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Original Article

The Effect of Omega-3 Rich Fish Oil on the Kidney Changes in Mice Induced by Azoxymethane and Dextran Sodium Sulfate

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ABSTRACT

Background: The study aimed to investigate the effect of omega-3 rich fish oil to kidney of mice induced by Azoxymethane (AOM) and DSS using histopathology parameters.
Method: The experimental mice were induced using 10 mg/kg AOM and 2% DSS for 2 weeks randomly allocated randomly into four groups as follows; Control Group: mice that not received fish oil, Low Dose Group: mice that received 1.5 mg/day fish oil, Medium Dose Group: mice that received 3 mg/day fish oil, and High Dose Group: mice that received 6 mg/day fish oil. The omega-3 rich fish oil was given for 12 weeks.
Result: The administration of high dose omega-3 rich fish oil was able to reduced necrosis and inflammation foci compared to the control group ($p<0.05$). Furthermore, the administration of low, medium, and high dose omega-3 rich fish oil was able to significantly reduced vascular edema and cell degeneration foci ($p<0.05$). The administration of medium and high dose of omega-3 rich fish oil were able to reduce the amount of fibrosis foci compared to the control group ($p<0.05$) compared to the control group.
Conclusion: The result suggested anti-nephrotoxic effect of omega-3 rich fish oil in mice induced by azoxymethane and DSS.
Key words: Omega-3, Fish oil, Histopathology, Kidney, Mice, Azoxymethane, Dextran sodium sulfate.

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INTRODUCTION

Colorectal cancer is a type of malignancy with a high incidence. According to cancer statistics in 2012, colorectal cancer has the third highest incidence with a high mortality rate. In fact, in developed countries the number of new cases of colorectal cancer has exceeded the number of new cervical cancers in women.[1]

Colorectal cancer occurs due to the interaction of external and internal factors. External factors include environment and food, while internal factors are genetic differences of each individual. Modeling of colorectal cancer in animals can be reached by inductions of Azoxymethane (AOM) and dextran sodium sulfate. In addition to its effects on colon, azoxymethane is also known to have an effect on the liver, lungs and kidneys.[2] Azoxymethane is known to be nephrotoxic and carcinogenic in the kidneys.[3]

The histopathological features of the kidneys of mice given azoxymethane are very diverse, ranging from inflammatory cell infiltration, tubular and glomerular degeneration, to the occurrence of renal carcinoma.[3] Two types of renal carcinoma most commonly found in mice given azoksimetan are renal cell carcinoma and renal medullary carcinoma.[2,3]

One factor that can influence the occurrence of colorectal cancer is food intake. Low-fiber and high-fat diets are known to be risk factors for colorectal cancer. In addition, read meat and burnt food are also risk factor for colorectal cancer. In contrast, a diet with sufficient fiber and high omega-3 fatty acids is a protective factor for colorectal cancer.[4]

The Ministry of Trade lists 10 potential Indonesian commodities, with fish and its processed goods being one of them.[5] One of the processed fish products that have potential in the health world is fish oil rich in omega-3. Omega-3 is a substance that has been shown to have immunomodulatory effects in the human body by reducing oxidative stress and potentiating the antioxidant system.[6] Although the amount is abundant in Indonesia and its effects are generally tested, the use of omega-3 fish oil by the Indonesian people is still very limited.

One study has proven the positive benefits of omega-3 administration in colorectal carcinogenesis by AOM and DSS.[6] Although it is well known that AOM and DSS can also cause damage to other organs such as the kidneys, there are still no studies examining the effects of omega-3 in preventing kidney damage. by AOM and DSS.[2,3] However, several studies have shown positive effects of omega-3 administration on kidney damage induced by other substances, such as cyclosporine-A and takrolimus.[7,8] Therefore, researchers want to know the effects of omega -3 in preventing kidney damage by AOM and DSS.

METHODS

Research design

This research is an *in vivo* experimental study using animal trials of Balb / c mice. The study protocol was approved by the Institutional Animal Care and Use Committee, Faculty of Medicine, Universitas Indonesia (ND 473/UN-2. F1/ETIK/PPM.00.02/2018, April 19, 2018).

Animals

The experimental animals were 16-week-old Balb / c mice with an average weight of 20 grams obtained from the FKUI Animal Pathology Anatomy Laboratory. Mice are acclimatized for one week before treatment. Mice were kept and treated

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according to *the Guide for the Care and Use of Laboratory Animals of Animal Care and Use Committee*. Mice are maintained under controlled temperature conditions of 25 ° C, humidity of 55% with a light / dark cycle of 12 hours. All mice were feeded using standard food and drink *ad libitum* mineral water.

