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The effect of Okra pods (*Abelmoschus esculentus* L.) methanol extract on white blood cell count, Phagocytic activity, and IFN-γ level in *Mus musculus* exposed sodium nitrite

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ABSTRACT

Okra is widely used as a vegetable and known have health benefits because it contains high antioxidants. The purpose of this study was to determine antioxidant potential of okra pods methanol extract (OPM) on immune response (white blood cell count, activity phagocytic, and IFN-γ level) of *Mus musculus* exposed sodium nitrite (NaNO 2). Twenty four male mice (BALB/c strain, 8-10 weeks, 25-30 g body weight were divided into 6 groups: normal control group (exposed distilled water), negative control (exposed NaNO 2 50 mg/kg body weight), and OPM treatment (exposed to $NaNO_2$ and OPM 50, 100, 200 and 400 mg/kg BW simultaneously). The mice was administrated orally both $NaNO_2$ and OPM once a day for 19 days. Finally treatment, the parameter analysis was performed statistically at P = 0.05. The results showed that the administration of OPM could increase white blood cell count (WBCs), phagocytic activity, and IFN-γ level significantly. The optimal dose of OPM is 50 mg/kg BW. The conclusion of this study is that the administration of OPM can increase the immune response (white blood cell count, activity phagocytic, and IFN-γ levels) of *Mus musculus* exposed $NaNO_2$.

Key words: *Okra pods, Sodium nitrite, Blood, IFN-γ level*

Introduction

Sodium nitrite is a chemical formed from the reduction of sodium nitrate used as a food preservation in preventing microbial growth in processed meat (salami, hot dogs, bacon, and corned beef) (Honikel, 2008), cheese and fish preparations (Cvetkovic *et al.,* 2019). In Indonesia, the use of nitrate and nitrite compounds as preservation is regulated in the head regulation of the Food and Drug Supervisory Agency (BPOM) with a maximum sodium nitrite limit of 30 mg/kg for processed or preserved meat and 20 mg/kg for cheese (BPOM, 2013). The use of sodium nitrite that exceeds the threshold can be detrimental to health.

Nitrites are known as a source of the formation of nitric oxide (NO) which plays a role in the immune, physiological and neurogicalcondition (Lundberg *et al.,* 2008). Nitric oxide reacts quickly with oxygen molecules to produce peroxinitrites that are highly reactive and can oxidize DNA, proteins and lipids. This can increase the production of ROS (Reactive Oxygen Species) such as O2-, H_2O_2, NO-, ONOO-, and OH- which can induce oxidative stress (Flora *et*

al., 2012). The main mechanism of nitrite toxicity is known through oxidative stress (Ansari *et al.,* 2017). Tissue damage due to oxidative stress can occur in hematopoeisis tissue, namely bone marrow and thymus. Pregnant mice exposed to sodium nitrite at a dose of 210 mg/kg BW/day for 14 days experienced chromosomal aberration in bone marrow in the mother and chromosomal aberration in the embryonic liver (El-Nahas *et al.,* 1984). Damage to the bone marrow can result in reduced white blood cells that play a role in the immune system (Gluhcheva *et al.,* 2012). Sodium nitrite of 10mM and 20mM can significantly reduce phagocytosis activity against Peseudomonas aeruginosa and Staphyllococcus epidermidis (Vassalo *et al.,* 2013). Sodium nitrite can cause apoptosis in T lymphocytes which results in decreased interleukin-2 and interferon γ secretion by Th1 cells and increased IL-4 secretion by Th2 cells (Abuharfeil *et al.,* 2000). Sodium nitrite consumed at low doses 24-50 mg/kgBB daily for 8 months causes tissue nitrosative damage, decreases the immune system (Alyoussef and Al-Gayyar, 2016), RBCs and WBCs levels (Helal *et al.,* 2008), and Th1 cytokine production (IL-2, IFN-γ, and tumor necrosis factor-β) (Ustyugova *et al.,* 2002).

