



# Wound Healing Response to Full-Thickness Skin Defect Sprague Dawley Rats Given Ozonated Black Cumin Oil Viewing from The Profile of Neutrophil and Macrophage Count



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## Keywords:

Full thickness skin defect  
Macrophage  
Ozonated Jinten Oil  
Neutrophil

## ABSTRACT

**Background:** The incidence of wounds keeps increasing and inadequate treatment can cause the wound to become infected. Ozone has antibacterial properties and can help activate pro-inflammatory agents in the wound healing process; one of the methods is oil ozonation, for example with black cumin oil. Topical administration of black cumin plays a role in wound healing because of its antibacterial and antifungal properties, and plays a role in increasing the epitalization process

**Objective:** To determine the effectiveness of applying ozonated black cumin oil (*Nigella sativa*) on the healing process of full-thickness skin defects in Sprague Dawley rats, in terms of the number of macrophage and neutrophil cells.

**Methods:** This research was an experimental study with a post-test only control group design method. Total sample of 48 Sprague Dawley rats was divided into 6 groups. The first group was given gentamicin ointment, the second group was given 0.9% normal saline, the third to the fifth group was given ozone black with doses of ozone respectively, namely 1400 mg/ml, 1800 mg/ml and 2200 mg/ml. Incision for taking the tissue samples was held on the 3rd and 7th day according to the treatment group. The tissue samples were subjected to histopathological examination with HE staining

**Results:** A significant decrease in neutrophil levels occurred at the dose of 1,400mg/ml ozonated black cumin oil on day 7 ( $p < 0.001$ ), dose of 1,800 mg/ml on day 3 ( $p = 0.021$ ) and day 7 ( $p < 0.001$ ), doses of 2,200mg/ml at day 3 ( $p = 0.020$ ) and day 7 ( $p < 0.001$ ) compared with a positive control group (given gentamicin). A significant decrease in macrophage levels occurred at a dose of 1,400mg/ml on day 3 ( $p = 0.037$ ) and 7 ( $p = 0.019$ ), a dose of 1,800 mg/ml on day 3 ( $p < 0.001$ ) and 7 ( $p = 0.020$ ), dose 2,200mg/ml on day 3 ( $p < 0.001$ ) and day 7 ( $p = 0.020$ ).

**Conclusion:** Administration of zonized black cumin oil resulted in a lower number of neutrophils in the tissue than the administration of gentamicin when given for 7 days. However, when given for 3 days, only 2,200 mg/ml of the neutrophil count was found to be lower than the administration of gentamicin

DIMJ, 2022, 3(1), 18-27 DOI: <https://doi.org/10.14710/dimj.v3i1.14955>

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## Article history:

Received 27-06-2022  
Accepted 27-07-2022  
Available online 31-07-2022

## 1. Introduction

Wound is a loss of skin or mucosal continuity caused by trauma. If wound care is not good, the risk of infection will be higher.<sup>1</sup> The incidence of injuries is increasing every year. A 2014 study of acute wounds recorded in hospitals amounted to 17.2 million both outpatients and inpatients.<sup>2,3</sup> Inadequate wound management can cause wounds to become infected by microorganisms and

become chronic wounds. It is estimated that the prevalence of chronic wounds is 2.21 out of 1000 in the general population.<sup>4</sup>

The skin has many functions, including provide a protective function against pathogenic microorganisms, prevent dehydration, and also cosmetic functions. When the skin layer is damaged, the human body has a physiological response to restore the integrity of the skin tissue through wound healing mechanisms. The wound healing process consists of three phases, namely the inflammatory, proliferative and maturation/remodeling

phases which usually take weeks to months to restore tissue integrity and form mature skin.<sup>5</sup>

In all phases of the wound healing cascade, collagen plays an important role, which is produced by fibroblasts and constitutes 25% of the total protein mass. Collagen also forms the structural framework of wound healing. It stimulates the adhesion, chemotaxis, and migration of certain cells such as macrophages and fibroblasts. Since there is less collagen and other components of the extracellular matrix in chronic wounds than in normal tissue or in acute wounds, the application of a collagen-based matrix is believed to modulate the chronic wound environment and contribute to wound healing in various ways.<sup>6</sup>