Treatment

Samples are kidneys from experimental animals that have been given intraperitoneal azoxymethane 10 mg / kg. A week later the mice were given standard food and drinks containing 2% dextran sodium sulfate every day for a week. After administering azoxymethane and dextran sodium sulfate, mice were given omega-3-rich fish oil every day for 10 weeks. Mice will be sacrificed 12 weeks after induction.

Male Balb / c mice are divided into 4 groups, namely:

A group of positive control mice induced with AOM and DSS, then not given omega-3-rich fish oil.

Groups of mice induced with AOM and DSS, then given low-dose omega-3 fish oil (1.5 mg per day).

A group of mice induced with AOM and DSS, then given a medium dose of omega-3 fish oil (3 mg per day).

Mice group induced with AOM and DSS, then given high-dose omega-3 fish oil (6 mg per day).

Azoxymetane and dextran sodium sulphate induction and omega-3 rich fish oil treatment

Azoxymethane is given in a single dose in mice through intraperioneal injection at a dose of 10 mg / kg body weight dissolved in 0.9% NaCl solution. Then, for one-week mice were given standard feed and drink mineral water. The following week the drink was replaced with mineral water containing 2% sodium sulfate dextran.[9] Administration of fish oil rich in omega-3 is done by force-feeding group mice treated with low doses (1.5 mg), medium (3 mg), and high (6 mg) every day. Administration of fish oil rich in omega-3 starts at the end of the administration of dextran sodium sulfate (end of week 2) until mice are sacrificed at 12 weeks after induction with azoxymethane (Figure 1). In addition, every 4 weeks the weight of mice is weighed to find out the body weight index. Body weight index is calculated by dividing the weight of the mice in the treatment group with the control group mice.

Tissue preparation

Mice were sacrificed after 12 weeks after administration of azoxymethane. Kidney mice are taken and cleaned with water, then then fixed by using 10% formalin phosphate buffer and ready to be made histopathological preparations.

Hematosiklin-Eosin (HE) staining

Kidney tissue pieces were made from Fixed Parafin Formalin Embedded (FFPE) and cut 4 µm thick slices and glued to the object glass for HE coloring. HE staining is done by the following steps. Preparation is initialized with three sequential dips in xylol for 5 minutes each followed by rehydration process using grading concentrations from absolute alcohol, 96% and 70% for 5 minutes each. The preparation was soaked in running water for 5 minutes. The preparation was dipped in a hematocycline solution (Meyer's solution) for 7 minutes and rinsed with running water for 10 minutes. The preparation is dipped in 2-3 saturated lithium carbonate, then rinsed with running water for 5 minutes. If the blue color was not sufficient, the preparation is dipped back into the hematocycline solution for 2 minutes, then rinsed in running water. The preparation was soaked in eosin for 1-2 minutes, then dehydrated using gradual concentration of 70%, 80%, 96%, and absolute alcohol for 3 minutes each. Clearing the preparation with xylol I, II, and II, then dripping the paste.

Interpretation of histopathological observations

Observation of kidney tissue in mice was carried out in ten visual fields randomly with 100x magnification of kidney tissue. Nephrotoxicity signs were assessed, namely the degree of degeneration of cell degeneration and the degree of fibrosis in ten visual fields with 100x magnification in the renal cortex. Degrees of cell degeneration and vascular congestion follow the method in the study by Tan *et al*. For cell degeneration: 0 = no degeneration or at least one field of view; 1 = mild degeneration in ≤20% of cells in one field of view; 2 = moderate degeneration in 21-50% cells; 3 = severe degeneration in> 50% of cells in one field of view. For vascular congestion: 0 = no vascular congestion focusses or very minimal; 1 = mild congestion (congestion focus includes one location); 2 = moderate congestion (congestion focus is found in several locations); 3 = severe congestion (congestion in almost all fields of view). The preparations to be assessed are blinding by random numbering by a third party (unknown to researchers and supervisors). So that both researchers and mentors do not know which treatment group is being assessed. The assessment was carried out by the researcher which was then validated by the research supervisor.

Data analysis

Data analysis was carried out using SPSS version 20 statistical test software on the Windows 7 operating system. The results of histopathological observation in the control group and test group were compared by calculating the significance value or p value. Meaningful results were obtained if the significance value was $p < 0.05$.

