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The Effect of Black Cumin Oil (*Nigella Sativa Oil*) Supplementation to Prevent Antiproliferative Action of Temozolamide on Hippocampal Neuronal Stem Cells

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Abstract

Hippocampus plays a pivotal role in learning and memory formation. Subgranular zone of the dentate gyrus in the hippocampus is the residence of neuronal progenitor cells (NPCs) that can proliferate and differentiate into newborn mature neurons in adulthood. This process is called adult hippocampal neurogenesis. Medications such as Temozolamide (TMZ) has been shown to give negative impact on neurogenesis. The aim of this study was to observe the effect of *Nigella sativa* oil (NSO) on NPCs proliferation after TMZ treatment.

This was an experimental study with cross sectional design. Female adult mice (n=20) were housed in 4 groups : control (aquadest per oral (p.o) + NaCl 0,9 % intra peritoneal (i.p)); P0 (aquadest p.o + TMZ i.p); P1 (TMZ i.p + MJH 0,1 ml p.o) ; P2 (TMZ i.p + MJH 0,2 ml p.o.). TMZ was administered at week-2 until week-5, 3 times/week (3 consecutive days). Meanwhile, NSO was administered on week-1 until week-9. On week-6, Brdu was administered i.p to label the proliferating cells in the subgranular zone at that time point. At the end of experiment (week-9) the brains were prepared and stained with 3-3' diaminobenzidine (DAB) to observe number of BrdU-positive cells. Ratio of BrdU cells in each dentate gyrus area was used as parameter.

We found significant decreased in Brdu-positive cells density in TMZ-treated group compared to control, including the groups with NSO treatment. This study showed that TMZ has negative impact on neuronal precursor cells proliferation that cannot be overcome by NSO treatment.

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1. Introduction

Hippocampus is a brain structure that plays a role in learning and memory, especially memory of location and space (spatial memory). This structure is highly susceptible to degenerative processes, aging processes and oxidative stress that can lead to decreased rate of neurogenesis and even cell damage to mature neurons. Toxic substances such as alcohol and drugs can also cause disruption of the neurogenesis process [1].

Temozolamide (TMZ) is a drug that can reduce neurogenesis. TMZ is used in chemotherapy of glioblastoma ([2], [3]). Decline in cognitive function is commonly reported as one of side effects of chemotherapy using TMZ[4]. A study in mice showed decrease in the number of stem cell proliferation and the formation of new neurons in the dentatus gyrate after four cycles of temozolomid chemotherapy [5].

Black cumin is has been recognized as medicinal plants since ancient healing practices. It has been used as universal remedy and as the primary agent of health care, therefore is known as all-in-one therapy [6]. Black cumin plant (*Nigella sativa* L.) is also known as habbah al-sauda, habbat al barakah [7], [8].

A growing number of studies has shown the effect of black cumin as an antioxidant [9], antiinflammatory [10], anticancer [11], antitumor [12], and supporting memory [13]. However, the effect of nigella sativa oil (NSO) in ameliorating negative effects of Temozolamide on hippocampal progenitor cells has never been reported.

2. Materials And Methods

Twenty healthy female mice, weighing ± 25 g, 9 weeks old were utilized in this study. The mice were obtained from and kept in animal facility, Universitas Sumatera Utara. The protocol of animal study followed animal welfare and ethics standard from The Medical Research Ethics Committee of the Faculty of Medicine Universitas Sumatera Utara with ethical approval number 377/TGL/KEPK FK USU-RSUP HAM/2017). Prior to experiment, mice were acclimatized for 1 week in 12 hours light/dark cycle and free access to food and water. The mice were housed in 4 groups of 5 mice each: control (aquadest p.o and NaCl 0,9 % i.p); P0 (aquadest p.o and TMZ i.p); P1 (TMZ i.p and NSO 0,1 ml p.o) ; P2 (TMZ i.p and NSO 0,2 ml p.o.). NSO is given from week 1 to week 6 while TMZ is given at week 2 to week 5 (3 times/week). At 6 weeks mice a BrdU (Sigma, St. Louis, MO) injection (i.p.) injection is performed to mark cell proliferation levels under TMZ. The mice belonging to the TMZ treatment group were injected with TMZ at a concentration of 25 mg kg – 1 (intraperitoneal injection, 2.5 mg ml – 1 in 0.9% NaCl) while controls were injected with the 0.9% NaCl vehicle. The animals were injected on 3 consecutive days every week for a total of 4 weeks (Fig.1).