The wound healing process is also influenced by effective wound care. Wound care plays an important role in the inflammatory phase of wound healing. A good wound care process should keep the wound edges clean, adjacent to the lining along the wound edges. The edges of the wound are marked by redness and slight swelling and disappear in about a week. The skin becomes closed even to become normal and the wound edges are fused.<sup>7</sup>

Ozone (O<sub>3</sub>) is a gas that occurs naturally in the earth's atmosphere, has a specific and strong odor, and is an allotropic form of oxygen. Ozone is a much stronger oxidant than oxygen, so it can oxidize many materials that are inert to oxygen under normal conditions.<sup>8</sup> Ozone is an unstable molecule that can decompose into singlet oxygen which is highly reactive against pathogenic organisms such as viruses, bacteria, and protozoa.<sup>9</sup> Some literature states that currently it is known that ozone can dissolve in plasma or water or serum or physiological saline and produce ROS (radical oxygen species) which can help activate pro-inflammatory agents, thereby accelerating the inflammatory process and wound healing.<sup>10</sup>

There has been no research in the form of clinical trials regarding the safety of using ozone in the treatment of various diseases and conditions that claim to be treated with ozone. The discussion of toxicity generally discusses the toxicity of ozone to the lungs.<sup>11-12</sup> Research on ozonated oil has also begun to be developed.<sup>13</sup> The oil that is most often used as a medium is oil from various types of fruits and vegetables, one of which is black cumin oil.<sup>14-15</sup>

Black cumin is called a blessed seed because it is known for its active substances that play a role in many diseases. A study proves that black seed oil has antibacterial and antifungal properties. Thymoquinone,

flavonoids and triterpenoids in black cumin oil can also increase the speed of wound healing.<sup>15</sup> Black cumin oil can also be given topically to increase the speed of wound regeneration in diabetic rats to accelerate the epithelialization process.<sup>16</sup>

There has not been much research on ozonated black cumin oil in the medical field. The combination of black cumin oil and ozone has a very promising wound healing effect, so the authors are interested in researching more deeply about the effect of ozonation of black cumin oil on the speed of the wound healing process.

## 2. Methods

This study uses an experimental type of research using a posttest only control group design. There were 6 treatment groups with 2 terminations for each treatment group, with 4 Sprague Dawley rats in each group, so the total sample used was 48 research samples. The first group was given gentamicin ointment. The second group was given normal saline 0.9%. The third group was given black cumin oil. The fourth group was given ozonated black cumin oil at a dose of 1400 mg/ml ozone. The fifth group was given ozonated black cumin oil at a dose of 1800 mg/ml ozone. The sixth group was given ozonated black cumin oil at a dose of 2200 mg/ml ozone.

Ozonated cumin oil was obtained from the Plasma Research Laboratory of Diponegoro University. The preparation of ozonated black cumin oil is obtained by dissolving ozone into the oil using a magnetic stirrer and a diffuser which is connected to an ozone generator. The ozone sample of cumin oil was taken and tested to determine the concentration of Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>) and its acidity level.

Making Full-Thickness wounds in experimental animals was carried out on day 1 by providing intra-peritoneal anesthesia using a mixture of Ketamine-Xylazine (ketamine 80 mg/kgBW; xylazine 10 mg/kgBW). Then a wound was made on the back of the rat with a diameter of 1 cm by cutting the skin from the back to the base of the panniculus carnosus. The wound site was gently cleaned of fatty tissue and connective tissue, and then irrigated with 0.9% Normal Saline solution. The wound was wrapped with hansaplast with non-adhesive segments that did not cover the wound.

Wound area measurements were carried out on the 3rd and 7th days. A ruler is placed next to the wound, then photographed using a digital camera. The wound area was

then measured using the ImageJ application (National Institutes of Health, United States).<sup>17</sup>

Tissue sampling was carried out on the 3rd and 7th days. Experimental animals were terminated using ether. The tissue sample is fixed. The tissue was taken by excision on the widest part of the wound by including normal skin tissue. The tissue was then fixed with 10% buffered formalin and made paraffin blocks. HE staining was used to count the number of neutrophils and the number of macrophages.

Data analysis was performed using computerized statistical software. The test used for group comparison in this study was the one-way ANOVA test. The data obtained were tested for normality using the Shapiro-Wilk test first. Subsequently, a one-way ANOVA test was performed, and a significant p value was obtained with a

p value of <0.05. So that the data was carried out post-hoc LSD test to compare between groups.