The effect of giving three doses of fish oil rich in omega-3 (1.5; 3; and 6 mg / day) to the occurrence of nephrotoxicity can be seen by looking at the degree of cell degeneration and vascular congestion. The data obtained is tested as an ordinal variable. Non-parametric Kruskal-Walli's analysis was performed to determine the difference in the degree of cell degeneration and significant vascular congestion between treatment groups. If the data obtained was statistically significant ($p < 0.05$), the Mann-Whitney test was performed in each combination of treatment groups. Mann-Whitney test is used to find out which groups have significant differences with other groups.

RESULT

Effects of omega-3 rich fish oil on kidney mouse cell degeneration

Histopathological observations on the degree of cell degeneration were performed at 100x magnification of kidney preparations with H & E staining. The degree of cell degeneration in each field of view from the 10 visual fields selected, is stated in low degree (1+), medium (2+), and heavy (3+). Cell degeneration in the renal cortex is characterized by enlarged cells and cloudy cytoplasm, and can be accompanied by vacuolization. (Figure 2)

The data of cell degeneration degree in each field of view obtained were then statistically analyzed using the Kruskal-Walli's test to determine the significant differences between groups. Independent variables are the four groups of fish oil rich in omega-3, namely: 1) control; 2) low dose (1.5 mg / day); 3) moderate dose (3.0 mg / day), 4) high dose (6.0 mg / day). The dependent variable is the degree of cell degeneration per field of view, which is assessed in 10 fields of view (x100) for each mouse on histopathological examination.

The Kruskal-Walli's test showed significant differences in the degree of cell degeneration between treatment groups ($p = 0.003$), with the mean rank of cell degeneration degrees 98.65 for the control group, 83.85 for

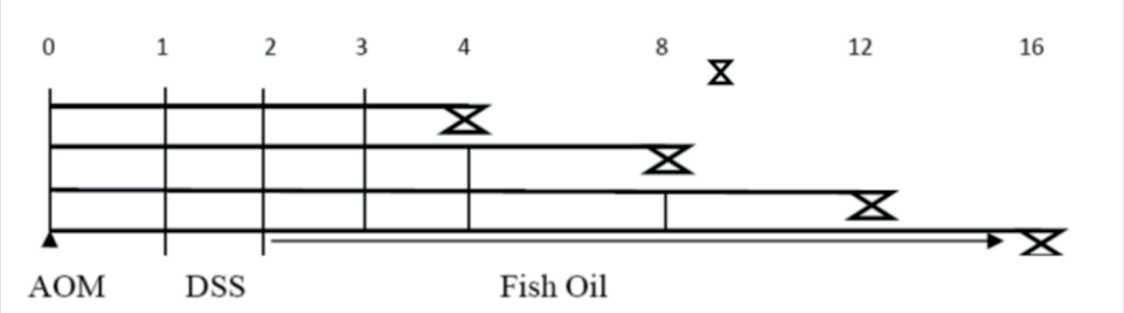

Figure 1: Research protocol. Intraperitoneally injected AOM 10mg/kgBW, 1% DSS was given through mineral water, fish oil was given orally. (⧖ means mice were sacrificed; number is referred to weeks).

