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Expression of VEGF-A And COX-2 mRNA in non-steroidal anti-inflammatory drugs treated rat primary colonic fibroblast

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ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) is often used to shorten recovery time after surgery, including after colon anastomosis surgery. Studies showed that NSAIDs might involve in the development of colon anastomotic leakage. However, the effect of NSAIDs in colon anastomosis leakage is still a subject of controversy. Studies indicated that selectivity of COX-2 might have a role in the deleterious effect of NSAIDs in colon anastomosis. Disruption of VEGF-A by NSAIDs also suspected to be the culprit in the development of anastomosis leakage during NSAIDs treatment. This study aimed to investigate the NSAIDs effect toward VEGF-A and COX-2 mRNA in rat primary colonic fibroblast. The *in vitro* study was conducted using fibroblast isolated from rat colon. The isolated fibroblast was divided into 4 groups of treatment i.e.controlgroup, acetaminophen group, metamizole group, and ketorolacgroup. After 48 h of treatment, the cell was harvested and the RNA was isolated. The expression of VEGF-A and COX-2 mRNA was conducted using semi-quantitative PCR(sq-PCR). Both VEGF-A and COX-2 were not expressed in untreated rat colon fibroblast. However, VEGF-A mRNA washighly expressed in the ketorolacgroup. Interestingly, COX-2 mRNA couldbe seen in the ketorolac and metamizole groups but not in the acetaminophen group. The COX-2 mRNA expression wasthe highest in ketorolac treated rat colon fibroblast. It can be concluded that the effect of various kinds of NSAIDs towards VEGF-A and COX-2 mRNA expression of colon fibroblasts is different. This condition is duetotheir different inhibitory selectivity towards COX-1 and COX2.

ABSTRAK

Non-steroidal anti-inflammatory drugs (NSAIDs) sering kali digunakan pada proses kesembuhan setelah proses operasi termasuk setelah operasi anastomosis kolon. Penelitian sebelumnya menunjukkan bahwa NSAID terlibat dalam perkembangan kebocoran anastomosis kolon. Namun, efek NSAIDs dalam kebocoran anastomosis usus besar masih menjadi subjek kontroversi. Studi menunjukkan bahwa selektivitas COX-2 mungkin memiliki peran dalam efek buruk NSAID pada anastomosis usus besar. Gangguan VEGF-A oleh NSAID juga diduga menjadi penyebab dalam pengembangan kebocoran anastomosis selam aperawatan NSAID. Penelitian ini bertujuan untuk menyelidiki efek NSAID terhadap mRNA VEGF-A dan COX-2 pada fibroblast kolon primer tikus. Studi in vitro dilakukan dengan menggunakan fibroblast yang diisolasi dari usus tikus. Fibroblast yang terisolasi dibagi menjadi 4 kelompok perlakuanya itu kelompok kontrol, asetaminofen, metamizole, dan ketorolac. Setelah 48 jam perawatan, seldipanen dan RNA diisolasi. Ekspresi mRNA VEGF-A dan COX-2 dilakukan menggunakan PCR semi-kuantitatif. Baik VEGF-A dan COX-2 tidak diekspresikan dalam fibroblast usus tikus yang tidak diobati. Namun, mRNA VEGF-A sangat diekspresikan pada kelompok ketorolac. Menariknya, COX-2 mRNA dapat dilihat pada kelompok ketorolac dan metamizole tetapi tidak pada kelompok asetaminofen. Ekspresi mRNA COX-2 adalah yang tertinggi pada fibroblast usus tikus yang diobati dengan ketorolac. Efek dari berbagai jenis NSAIDs terhadap ekspresi mRNA VEGF-A dan COX-2 dari fibroblast usus berbeda. Keadaan ini karena selektivitas penghambatan yang berbeda terhadap COX-1 dan COX2.