### 3. Results

Black cumin contains bioactive substances that have many medical effects, from antidiabetic, anti-cancer, antibacterial, to antioxidant effects. These effects are known to accelerate wound healing.<sup>18-25</sup> The active substance in *Nigella sativa*, namely thymoquinone, can reduce the production of ROS and inhibit the COX and 5-LPO pathways of arachidonic acid metabolism, causing a decrease in the rate of inflammation, thereby accelerating wound healing.<sup>16,23,25-27</sup>

Table 1. Average count of Neutrophil Group

Neutrophil Group	Average count	
	3 days interval	7 days interval
Group K+	36,20 ± 3,09	45,00 ± 2,41
Group K-	40,90 ± 7,47	46,80 ± 3,40
Group M	35,60 ± 6,94	37,85 ± 1,16
Group A	33,45 ± 1,45	35,65 ± 1,06
Group B	30,40 ± 1,51	33,10 ± 1,35
Group C	25,85 ± 0,91	30,05 ± 0,70

## Neutrophil count

The difference of neutrophil count between 3 days and 7 days is shown by table 1. Neutrophil count in all groups at 7-days interval is more than neutrophil count at 3-days interval. (Table 1)

### Comparison of the number of neutrophils in the group given gentamicin and ozonated black cumin oil

The administration of gentamicin alone did not differ in the number of neutrophils after treatment for 3 days compared to the administration of black cumin oil alone. However, the number of neutrophils given black cumin oil for 7 days was found to be lower than the administration of gentamicin.

Comparison between rats treated with positive control and ozone dosed black cumin oil 1,400 mg/ml, 1,800 mg/ml, the number of neutrophils in treatment for 3 days did not differ ( $p = 0,393$  ;  $p = 0,082$ ), whereas in treatment for 7 days, the number of neutrophils was lower ( $p < 0,001$ ). In the administration of ozonated black cumin oil at a dose of 2,200 mg/ml ozone, the number of neutrophils was lower than the administration of gentamicin for 3 days ( $p = 0,04$ ) and 7 days ( $p < 0,001$ ).

### Comparison of the number of neutrophils in the group given 0.9% NaCl with ozonated black cumin oil

Comparison between rats with negative control and cumin oil alone, there was no difference in neutrophil levels in the 3 day treatment. In the 7-day treatment, the neutrophil levels in the group given black cumin oil were lower than those given 0.9% NaCl.

In the administration of ozonated black cumin oil doses of ozone 1,400 mg/ml, 1,800 mg/ml, and 2,200 mg/ml, for 3 days ( $p = 0,029$ ;  $p = 0,004$ ;  $p < 0,001$ ) and 7 days ( $p < 0,001$ ), the number of neutrophils was lower than the administration of 0.9% NaCl.

### Comparison of the number of neutrophils in the group that was given black cumin oil alone with ozonated black cumin oil

Comparison between rats treated with black cumin oil only and ozone treated with ozone dose of 1,400 mg/ml, the number of neutrophils in the treatment for 3 days ( $p = 0,503$ ) and 7 days ( $p = 0,123$ ) was not different.

The administration of ozonated black cumin oil at a dose of 1,800 mg/ml of ozone showed that the number of neutrophils in the treatment for 3 days was not different ( $p = 0,116$ ), while in the treatment for 7 days, the number of neutrophils was lower ( $p = 0,003$ ). When giving ozone treated black cumin oil at a dose of 2,200 mg/ml, it was found that the number of neutrophils was lower than the administration of black oil alone for 3 days ( $p = 0,006$ ) and 7 days ( $p < 0,001$ ).

## Macrophage count

The difference of macrophage count between 3 days and 7 days is shown by table 2. Macrophage count in all groups at 7-days interval is more than macrophage count at 3-days interval.

### Comparison of the number of neutrophils in the group given gentamicin and ozonated black cumin oil

The administration of gentamicin alone did not differ in the number of macrophages after treatment for 3 days compared to the administration of black cumin oil alone. However, the number of macrophages in the group given black cumin oil for 7 days, was found to be lower than the group given gentamicin.