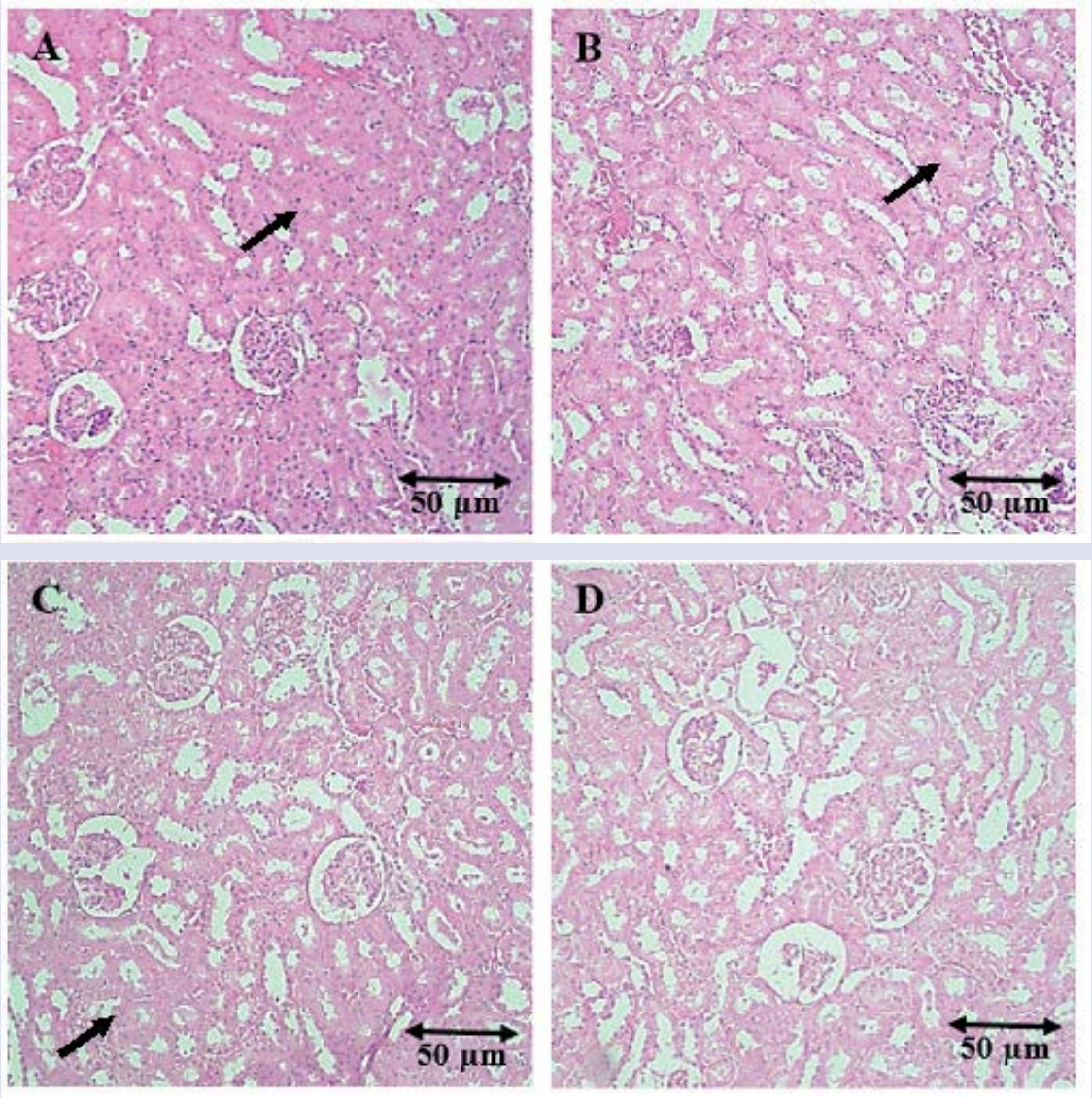

Figure 2: Assessment of the degree of cell degeneration in the kidneys of mice with H & E staining (100x magnification). Black arrows indicate cell degeneration. Figures (a), (b), (c) and (d) were taken from the control group, low dose, moderate dose and high dose, respectively. (a) shows degeneration in> 50% cells (degree 3+); (b) there is degeneration at 21-50% cells (degree 2+); (c) shows degeneration in <20% cells (degree 1+); (d) there is very minimal cell degeneration (degree 0).

the low dose group, 72.6 for the moderate dose group, and 66.90 for the High Dose group.

The degree of cell degeneration between treatment groups was then compared using the Mann-Whitney test. Mann-Whitney test results showed a significant difference in the degree of cell degeneration between the control and moderate dose groups ($p = 0.005$) and between the control and high dose groups ($p = 0.001$). There was no significant difference in the degree of cell degeneration between the Control and Low Dosage groups ($p = 0.066$). There is no significant difference between giving a dose with another dose to the degree of cell degeneration.

Effects of omega-3 rich fish oil on vascular congestion of kidney mice

Histopathological observations on the degree of vascular congestion were performed at 100x magnification of kidney preparations with H & E staining. The degree of vascular congestion in each field of view from the 10 visual fields selected is expressed in low degrees (1+), moderate (2+), and weight (3+). Vascular congestion in the renal cortex is characterized by a buildup of red blood cells in the blood vessels. (Figure 3)

Data on the degree of vascular congestion in each field of view obtained were then analyzed statistically using the Kruskal-Walli's test to determine the significant differences between groups. Independent variables are the doses of fish oil rich in omega-3, namely: 1) control; 2) low dose (1.5 mg / day); 3) moderate dose (3.0 mg / day), 4) high dose (6.0 mg / day). The dependent variable is the degree of vascular congestion per field of view, which is assessed in 10 fields of view (x100) for each mouse on histopathological examination.