At the end of experiment, mice were killed with high dose Ketamin/Xylazine and perfused transcardially with NaCl 0,9 % followed by 10 % buffered formalin solution. Brains were removed by opening the skull and fixed in 10% buffered formalin solution for at least 72 hours in 4°C and then immersed in 30% sucrose before cryosection [14]. Brains were cut into 40 μ m coronal sections in -20°C using cryostat Leica CM1520 (Leica, Buffalo Grove, IL, USA). Serial sections of the brains were stored at 4°C in cryoprotectant solution and BrdU labelling as proliferation marker was stained with immunohistochemistry methods using Mouse/Rabbit PolyVue PlusTMHRP/DAB Detection System (Diagnostic BioSystem, Cat.No.:PVP1000D) .

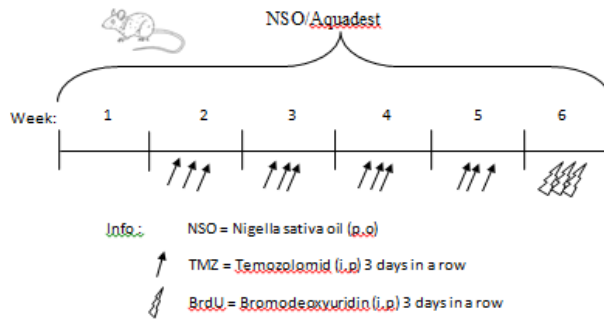


Fig. 1. Experiment Scheme.

For BrdU immunostaining, the sections were incubated 2 M HCl solution for 30 minutes in 37° C. After washing steps with PBS, the tissues were treated with hydrogen peroxide containing solution for 15 minutes in room temperature. Blocking solution containing serum and TritonX 2 % were applied before incubation with clone BU-33 anti BrdU primary antibody (Sigma, Cat. No. B8434). After 12 hours incubation with primary antibody in 4° C followed by extensive washing with PBS, the sections were immersed in secondary antibody Poly Plus Mouse/Rabbit HRP Label for 2 hours in room temperature followed by DAB Plus chromogen for 5 minutes at room temperature. The sections were mounted in Aurora Ultraplus coated slide and dehydrated in xylene before covered with glass coverslip

A cell was counted as being in the subgranular zone (SGZ) of the dentate gyrus if it was touching or in the SGZ. Cells that were located more than two cells away from the SGZ were classified as hilar. The area to be counted was first identified by 200x magnification under the microscope and counting was performed at 400x magnification. Images of each slide were taken at 50x magnification using StereoInvestigator software. All images were taken using the same brightness and contrast settings. Cell density measurements were performed with ImageJ [16].

Statistical Analysis, the results are presented as mean \pm standard error of the mean SEM. The data obtained is the number of positive cell BrdU per mm² area of dentate gyrus. The statistical test was done, from the test result it was found that the data did not find distributed equality then the non-parametric comparison test was done by Kruskal-Wallis. The p value <0.05 is determined as a reference to determine the level of statistical significance.

3. Results and Discussion

To observe the effect of NSO on the proliferation of hippocampal stem cells during exposure to TMZ, the animals were administered BrdU ip a week after the last injection of TMZ to label cells currently in the proliferative phase at the time ([3], [17]). The number of BrdU-positive cells in the subgranular and granular areas of the dentate gyrus was divided by the area of the observed dentate gyrus to compare the stem cell density (stem cell number / mm²) of each group (Fig. 2)

TMZ treatment 25mg/KgBB can inhibit cell proliferation in DG hippocampus. Her research on adult mice showed as much as 90% decreased stem cell proliferation and the formation of new neurons in the hippocampus dentatus after four temozolomid cycles [5]. This decrease in neurogenesis affects [18]. The TMZ study in the adult male Sprague-Dawley rat caused the decline in new neurons labeled BrdU produced in the hippocampus that impacted memory spatial and learning disorders [19]. There was a significant decrease in the number of stem cells / mm² in the hippocampal dentate gyrus after having exposure with TMZ for 4 weeks compared with the control group. This decrease in stem cell counts was observed in all treatment groups, including the group

given NSO. There was no significant difference in the number of stages of hippocampal stool between the group given TMZ alone (P0) and the group given NSO with a dose of 0.1 ml and 0.2 ml (P1 and P2), and also no significant differences between P1 and P2 groups were shown in Table 1.

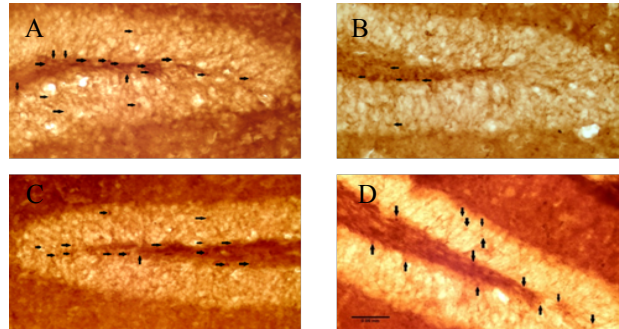


Fig. 2 Microscopic picture of the hippocampus dentatus gyrus (400x magnification) with BrdU marker staining. The arrows show positive cells with BrdU in each group; Control (A); TMZ (B); TMZ + NSO 0.1 ml/KgBB (C) and TMZ + NSO 0.2 ml /KgBB (D)