The number of macrophages in the group given ozone dosed black cumin oil 1,400 mg/ml, 1,800 mg/ml, 2,200 mg/ml, was found to be lower in the treatment for 3 days ( $p = 0,037$ ;  $p < 0,001$ ;  $p < 0,001$ ) and 7 days ( $p < 0,001$ ;  $p < 0,001$ ;  $p < 0,001$ ) compared to gentamicin.

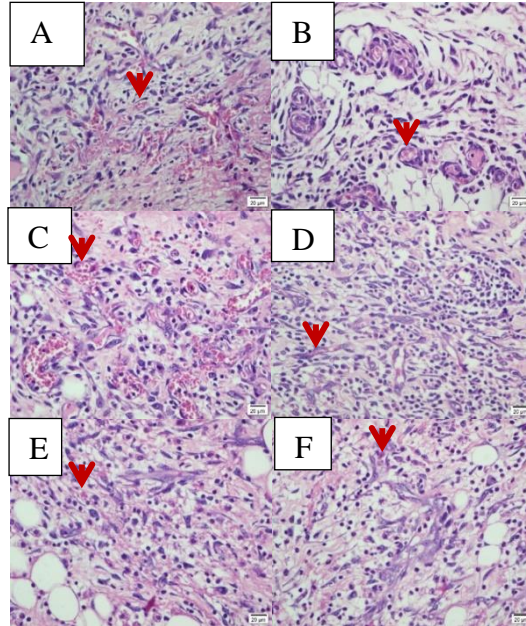


Figure 1. Histopathological features of neutrophil count after 3 days intervention (HE staining, 100x magnification). Red arrows show neutrophil cells in tissue. (A) Group K+3, (B) Group K-3, (C) Group M3, (D) Group A3, (E) Group B3, (F) Group C3

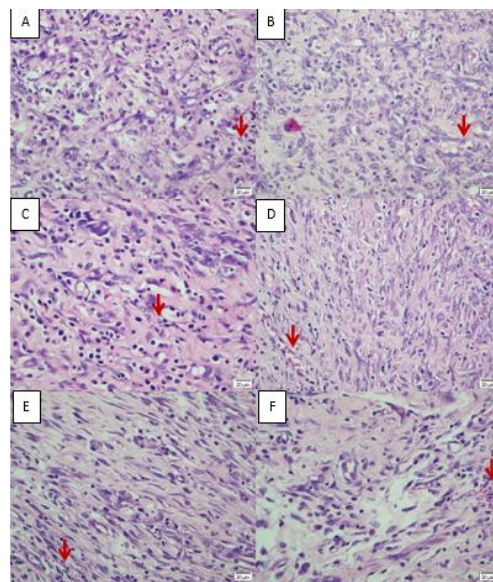


Figure 2. Histopathological features of neutrophil count after 7 days intervention (HE staining, 100x magnification). Red arrows show neutrophil cells in tissue. (A) Group K+7, (B) Group K-7, (C) Group M7, (D) Group A7, (E) Group B7, (F) Group C7

### Comparison of the number of neutrophils in the group given 0.9% NaCl and ozonated black cumin oil

Comparison between the rats treated with negative control and cumin oil alone, it was found that macrophage levels were lower in the 3 days and 7 days treatment compared to 0.9% NaCl administration.

In the administration of ozonated black cumin oil doses of ozone 1,400 mg/ml, 1,800 mg/ml, and 2,200 mg/ml, for 3 days ( $p = 0,001$ ;  $p < 0,001$ ;  $p < 0,001$ ) and 7 days ( $p < 0,001$ ;  $p < 0,001$ ;  $p < 0,001$ ), the number of macrophages was lower than the administration of 0.9% NaCl.