The Kruskal-Walli's test showed a significant difference in the degree of degeneration of vascular congestion between treatment groups ($p = 0.017$), with the mean rank was 99.26 for the control group, 74.76 for

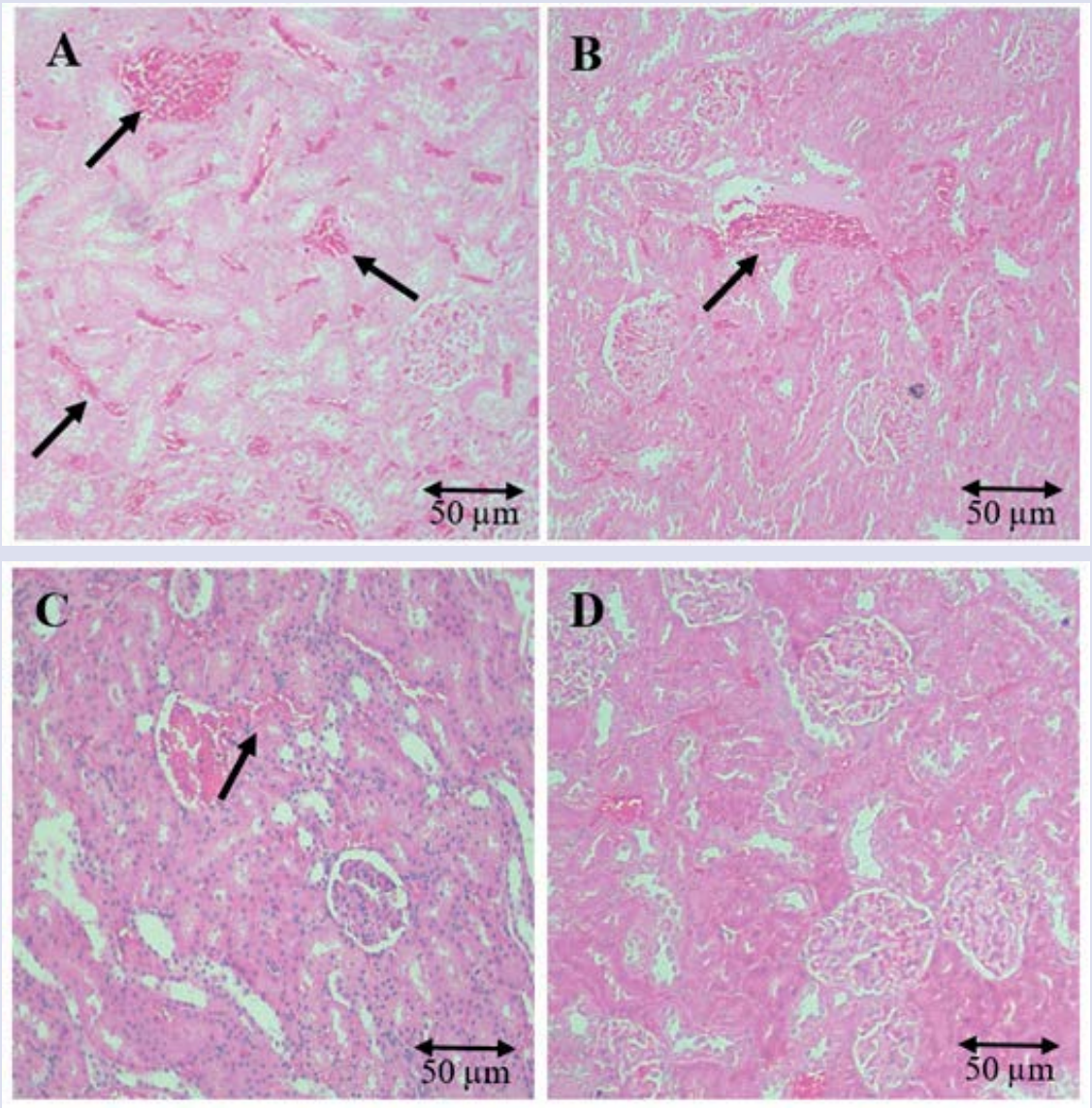

Figure 3: Assessment of the degree of vascular congestion in the kidneys of mice with H & E staining (100x magnification). Black arrows show vascular congestion. Figures (a), (b), (c) and (d) were taken from the control group, low dose, moderate dose and high dose, respectively. (a) visible vascular congestion in all fields of view (degree 3+); (b) shows vascular congestion at some focus in one field of view (degree 2+); (c) visible vascular congestion in one focus in one field of view (degree 1+); (d) there is very minimal vascular congestion (degree 0).