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are a group of drugs that has not only an anti-inflammatory effect but also analgesic effect by their action as cyclooxygenase (COX) inhibitor. They are often used as analgesic after surgery to reduce hospitalization duration. The NSAIDs are also often used to reduce the risk of anastomotic leakage.[1]

The COX-1 and COX-2 are cyclooxygenase isoforms. The COX-1 gene has an important role as a housekeeping promoter. Meanwhile, the COX-2 gene acts as an initial response gene. The COX-2 will convert arachidonic acid to prostaglandin E2 (PGE2), an important proinflammatory substance, which will cause inflammatory symptoms such as pain, fever, and swelling. NSAIDs block conversion of arachidonic acid into prostaglandin E2, which is responsible for pain and fever associated with inflammation by inhibiting COX. NSAIDs administration in post-operative patients inhibits the COX-2 expression as well as prostaglandin E2 and Vascular endothelial growth factor A (VEGF-A). VEGF-A plays a huge role in neovascularization, including vascular smooth muscle cell proliferation and fibroblasts.[2] VEGF-A is a special mitogen for endothelial cells that stimulates vasculogenesis directly. Besides, VEGF-A results in the secretion of the basic fibroblast growth factors in an autocrine manner, and also promotes cellular replication such as vascular smooth muscle cells and fibroblast.[3]

Recent study shows that there is an interference of anastomosis healing in the digestive tract, with one study suggesting an increased risk of up to 60% in a patient undergoing non-elective colorectal surgery.[4] The NSAIDs that are suggested to interfere the anastomosis wound healing are ketorolac and metamizole, whereas acetaminophen adds the least risks of leakage.[5]

During inflammation, the presence of COX-2 induces tissue expression of VEGF-A. Therefore, NSAIDs administration post-resection blocks the action of COX-2 thus it also suppresses the VEGF-A expression. Although the NSAIDs mechanism in anastomosis wound healing inhibition still unclear, it is thought that the anastomosis healing inhibition is happening through inhibition of fibroblast migration, angiogenesis, and collagen production.[6,7] For that reason, this study purposed to investigate the effect of some NSAIDs administration towards VEGF-A and COX-2 mRNA in rat primary colonic fibroblast.

MATERIALS AND METHODS

Fibroblast culture

For *in vivo* studies, we used 3-month-old Wistar rats with250–300 g body weight (BW). Rats were obtained from the Department of Pharmacology and Therapy Faculty of Medicine, Public Health, and Nursing,Universitas Gadjah Mada, Yogyakarta, Indonesia. Our research protocol referred to the provisions of the principles of handling experimental animals and has obtained ethical permission about research using experimental animals from our institution's ethics commission. All animals in our study were maintained in international standard animal facility in the best possible conditions and got the best possible care from skilled and experienced animal caregiver. They were acclimatized for 7 days with controlled room temperature and received a regular 12/12 h lighting cycle. Experimental animals were given standard feed and water ad libitum. For the *in vitro* studies, fibroblasts were primary isolated from the colons of healthy Wistar rats.

Treatment of fibroblast culture

The fibroblast was isolated from rat colon. The fibroblast was treated with various kind of treatment. Treatment groups were as follows: group 1 was treated with sterile water, group 2 was treated with acetaminophen, group 3 was treated with metamizole, and group 4 was treated with ketorolac. The concentrations ofacetaminophen were 5, 50 and 250 µg/mL (P5, P50, and P250). The

concentrations of metamizole were 5, 50 and 250 µg/mL (M5, M50, and M250). The concentrations of ketorolac were 1, 5 and 25 µg/mL (K1, K5, and K25). Incubation with the treatment was done for 24 h.