Table 2. Average count of macrophage group

Macrophage Group	Average count	
	3 days interval	7 days interval
Group K+	32,95 ± 2,34	44,90 ± 1,51
Group K-	36,85 ± 1,93	46,80 ± 1,24
Group M	35,85 ± 3,41	37,85 ± 0,93
Group A	31,80 ± 1,75	34,05 ± 0,60
Group B	29,30 ± 1,14	31,55 ± 2,63
Group C	23,90 ± 20,2	25,40 ± 0,52

### Comparison of the number of neutrophils in the group that was given black cumin oil alone with ozonated black cumin oil

Comparison between rats treated with black cumin oil alone and ozone-treated black cumin oil at a dose of 1,400 mg/ml, the number of neutrophils in the treatment for 3 days was not different ( $p = 0,138$ ), whereas in the treatment for 7 days ( $p = 0,001$ ), the number of neutrophils was lower. Administration of zoned black cumin oil at doses of 1,800 mg/ml and 2,200 mg/ml of ozone, the number of neutrophils was lower than the administration of black oil alone for 3 days ( $p < 0,001$ ;  $p < 0,001$ ) and 7 days ( $p < 0,001$ ;  $p < 0,001$ ).

At the time of injury, the tissue under the skin is exposed to an environment with many microbes such as the skin itself. Microorganisms can invade tissues which induce a local immune response and cause inflammation leading to tissue damage and delayed wound healing. One consequence of infection is prolonged inflammation. The use of appropriate antibiotic-dressing can help fight infection and the formation of biofilms, so that the application of antimicrobials to wounds can reduce the duration of inflammation and accelerate wound healing.<sup>28</sup>

In the mean data, the use of topical gentamicin showed lower levels of neutrophils and macrophages than the negative control, although not significantly different. This could be due to the bactericidal effect of gentamicin which reduces inflammation by simply killing bacteria around the wound.<sup>29</sup>

## 4. Discussions

In a study conducted by Hibono (2017), administration of black cumin oil on full-thickness wounds of diabetic rats accelerated wound healing compared to the administration of the antibiotic mupirocin.<sup>16</sup> In all treatments for 7 days, there was a decrease in the levels of neutrophils and macrophages with the administration of black cumin oil. This is related to the active substance content, namely thymoquinone which can inhibit fMLF-induced phosphorylation of ERK 1/2 and p38 MAPK.

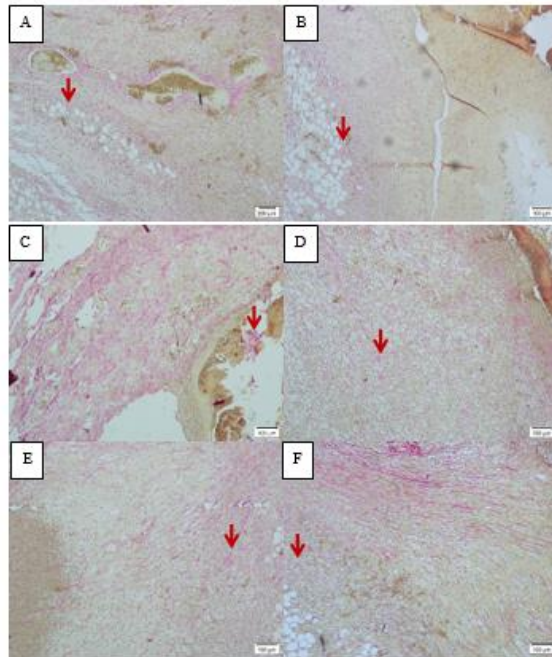


Figure 3. Histopathological features of macrophage count after 3 days intervention (HE staining, 400x magnification). Red arrows show macrophage cells in tissue. (A) Group K+3, (B) Group K-3, (C) Group M3, (D) Group A3, (E) Group B3, (F) Group C3