Okra (*Abelmoschus esculentus* L.) is also known to have high antioxidants (Kumar, 2013). The leaves, flowers, fruits and okra seeds contain phenolic compounds that can act as antioxidants (Liao *et al.,* 2012). Quercetin and routine act as efficient radical inhibitors and have a neuroprotective effect on ischemia and reperfusion-induced brain injury (Cho *et al.,* 2006; Pu *et al.,* 2007). Crude extracts of okra polysaccharides are also known to have immunomodulatory activity (Wahyuningsih *et al.,* 2018). Sheu and Lai's study (2012) showed that administration of 100 μg/mL of okra polysaccharide could significantly increase the secretion of Th-1 cytokines, IL-12 and IFN-γ, significantly in rat bone marrow hematopoeisis cell culture. Vitamin C or ascorbic acid contained in okra has the potential to eliminate ROS which makes it a potential detoxification agent (Das and Saha, 2011; Tariq, 2007). In addition to vitamin C, flavonoid compounds can also prevent or eliminate oxidative stress by breaking the chain of free radical reactions (Terao, 2009; Rice-Evans, 2001).

This study was conducted to determine the effect of okra fruit methanol extract to reduce oxidative effects due to sodium nitrite exposure by knowing the white blood cell count, phagocytic activity and levels of IFN-γ *Mus musculus*.

Materials and Methods

Materials and Chemicals

Okra pods were obtained from traditional markets in Surabaya, Indonesia, in January 2019. *Saccharomyces cereviseae* was purchased from the Microbiology Laboratory, Airlangga University, Surabaya, Indonesia. Interferon-γ enzyme-linked immunosorbent assay (ELISA) kit were obtained from BioLegend (BioLegend, Inc., San Diego, USA). All other chemicals and solvent used were of analytical reagent grade.

Preparation of OPM

Two kg of okra pods were cleaned and cut into small pieces, then air dried for about 7 days, then crushed with a blender to produce a coarse powder. Coarse powder is 196 g and stored in an airtight container. The coarse powder is macerated (immersed) in 750 mL of pure methanol pro analysis for 24 hours (repeated 3 times) and continuously stirred. The results of measurements and collected ± 2250 mL of the concentrate evaporated at 60 ºC in a rotary evaporator to ± 80 mL. The results of the evaporation were carried out freeze-drying to remove the solvent. The result of freeze-dring were collected 52.3 gr OPM powder.

Animals

Adult male mice strain BALB/c, 8-10 weeks, 25-30 gr were provided from the Laboratory Animal, Faculty of Pharmacy, Airlangga University, Surabaya. The ethical test was conducted at the Faculty of Veterinary Medicine of Airlangga University, Surabaya, Indonesia (2.KE.138.06.2019).

Experimental Design

After two weeks of acclimation, mice were divided randomly into six groups (KN: normal control only given distilled water; K-: negative control exposed to sodium nitrite 50 mg/kg bw; P1, P2, P3, and P4 were exposed to sodium nitrite and OPM doses 50, 100, 200, and 400 mg/kg bw. Sodium nitrite and OPM were given oral gavage for 19 days, the 20th day, the mice were sacrificed. Whole blood sample ere obtained in heparinized tube. Serum were collected from centrifuged whole blood at 3000 rpm and 4ºC for 10 min. The peritoneal fluid taken pre-

viously has been infected with *S. cereviseae.*

Measurement of White Blood Cell Counts

Determination of the number of white blood cells was obtained using automated hematology analyzer ABX Micros 60 at the Optima Health Laboratory, Rungkut, Surabaya.

Phagocytic activity

The mice infected by 0.2 mL *S. cereviceae* suspension intraperitoneally. One hour later, mice were injected with 3% EDTA solution of 3 mL. Intraperitoneal fluid was collected as much as 2 mL. Phagocyte observation was carried out by making an intraperitoneal fluid smear with Giemsa staining. Apply fixation with methanol and colored using Giemsa coloring. The colored polishing is reviewed under a 400x magnification microscope. Phagocytic activity can be determined by the percentage of the number of phagocytes that actively carry out phagocytosis of 100 phagocytes.

IFN-γ Level Measurement

IFN- levels were carried out by following the protocol in the IFN-γ ELISA kit (BioLegend, Massachusetts, USA). The absorbance were measuredusig ELISA reader at 450 nm.

Statistical Analysis

The data obtained were analyzed using the Statistical Package for Social Sciences (SPSS) 21.00 for windows software. The data was approved by using the normality test using the Kolmogrov-Smirnov test. Homogeneity test uses the Levene test. Furthermore, one-way analysis of variance (ANOVA) was tested so that the effect of each treatment group could be known. Then, Duncan test was performed with $\alpha = 0.05$.

