plays a role in the neovascularization process is the monocyte cells. EPCs indirectly differentiate monocyte cell formation to form endothelial cells, but migrate to perivascular space and secrete proangiogenic cytokines, such as vascular endothelial growth factor (VEGF). The EPC vasculogenic function is mediated by hypoxia-inducible factor-1α (HIF-1α) through the activation of chemokine type 4 receptor (CXCR-4). In addition, HIF-1α also induces stromal cellderived factor-1 (SDF-1) in ischemic endothelial cells. The concentration gradient of SDF-1 in ischemic tissue and its interaction with CXCR-4 receptors can trigger migration and incorporation of EPC in ischemic tissue useful for vascular healing [20]. This is thought to be the cause of the high number of endothelial and monocyte endothelial VEGF expression post-extraction of the tooth in the P2 group of the 3rd day.

According to El Hady et al., (2015) in a hypoxic state, the angiogenic molecule is secreted by tissue around the wound and stimulates the proliferative process as well as the growth of endothelial cells. This process includes four stages of protease production by endothelial cells resulting from the degradation of basal laminae of existing blood vessels, chemotaxis, proliferation, remodeling and differentiation. VEGF is the main component that plays a role in the process. In addition, administration of Thymoquinone in the P1 group is also likely to be the cause of the increasing number of blood vessels on day 7. According to Omran (2014) Thymoquinone plays a role in accelerating the formation of new blood vessels (angiogenesis) in the wound healing process through increased protease activity and endothelial cell migration. Proteases work by destroying damaged tissue around the wound as an endothelial cell migratory pathway, promoting migration and endothelial cell proliferation, and directly forming vascular tubules from endothelial cell populations that are developing.

On the 10th day post extraction all groups experienced a decrease in the number of new blood vessel formation. According to Khullar et al., (2012) on day 10-14 post extraction of the tooth, the blood clot (blood clot) on the tooth socket becomes small. Furthermore, new sinusoids begin to form along the socket wall and new bone trabeculae begin to be observed at the base of the socket. At this stage, angiogenesis begins to be suppressed in number. The rate of growth factor decreased followed by decreased inflammatory tissue. Endogenous angiogenesis inhibitors become dominant.

Pericytes that function to stabilize endothelial cells secrete various inhibitors activated by TGF-β that inhibit vascular proliferation. According to William et al., (2003) epidermal interferon-β also inhibits angiogenesis at this stage. Endostatin-splitting results of collagen XVIII appear around the basement membrane and inhibit wound vascularization. Another vasostatin molecule also plays a role in decreasing vascularization of the wound.

The results of the study on the group of DM mice treated with Thymoquinone extract showed a mean of randomized BGL score reached the lowest point on the 17th day after treatment (day 10 post extraction). These results suggest that the longer the administration of Thymoquinone extract, the hypoglycemic effect will be more visible [21]. This is consistent with Fararh et al. (2005) which states that on the 10th day post Thymoquinone administration there is a decrease in BGL and on the 20th day and the 30th day post-administration, BGL decreases. The anti-diabetic effect of Thymoquinone comes from suppressing the production of gluconeogenesis regulating enzymes such as phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase and fructose-1,6biphosphatase by Thymoquinone resulting in decreased hepatic glucose production [22]. In addition, according to Abdelmeguid et al., (2010) states that Thymoquinone can also trigger the regeneration of pancreatic β cells of diabetic rats. The presence of this regeneration is accompanied by levels of antioxidant enzyme superoxide dismutase (SOD) which returns to normal. This enzyme is the first defense against reactive oxygen species (ROS). The SOD enzyme is an antioxidant that works by inactivating free radicals (superoxide, hydrogen peroxide) by converting them into water and oxygen molecules [23]. The presence of regenerative effects of pancreatic β cells is also thought to play a role in the decrease in P1 group 10 days post extraction.

The results of BGL measurements in the metformin-treated group of diabetic mice (P2) showed that the mean BGL was elevated in the P2 group on day 10. The increase in the value of BGL is not in accordance with the research of Cheng et al. (2006) who stated that giving metformin decreased BGL in diabetic rats after giving metformin (100 mg / kg BW rat) 3 times for 3 days. Metformin is an oral hypoglycemic drug (OHD) of the biguanid group that works by suppressing gluconeogenesis. Metformin works by lowering mRNAs that encode the PEPCK enzyme that initiates gluconeogenesis by catalyzing the decarboxylation and phosphorylation reactions to convert the okasaloacetate to phosphoenolpyruvate [24,25]. This condition will inhibit gluconeogenesis so that BGL increases postadministration of metformin.

In this study rats were fed and drank liberally / ad libitum because in DM patients, metformin intake was performed simultaneously with mealtimes to prevent gastrointestinal side effects such as nausea and nausea [26]. The presence of food may in fact decrease the biodaibility of metformin, which is indicated by an average peak concentration (Cmax) of 40% lower, area under the lower curve (AUC), and takes 35 minutes longer to reach Cmax (Tmax) [27]. This condition shows a decrease in the concentration and the amount of metformin in the blood so that the suspected effect decreases and causes an increase in BGL.

Results of the study of aquadest treated group of diabetic rats showed a lower mean value of BGL on day 10 and 14 after treatment than the mean BGL on 7th day after treatment [28,30]. The presence of a decrease in BGL in this group is thought to be due to a different body response and adaptation mechanism in the experimental animals.

According to Wilson and LeDoux (1989), STZ works by forming highly reactive free radicals that can cause damage to cell membranes, proteins and DNA, causing a disruption of insulin production by the Langerhans pancreatic beta cells. Sulistyowati et al., (2013) states the destruction of pancreatic beta cells by partial STZ induction so that insulin can still be produced and can lower blood sugar levels.

Conclusion

Based on this study, it can be concluded that giving Thymoquinone extract can effectively increase the process of formation of new blood vessels (angiogenesis) socket post extraction of tooth in diabetic mouse model and effectively decrease BGL diabetic rat. Further researches with a longer time until the wound healing process is completed is needed to analyze the effect of giving Thymoquinone on the speed of wound healing in diabetic conditions.

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