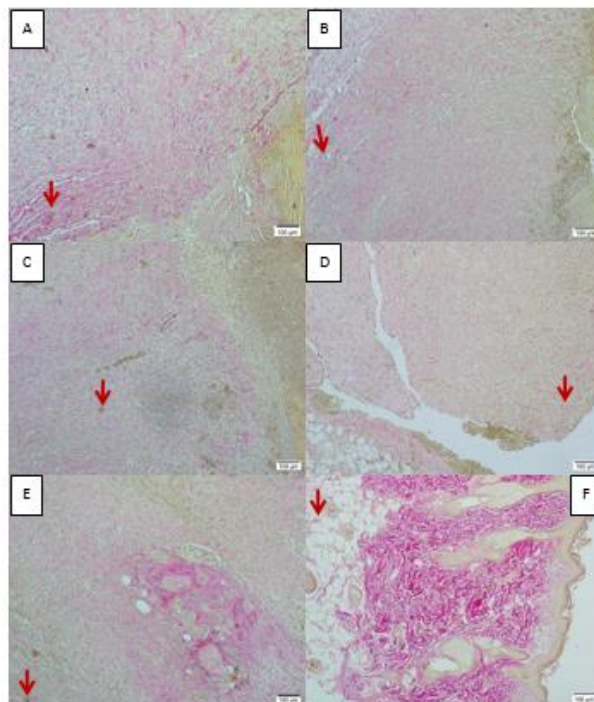


Figure 4. Histopathological features of macrophage count after 7 days intervention (HE staining, 400x magnification). Red arrows show macrophage cells in tissue. (A) Group K+7, (B) Group K-7, (C) Group M7, (D) Group A7, (E) Group B7, (F) Group C7

ERK 1/2 is associated with macrophage development, and macrophage chemoattractant production. A study in mice with myeloid lacking both ERK1/2 by Richardson et al. (2015), showed that these precursor cells could not survive or differentiate into macrophages thereby reducing macrophage production. Research on the glioblastoma model by Lailler et al. (2019), there was an increase in infiltration of tumor-associated macrophages in tumors with an increase in ERK1/2 phosphorylation. In this study it was also found that glioblastomas produced chemoattractants from monocytes, such as CCL2/MCP1 and CSF1/M-CSF, in large quantities and inhibition of ERK1/2 greatly reduced this production. This explains the reduction in macrophage levels in the administration of cumin oil. black which contains the active compound thymoquinone.

P38 MAPK has a function in the regulation of neutrophil degranulation, its inhibition causes reduced ROS activity and neutrophil degranulation thereby reducing tissue damage. In addition, inhibition of MAPK signaling may reduce neutrophil recruitment to tissues.<sup>40</sup>

Administration of ozone at a dose of 2,200 mg/ml in black cumin oil showed significant results in reducing levels of neutrophils and macrophages compared to all other treatments. This indicates that in this study, the administration of ozonated black cumin oil at a dose of 2,200 mg/ml of ozone was optimal in reducing the levels of neutrophils and macrophages. The decrease in the levels of neutrophils and macrophages was greater in the administration of ozone compared to the absence of ozone, possibly due to the increased production of ROS in the tissue. ROS can attack pathogens, reduce pathogens that can trigger inflammation, and stimulate wound healing. This reduces the need for tissues to recruit neutrophils and macrophages.

The results of this study are in line with research by Rizaoglu (2018), the administration of zoned black cumin oil on full-thickness wounds also showed faster wound healing compared to wounds that were not given anything.<sup>15</sup>

Overall, there was an average increase in the number of neutrophils and the number of macrophages on 7 days of treatment when compared to the number of neutrophils and macrophages on 3 days of treatment for each group. This can be related to the proliferative phase which lasts from the first 48 hours to up to 14 days after the formation of the wound

The limitation in this study was that the macrophages found in the tissue after treatment could not be distinguished by subtype (M1/M2). In this study, the length of the wound was not measured, so it could not be linked between the levels of neutrophils and macrophages on wound closure.

## 5. Conclusion

Administration of zoned black cumin oil resulted in a lower number of neutrophils in the tissue than the administration of gentamicin when given for 7 days. However, when given for 3 days, only 2,200 mg/ml of the neutrophil count was found to be lower than the administration of gentamicin. Administration of zoned black cumin oil also caused the number of macrophages in the tissue to be lower than the administration of gentamicin.

## Ethical Approval

All procedures have been approved by the issuance of ethical clearance from the Health Research Ethics Commission of The Faculty of Medicine, Diponegoro University, Semarang.

## Conflicts of Interest

The authors declare that there was no conflict of interest.

## Funding

No specific funding was provided for this article

## Author Contributions

Conceptualization, IRS, MTA, and MN; methodology, IRS; software, IRS; validation, MTA and MN; formal analysis, IRS; investigation, IRS; resources, IRS; data curation, IRS; writing—original draft preparation, IRS; writing—review and editing, IRS, MTA, MN; visualization, IRS; supervision, MTA, MN

## Acknowledgments

Thanks to God Almighty who has given the author the strength to complete this article. Sincere thanks to all those who have helped with this research. The author realizes that this research is still far from perfect, therefore constructive criticism and suggestions are expected for better writing.



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