Result

White Blood Cell Count

White blood cell count increased significantly in the treatment group P1 (11.950 ± 2.883 cells/mm^3), P2 (10.875 ± 2.405 cells/mm^3), P3 (12,637 ± 1,970.35 cells/mm^3), and P4 (11,775 ± 1241.97 cells/mm^3) when compared to the KN group (10,125 ± 1,835.53 cells/mm^3) and K- (6.875 ± 2.467.62 cells/mm^3) ($P < 0.05$) (Fig. 1).

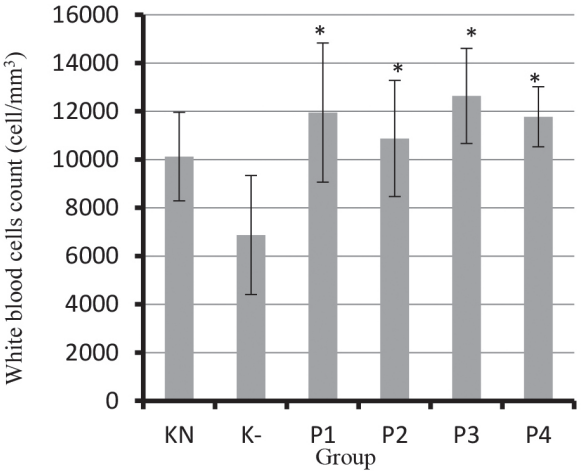

Fig. 1. Effect of OPM on the number of white blood cells induced by sodium nitrite mice between treatment groups (cell/mm^3). KN = normal control; K- = negative control (exposure to sodium nitrite 50 mg / kg BW); OPM treatment groups P1, P2, P3, P4 (dose 50,100, 200,400 mg / kgBB. *) significant differences based on Duncan's test ($\alpha = 0.05$).

Phagocytic activity

Phagocytic activity increased significantly in the treatment groups P1 (50.80 ± 2.63%), P2 (46.87 ± 5.71%), P3 (48.17 ± 3.84%), P4 (48.32 ± 6.69%.) When compared with the KN group (41.93 ± 3.95%) and K- (31.18 ± 2.75%) ($P < 0.05$) (Figure 2).

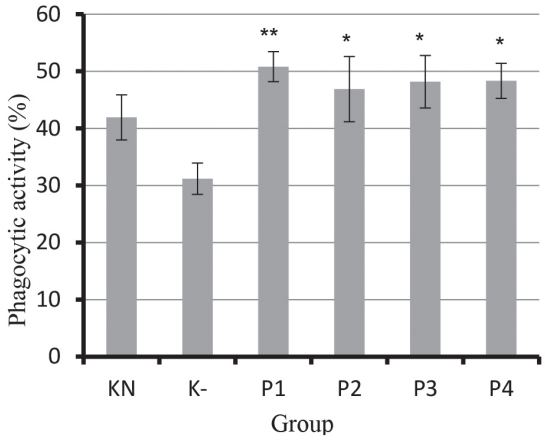

Fig. 2. Effect of OPM on phagocytic activity of mice induced by sodium nitrite (%) between treatment groups. KN = normal control; K- = negative control (exposure to sodium nitrite 50 mg / kg BW); OPM treatment groups P1, P2, P3, P4 (dose 50,100, 200,400 mg / kgBB. *) significant differences with negative controls; **) significant difference with negative control and normal control based on Duncan's test ($\alpha = 0.05$).

IFN-γ levels

IFN-γ levels increased significantly in the treatment group P1 (1 136.75 ± 21.46 pg/mL), P2 (123.63 ± 13.44 pg/mL), P3 (119.88 ± 19.93 pg/mL), and P4 (113.60 ± 23.84 pg / mL) when compared to the KN group (105.50 ± 8.89 pg / mL) and K- (78.63 ± 8.26 pg / mL) ($P < 0.05$) (Figure 4).