Genomic identification

Treated fibroblast cells were collected and centrifuged. All the supernatants were removed and isolated with FavorPrepTM Tissue Total RNA Purification Mini Kit (Favorgen, FATRK001-1) 2.2. The cDNA was made with Complementary DNA (cDNA): High capacity cDNA Reverse Transcription Kit (Applied Biosystem, LT-22041). The VEGF-A mRNA expression was examined by PCR with a forward primer (5'-GCT CTC TTG GGT GCA CTG GA-3') and reverse primer (5'-CAC CGC CTT GGC TTG TCA CA-3'). PCR was then performed with 40 cycles configuration, with a temperature of 95°C for 5 min, continued with 95°C for 60 sec, 57°C for 60 sec, 72°C for 60 sec, and 72°C for 10 min, with final extension phase at 4°C for 10 min. The COX-2 mRNA expression was examined by PCR with a forward primer (5'-TGCGATGCTCTT CCGAGCTGTGCT-3') and reverse primer (5'-TCA GGAAGTTCCTTATTTCCTTTC-3'). The PCR thermal cycling conditions were as follows: 4 min at 95°C, 4 min at 95°C, 1 min at 62°C, 1 min at 72°C repeat for 40 cycles, 4 min at 72°C followed with another 10 min at 4°C. The GAPDH was used as the housekeeping gene with a forward primer (5'-TGG GAA GCT GGT CAT CAA C-3') and reverse primer (5'-GCA TCA CCC CAT TTG ATG TT-3'). The PCR process was followed with electrophoresis. The PCR product was run in agarose gel 1.5 %. The result was then documented and analyzed by densitometry analyzer using Image J software.

RESULT

The electrophoresis result of VEGF-A cDNA PCR product showed only one band in the region of K25 administration at 380 bp, and no other bands wereobserved in the other regions (FIGURE 1). This has been confirmed by ImageJ software, and this does not represent any anomalies in the PCR nor the electrophoresis technique, as there is an expression of GAPDH mRNA of similar homogeneity at 78 base pairs. GAPDH is included in this study as a housekeeping gene, where its expression is remaining constant in the cells under investigation (FIGURE 2A).

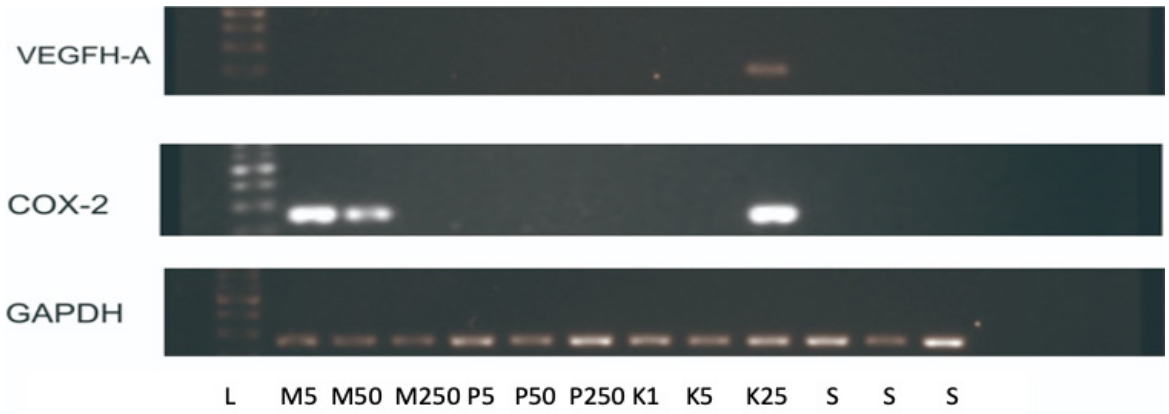
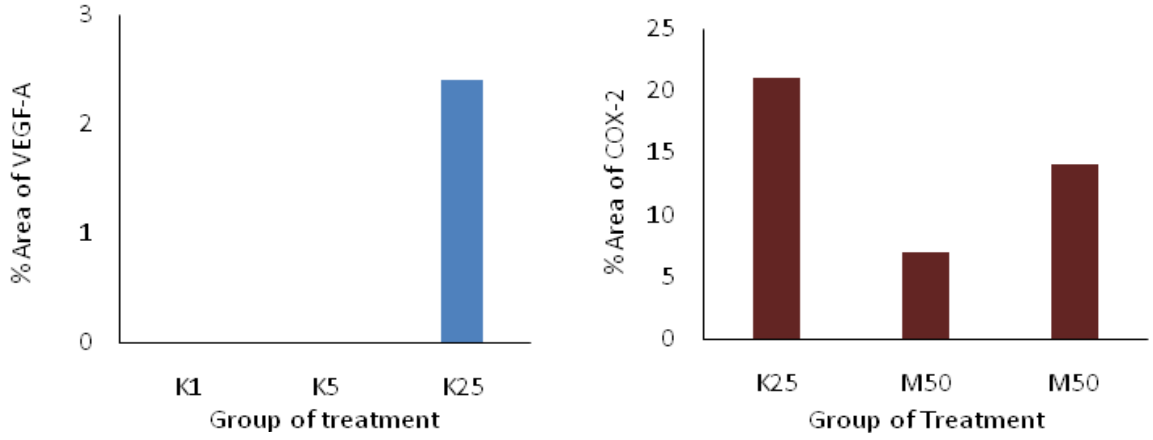