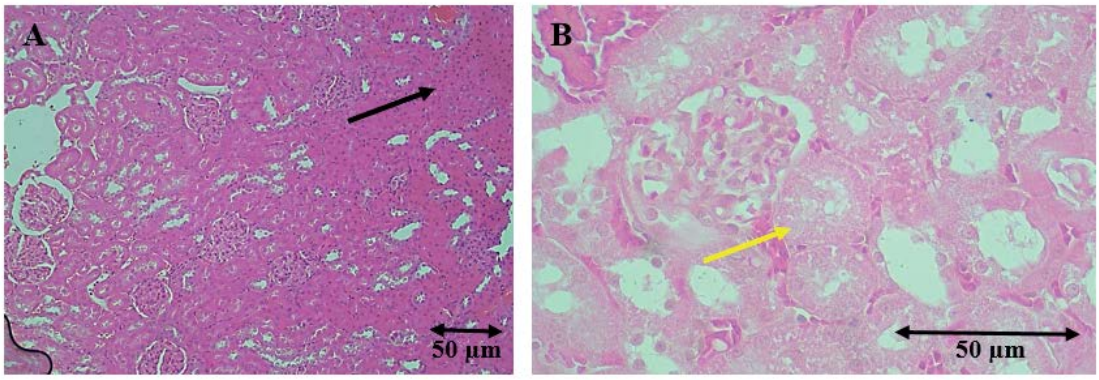

Figure 4: Other histopathological features found after administration of azoxymethane and DSS. (a) Black arrows show inflammation characterized by the number of monomorphonuclear inflammatory cells (100x magnification); (b) Yellow arrows indicate the occurrence of necrosis in the renal tubules, which is characterized by damage to cell integrity and cell nucleus that is already invisible (400x magnification).

the low dose group, 76.39 for the group moderate dosage, and 71.59 for the high dose group.

The degree of vascular congestion between treatment groups was then compared using the Mann-Whitney test. The results showed a significant difference in the degree of cell degeneration between groups between the Control and Low Dosage groups ($p = 0.019$), between the Control and Moderate Dosage groups ($p = 0.015$), and between the Control and High Dose groups ($p = 0.005$) respectively. No significant difference observed between giving a dose with another dose to the degree of vascular congestion.

Description of other histopathological changes in the kidney

In addition to cell degeneration and vascular congestion which is used as a variable in determining the effects of fish oil rich in omega-3, there are also several other histopathological features, namely necrosis and inflammation in kidney tissue of mice. Both of these histopathological features occurred in some mice in the control group, low doses, and moderate doses. Necrosis occurs in some kidney tissues from 3 mice in the control group, 1 low-dose group mice, and 1 high-dose group mice. Inflammation occurred in part of the kidneys of mice from 1 control group mice and 1 low-dose mice. (Figure 4)

DISCUSSION

Effects of omega-3 on the degrees of kidney degeneration postinduction AOM / DSS

The results showed that medium and high doses of omega-3 fish oil were able to reduce the degree of cell degeneration in the kidneys of mice induced by AOM and DSS. Administration of low doses of omega-3 fish oil cannot significantly reduce the number of fibrotic foci in the kidneys of mice. The effect of fish oil rich in omega-3 doses in reducing the degree of cell degeneration is not better than high doses, and vice versa.

Cell degeneration can occur due to damage in mitochondria, cessation of ATP production, and sodium pump failure causes an increase in intracellular osmotic pressure.[10] This change leads to increased permeability of the cellular membrane.[10] Mitochondrial damage can be caused by azoxymethane and its metabolites.[11]

In addition, the formation of free radicals during the inflammatory process can also cause cell degeneration.[10] Several studies have studied the effects of omega-3 antioxidants on the kidneys. Garrel *et al* showed that omega-3 increased the activity of the enzyme dismutase superoxide in mitochondria. In a study that studied *cadmium-induced nephrotoxicity*, administration of omega-3-rich fish oil improved mitochondrial function.[12]

Free radicals can cause fat peroxidation in cell membranes. Cell membranes which are mostly composed by fat become impaired in function. Increased permeability of cell membranes, so that sodium and water can enter more easily and cause cell swelling.[13] Omega-3 oils are known to not increase the occurrence of fat peroxidation, but are thought to reduce damage to cells due to fat peroxidation.[14]