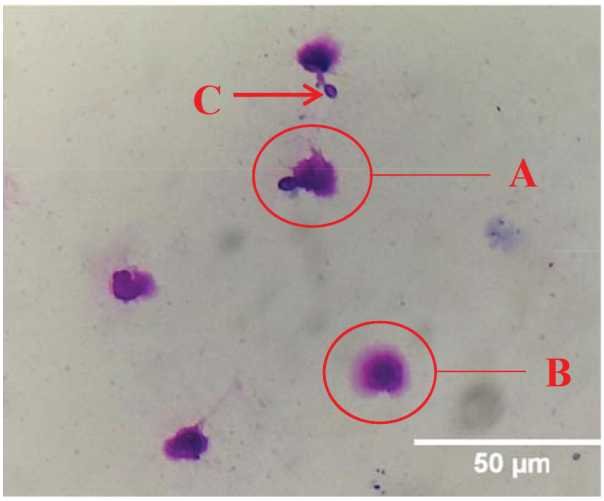

Fig. 3. Difference between active and inactive phagocytes, Giemsa staining, 400x visibility. A: active phagocytes; B: non-active phagocytes; C: Saccharomyces cereviceae.

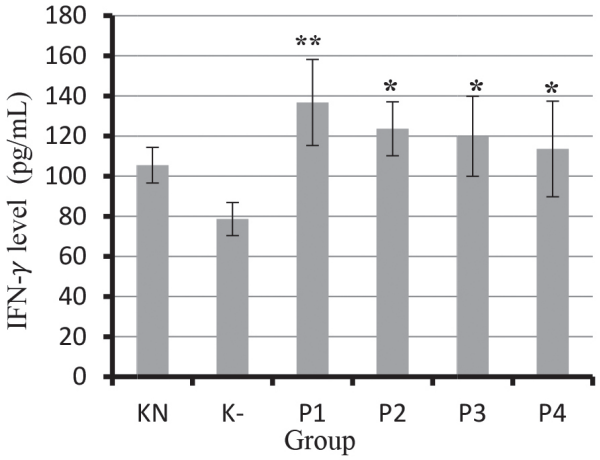

Fig. 4. Effect of OPM on IFN-γ level of mice induced by sodium nitrite (pg/mL) between treatment groups. KN = normal control; K- = negative control (exposure to sodium nitrite 50 mg / kg BW); OPM treatment groups P1, P2, P3, P4 (dose 50,100, 200,400 mg / kgBB. *) significant differences with negative controls; **) significant difference with negative control and normal control based on Duncan's test ($\alpha = 0.05$).

Discussion

Sodium nitrite is toxic because it can cause methemoglobinemia, a condition in which hemoglobin is oxidized to methemoglobin which releases oxygen (Cvetkovic *et al.*, 2019). The condition of methemogobinemia causes hypoxia in cells and tissues, triggering oxidative stress and leads to cellular damage. Sodium nitrite consumed in low doses for a long time can result in histopathological changes, tissue damage due to nitrosative stress, lipid peroxidation in the liver and kidneys, chromosomal damage, risk of colon cancer, hypoxia, vasodilation, methaemoglobin, and decrease the immune system (Suparmi *et al.*, 2016).

Giving sodium nitrite can reduce white blood cells count (WBCs), phagocytic activity and IFN-γ levels. WBCs in the K-group showed decrease (6.875 cells/mm^3), but not significantly compared to KN group (10,125 cells/mm^3). This shows that sodium nitrite at a dose of 50 mg / kg BW can reduce WBCs in mice. A decrease in WBCs can occur because white blood cells migrate to damaged or inflamed tissue, so the total number of white blood cells in the blood decreases. However, the white blood cell count in the K- group can still be said to be within the normal range of healthy blood of mice according to McGarry *et al.* (2010) which is 5000-13000 cells/mm^3. The results of this study are also supported by in vivo research by Gluhcheva *et al.* (2012), that oral administration of sodium nitrite at a dose of 50 mg/kg BW for 20 days can reduce the total number of white blood cells and the number of lymphocytes.

The decrease in WBCs in the K-group was relatively insignificant with the KN group and remained in the normal range, which means that sodium nitrite was still tolerable by the body. This can occur because the treatment period is too short or the dose used is too low, because in general food preservatives will show a negative impact after years of use d. Decreased WBCs can also occur due to damage to hematopoiesis tissue such as bone marrow, spleen and liver, so that the production of new blood cells is reduced (Gluhcheva *et al.*, 2012).