Table 1. The density of proliferative hippocampal neuronal stem cells (BrdU-positive cells) in dentate gyrus

Group	Numbers of stem cells	Area (mm ²)	Density (cells/mm ²)
Control	19.03 ± 1.65	0.08 ± 0.006	299.9 ± 29.58
P0	15.83 ± 1.48	0.1 ± 0.007	178.7 ± 16.19
P1	14.5 ± 1.09	0.07 ± 0.002	206.9 ± 16.19
P2	12.62 ± 1.31	0.09 ± 0.006	163.8 ± 20.18

These observations showed that there was a decrease in stem cells in DG hippocampus after having exposure with TMZ for 4 weeks compared with control group. There was no significant difference in the number of positive BrdU-positive stem cells in groups given TMZ alone (P0) with the group given NSO with 0.1 ml and 0.2 ml (P1 and P2) doses, and no significant differences were found between the P1 and P2 groups (fig. 3). The decrease in the number of hippocampal stem cells is in line with those reported by Garthe [5].

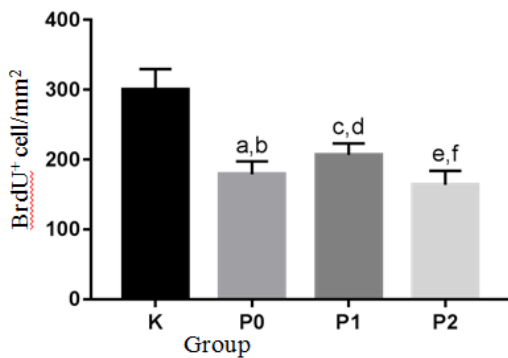


Fig. 3 Change in number of dentatus gyrus stem cells in hippocampus after being given TMZ with BrdU marker (mean value ± SEM; n = 5) a = K vs P0 (p <0.05); b = P0 vs. P1 (p > 0.05); c = K vs P1 (p > 0.05); d = P1 vs P2 (p > 0.05); e = K vs P2 (p <0.05); f = P0 vs P2 (p > 0.05)

One of TMZ's action mechanisms is to increase the production of free radicals (ROS) so as to induce DNA damage and apoptosis [23]. Meanwhile, NSO contains a number of compounds that are antioxidants that can neutralize the free radicals that are formed. According to the researcher that by giving TMZ together with NSO, the TMZ antiproliferative effect can not be solved by NSO. Bararti administration of NSO during treatment with TMZ was not able to overcome the antiproliferative effect of TMZ on hippocampal stem cells.

In this study, it remains to be seen whether the effects of TMZ on stem cells hamper only the proliferation of cells or to cause apoptosis. Labeling of cells with BrdU can only indicate the number of cells currently in the "S" phase of the cell cycle. Cells that are not labeled with BrdU mean being out of phase "S" [20].

TMZ causes the cell cycle to stop at the G2 / M phase [21]. Thus it will certainly decrease the number of cells that will bermitosis to then re-enter the cell cycle. Therefore, it can be explained after giving TMZ will also decrease the number of cells that will enter phase S in the next cell cycle so that the cell proliferation rate decreases.

Research conducted on cancer cells found that phenol compounds can increase the antiproliferative effect of cytotoxic drugs in cancer cells, but not in healthy cells [22]. To prove that NSO does not decrease the proliferation of hippocampal stem cells and whether NSO can help restore cell proliferation rate, Ki67 is examined, a marker of proliferation, at the end of the study, 5 weeks after TMZ administration.

From the literature is known that one of TMZ's mechanism of action is to cause the lesion in DNA that is due to the occurrence of DNA alkylation in the position of O6 guanine. The repair mechanism of DNA in this code can occur by increasing the expression of the MGMT enzyme that can release the alkyl group [23]. The neuroprotective effect of NSO compounds is thymoquinone against brain damage due to oxidative stress in mouse hippocampus occurs through its antioxidant mechanism. This is indicated by the improvement of morphological damage of neurons, and improvement of antioxidant parameters to normal levels ([24], [25]). The active ingredient of black cumin oil is thymoquinone. Thymoquinone, a major element of essential oils of *Negella sativa* seeds, is reported to have antioxidant properties that can enhance protection and resistance to oxidative stress. In another study showed that *nigella sativa* oil and thymoquinone can repair the morphological damage of neurons in mouse hippocampus due to exposure to toluene [26].

4. Conclusion

Based on data obtained from this research, it is concluded that: The decrease in hippocampus stem cells of mice (antiproliferative effects) due to TMZ can not be solved by NSO. This is evident from the number of positive BrdU stem cells (at week 5) in all groups given TMZ significantly lower than the control group.

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