In addition to increasing the number of free radicals, tissue hypoxia also causes cell degeneration. Azoximetan is known to cause vascular changes in kidney tissue in the form of dilation, edema, and congestion.[3] These three changes, especially congestion, can reduce the supply of oxygen to kidney tissue and cause hypoxia. Hypoxia causes reduced ATP production, which is needed to maintain cell osmolarity through the Na + / K + .[15] pump function Omega-3 is known to improve hypoxia in kidney tissue that has been chronically damaged through immuno-medulatory pathways. [16]

TGF-β is also associated with the occurrence of cell degeneration through apoptotic pathways and transdifferentiation in chronic kidney disease.[17] Azoxymethane and its metabolites can trigger an increase in Omega-3 TGF-β.[18] receptors known to reduce the expression of TGF-β receptor gene in kidney cells.[19]

The results of this study were consistent with the results of previous studies that showed impact of omega-3 administration to reduce the degree of cell degeneration in the kidneys through various mechanisms.

Omega-3 effect on the degree of vascular congestion after AOM / DSS induction

The results showed that the administration of fish oil rich in omega-3 doses of low, medium, and high can reduce the degree of vascular congestion in the kidneys of mice induced by azoxymethane and DSS. There is no difference between administering a dose with another dose in reducing the degree of vascular congestion.

Vascular congestion can be caused by active or passive processes. Active processes that can cause vascular congestion include inflammatory reactions. While the passive process of venous congestion is caused by obstruction of venous return. Renal tubular cell swelling due to hydropic degeneration can contribute to venous return flow obstruction.[15] Enlarged tubular cells can suppress surrounding blood vessels and cause obstruction.

During inflammatory reactions, vasoactive amines such as histamine and serotonin, and also eicosanoids such as prostaglandin E2 and leukotriene B4 were relased by macrophages to modify local vascularization. Macrophages and endothelial cells can also release NO in response to pro-inflammatory cytokines. Mediators this can cause vasodilation and increase the permeability of capillary blood vessels that transfer of blood plasma from vessels to tissues.[20]

Azoxymethane can increase NO secretion by endothelial cells by increasing the enzyme expression of inducible-NO synthase (iNOS).[21] In addition, azoxymethane also increases the expression of the cyclooxygenase-2 (COX-2) gene that plays a role in prostaglandin production.[22] Another study has shown a dose dependent between DHA administration (one type of omega-3) to iNOS reduction.[23] In this study no dose-dependent relationship between vascular congestion and omega-3-rich fish oil was found. This can be caused by the extraction of fish oil which is not fully composed of DHA, and also the mechanism of iNOS which is not directly related to the occurrence of vascular congestion.

The results of this study are consistent with several other studies that show that administration of omega-3 can reduce vascular congestion in kidney tissue through various pathways described above.

Description of other histopathological changes in kidney mice

Inflammation and necrosis found in the kidneys of mice in accordance with the results of previous studies regarding the effects of azoxymethane on renal histopathology of mice.[2,24,25]

Inflammation was found in the kidney tissue of the control group mice more consistently (3 mice) than in the treatment group (1 low-dose mice group and 1 high-dose mice group). This indicates the effect of omega-3 in inhibiting inflammation. EPA and DHA can inhibit leukocyte chemotaxis, adhesin expression, adhesin-leukocyte-endothelial interactions, production of arachidonic acid derivative mediators such as prostaglandins and leukotrienes, as well as the production of pro-inflammatory cytokines (TNF-α, IL-1, and IL-6).[6] The mechanisms of EPA and DHA in inhibiting inflammation have been widely studied, and it is concluded that there are a variety of pathways that can contribute to these effects. The first needle is through the GPR-120 receptor, where EPA and extracellular DHA can inhibit the activation of the NF-κB gene which functions to increase the synthesis of pro-inflammatory proteins.[26] The second needle is through intracellular EPA and DHA bonds to PPAR-γ which also inhibits NF-κB gene activation.[27] The third pathway is eicosanoids formation by EPA and DHA indirectly reduced the formation of inflammatory mediators of arachidonic acid derivatives, such as prostaglandins and leukotrienes.[28] The results of this study were consistent. These findings were consistent with the results of previous studies on immuno-omega-3 modulator.

Necrosis was found in the kidney tissue of the control group mice (1 mouse) and the low dose group (1 mouse). No necrosis in kidney tissue was found in mice in the medium and high dose groups. These findings indicate the effect of fish oil rich in omega-3 in reducing the occurrence of necrosis in the kidneys of mice induced by AOM and DSS. Research by Singer *et al* demonstrated that administration of fish oil reduced the degree of necrosis in rat kidneys due to severe inflammatory processes and reperfusion after ischemia.[29] This finding is consistent with the results of previous studies on the effects of omega-3 in reducing necrosis in kidney tissue.