The reduction in the percentage of phagocytic activity in the K- group treated by sodium nitrite was significantly compared with the KN group. This occurs because sodium nitrite can increase free radicals (ROS and RNS) which trigger conditions of oxidative stress and nitrosative stress in hematopoeisis tissue so that it can reduce the pro-

duction of new blood cells including white blood cells that function in the body's immunity (Gluhchevaa *et al.,* 2012). This decrease in phagocytic activity was also supported by a decrease in the total white blood cell count due to sodium nitrite administration which was also observed in this study. This is also supported by the results of Abuharfeil's research (2001) showing that administration of sodium nitrite at a dose of 100 mg/kgBW for 3 weeks can suppress the immune system. Sodium nitrite is absorbed by the body through the digestive tract which is absorbed by the body by as much as 40% while 60% is not known with certainty, it can be suspected that other sodium nitrite metabolism in the form of nitric oxide and nitrosamines can suppress and decrease immune function by suppressing lymphocyte response to phytoaglutinin, causes apoptosis of T lymphocytes in the spleen, and inhibits the secretion of interleukin-2, interleukin-4, and IFN-γ (Abuharfeil, 2001). Giving sodium nitrite can inhibit the activity of macrophages in digesting and killing bacteria. This statement is proven in in vitro studies on rabbit alveolar macrophage cells that sodium nitrite at a dose of 10 mM to 20 mM can reduce the percentage of phagocytic activity of *Peseudomonas aeruginosa* and *Staphyllococcus epidermidis* significantly. It can be said that sodium nitrite suppresses macrophages to digest or kill bacterial cells (Vassallo *et al.,* 2013).

The decrease in IFN-γ levels in the K- group (78.63 pg/ml) after exposure to sodium nitrite was significantly compared to KN group (105.50 pg / ml). In addition, the K- group showed the lowest average IFN- γ level. This shows that sodium nitrite can reduce the immune system, which is characterized by a decrease in IFN-γ levels after exposure. Exposure to nitrites in the body apparently can reduce the response of Th-1 cells that secrete several types of cytokines such as IFNγ, IL-2 and TNF-β. Nitrates and nitrites that enter the body can disturb the balance of Th-1 to stimulate the Th-2 response, so exposure to these compounds can reduce the immune response. This statement is in accordance with research conducted by Ustyugova *et al.* (2002) that the administration of nitrates or nitrites decreases the cytokines secreted by Th-1 namely IFN-IL, IL-2 and TNF-β. The study was conducted in vitro using human peripheral blood mononuclear cell culture with a dose of sodium nitrite used was 10 ppm. The results of the study that IFN-γ was decrease significantly after administration of sodium nitrite.

The treatment group giving okra P1, P2, P3 and P4 of 50 mg/kg bw, 100 mg/kg bw, 200 mg/kg bw, and 400 mg/kg bw OPM doses showed a significant increase compared to the K- and has no real difference when compared to the KN group. This shows that the administration of okra fruit methanol extract can increase the number of white blood cells. The results of this study are also supported by the research of Adeyomo-salami and Emmanuel (2015) proving that the administration of methanol extract of *Paullinia pinnata* (Linn.) Leaves at a dose of 50 mg /kg bw, 100 mg / kg bw and 200 mg/kg bw can increase WBCs of white mice when compared to KN group.

An increase in WBCs is at the normal high limit as in the treatment groups P1, P2, P3, and P4 shows that the immune system produces sufficient total number of leukocytes in the blood circulation (Vieira, 2011). In addition, the increase that occurs due to methanol extract of okra fruit which is rich in antioxidants can repair damaged tissue by donating H atoms so that it will reduce the amount of ROS and oxidative stress on cells and tissues can be overcome (Flora *et al.,* 2012). Okra is known to contain a variety of good nutrients and is very important for health. The content of vitamins and other components in okra is an important component in increasing the number of white blood cells. The body needs vitamins like vitamin B12 and folic acid to support the formation of white blood cells (Koiso *et al.,* 2011). Okra is a plant that is known to be rich in fiber, potassium, magnesium, manganese, vitamin C, folic acid, vitamins B1 and B6, vitamin K and bioactive components such as flavonoids, especially quercetin (Bawa, 2016). Research by Wahyuningsih *et al.,* (2018) that okra extract can function as an immunomodulator. Immunomodulator is a substance or material that can restore the function of an impaired immune response (immunorestoration), improve and strengthen the immune response (immunostimulation), and suppress excessive immune responses (immunosuppression) (Murphy *et al.,* 2012).