CONCLUSIONS

The conclusion of this study is: Administration of fish oil rich in omega-3 at moderate and high doses can significantly reduce the degree of cell degeneration on the kidneys of mice induced by azoxymethane and DSS.

Administration of fish oil rich in omega-3 at low, medium and high doses can significantly reduce the degree of vascular congestion in the kidneys of mice induced with azoxymethane and DSS.

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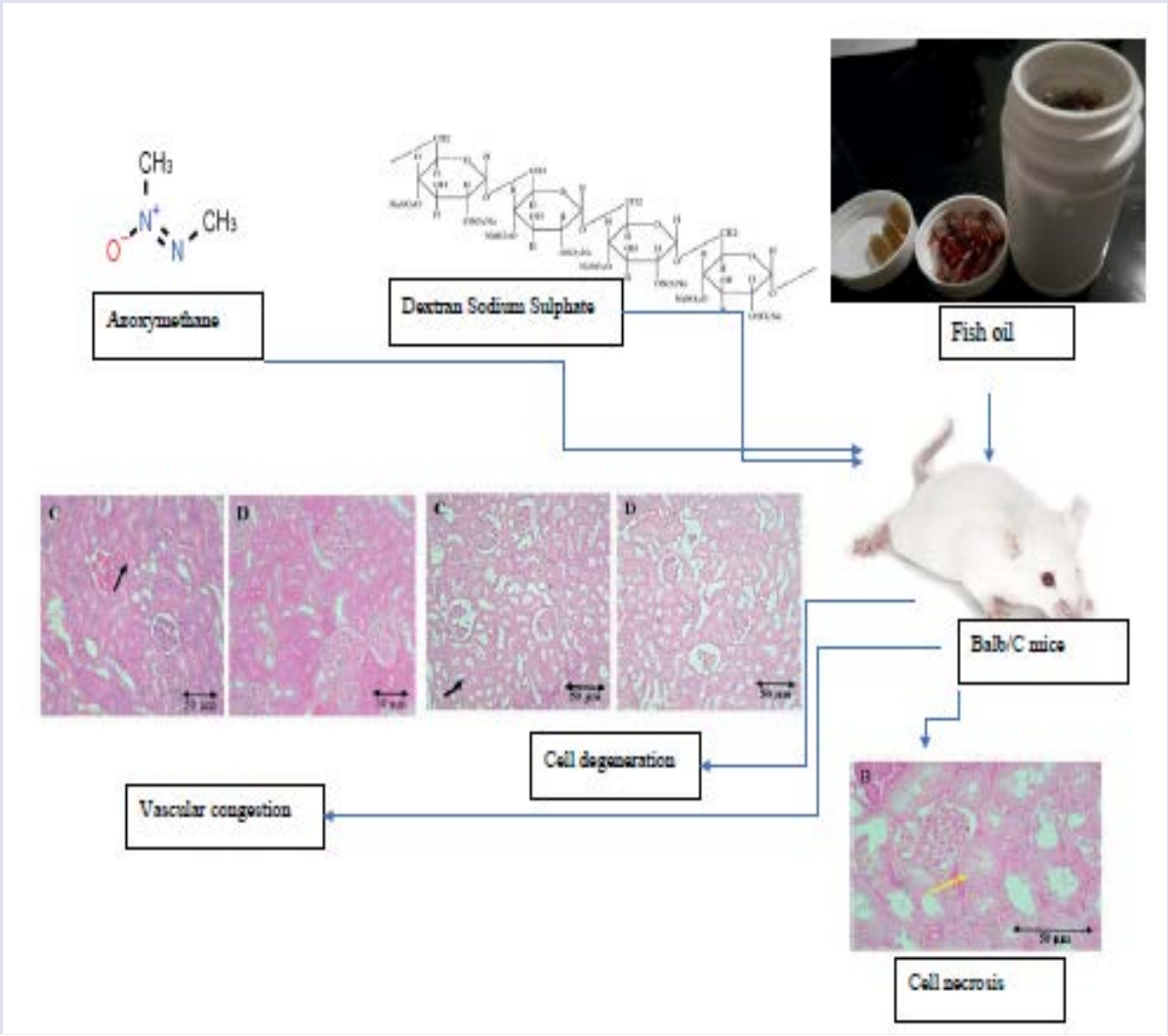
GRAPHICAL ABSTRACT



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