The treatment group P1 by giving OPM at a dose of 50 mg/kg bw has the highest increase in the percentage of phagocytic activity with a value of 50.80% significantly different when compared to the KN group (41.93%) and K- (31.18%). This shows that the administration of okra fruit methanol ex-

tract which contains antioxidants has the potential as an immunostimulant by increasing and strengthening the immune response. The content of okra polysaccharides is as an immunomodulator (Wahyuningsih *et al.,* 2018) which can activate immunocompetent cells to increase body immunity. The increase in the body's immune system is thought to be an action of preparation and prevention due to the entry of antigens in the body (Jang *et al.,* 2009).

The treatment groups P2, P3, and P4 also showed a significant increase in the percentage of phagocytic activity when compared to the K-group, but not significantly different from the KN group. It can be said that the active compound in okra fruit which acts as an antioxidant is able to regulate the immune system because it can restore the immune response to normal conditions. The content of flavonoids in okra fruit has the potential to increase the lymphokin section by T cells that stimulate phagocytes to carry out phagocytosis (Nugroho, 2012). This is in accordance with a study conducted by Nurkhasanah *et al.,* (2017) showing that the flavonoid content in *Zingiber cassumunar* can increase phagocytic activity and phagocytic capacity in cell culture of mice intraperitonel cells. The content of flavonoids in okra flavonoids has anti-tumor, antioxidant, immunostimulant, anti-inflammatory, analgesic, anti-viral, anti-fungal and anti-bacterial effects (Knight, 200). Flavonoid compounds have been shown to increase IL-2 and lymphocyte proliferation (Nopitasari, 2006). Lymphocyte proliferation will affect CD4 + cells, which will cause Th1 cells to be activated (Ukhrowi, 2011). Th1 cells that have been activated will affect SMAF (Specific Macrophage Activating Factor). SMAF (Specific Macrophage Activating Factor) are multiple molecules, one of which is IFN-γ. IFN-γ can indicated with macrophages activity, so that macrophages will experience increased phagocytic activity. This will cause macrophages to kill antigens more quickly (Bratawidjaja, 2006).

The treatment group P1 showed the highest average IFN-γ level (136.75 pg/mL) when compared to other treatment groups and significant compared with the KN group (105.50 pg/mL). This shows that the methanol extract of okra fruit can improve the immune response. The content of phenol and polyphenol compounds in okra fruit can be potential as an immunomodulator (You *et al.,* 2013). This statement is also proven by in-vivo research Muchtaromah *et al.* (2019) polyphenol content in *Calotropis gigantea* ethanol extract dose of 150 mg / kg bw for two weeks can increase IFN-γ levels (136.75 pg/mL) in mice exposed to 7,12-dimethylbenz (α) anthracene significantly when compared to the group normal control (105 pg/mL). The treatment groups P2, P3, and P4 was decreased in IFN-γ level along with the increase in the dose of okra extract when compared with the P1 group, but an increase when compared to the KN group insignificantly. This shows that the methanol extract of okra can increase IFN-γ levels in mice experiencing oxidative stress due to exposure to sodium nitrite. This result is also supported by research by Wahyuningsih *et al.* (2018) that the administration of crude extracts of okra polysaccharides can significantly increase NK cell activity and secretion of IFN-γ cytokines.

OPM (*A. esculentus*) is known to contain antioxidant components. OPM contains catechin, quercetin, epicatechin, procyanidin B2, and routine (Khomsug *et al.,* 2010). These compounds are known to function as antioxidants. The mechanism of action of OPM is given by donating H atoms thereby reducing ROS levels (Flora *et al.,* 2012). This is consistent with this study, that the administration of okra fruit methanol extract can improve the oxidative and nitrosative effects of sodium nitrite exposure by increasing WBCs, phagocytic activity and IFN-γ levels.

Conclusion

Giving Okra pods (*Abelmoschus esculentus* L.) methanol extract increased the number of white blood cells, phagocytic activity, and IFN-γ *Mus musculus* levels after induced sodium nitrite.

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