FIGURE1. Electrophoresis result of VEGF-A, COX-2, and GADPH mRNA expression. M5, M50and M250are colonic fibroblast that have been treated with metamizole at a concentration 5, 50 and 250 µg/mL respectively. Likewise, P5, P50 and P250 are colonic fibroblast that have been treated with acetaminophen at a concentration 5, 50 and 250 µg/mL respectively. K1, K5, and K25 represents colonic fibroblast that have been treated with ketorolac at a concentration 1, 5 and 25 µg/mL respectively. S is colonic fibroblast without any treatment.

The COX-2 mRNA has been expressed on the M5, M50 and K25. The targeted band is 480 bp. In addition, GAPDH was expressed in all groups. The result was showed in FIGURE 1. Image J software was used to quantify and measure the band density of COX-2 and GAPDH. The density of COX-2 mRNA expression for M5, M50, and K25 are 30.3, 56.5, and 87.9 respectively. Then it was compared with the density of GAPDH to validate the result. The ratio of COX-2 mRNA and GAPDH expression in M5, M50, and K25 are 73, 14.3, 21.3. The following chart is made according to the ratio of GAPDH and COX -2 mRNA expressions (FIGURE 2B).



A **B**

FIGURE 2. Density of the electrophoresis bands of the (**A**) VEGF-A mRNA of different NSAIDs as measured by ImageJ software. K1, K5, and K25 represents colonic fibroblast that have been treated with ketorolac at a concentration 1, 5 and 25 µg/mL respectively. Density of the electrophoresis bands of the (**B**) COX-2 mRNA of different NSAIDs as measured by ImageJ software. M5 and M50 are colonic fibroblast that have been treated with metamizole at a concentration 5 and 50 µg/mL respectively. K25 represents colonic fibroblast that have been treated with ketorolac at a concentration 25 µg/mL.

DISCUSSION

VEGF-A expression is absent in normal tissue and its up-regulation is provoked by mechanical injury to stimulate wound healing at normal tissue condition.[8] This supports the findings in this study. The fibroblast culture was obtained from the colon of a healthy rat. Therefore, no VEGF-A mRNA was expressed. External factors that can cause induction of VEGF-A mRNA expression include COX-2, pro-inflammatory cytokines, growth factors, hormones, and cellular stresses.[9] Ischemia and hypoxia are characteristics of wound injury. Hypoxia, a condition of low oxygen concentration, is said to be the principal regulator of VEGF-A expression.[9,10] Hypoxia - inducible factor-1α (HIF-1α), a regulator of cellular responses to physiological and pathological hypoxia is known as VEGF-A expression stimulator. The accumulation of HIF-1α protein due to hypoxia during inflammation will cause the activation of VEGF-A expression.

COX-2 mRNA is also not expressed on control because normally COX-2 is expressed under a certain condition such as injury. COX-2 expression is different from COX-1 expression that happens continuously.[11] To explain the non-existence of COX-2 on control sample it could be said that there was no inducer or promoter of COX-2 promotion. In the NSAID treated fibroblast, the inflammation is triggered

through the administration of NSAIDs and also might happen due to the blockage of COX-1. If the COX-1 that has a protective role for the fibroblast is blocked, the fibroblast damage will occur and the inflammatory response will follow.[12] Previous study has also proved that NSAIDs might trigger a flare in inflammatory bowel disease patients but the process remains unclear.[13] Thus, we suggest that the NSAIDs administration triggers the inflammation response in this experiment. It is proven that acetaminophen resembles the selective COX-2 inhibitor.[14] Therefore, there is no expression of COX-2 mRNA in the acetaminophen group.

Metamizole shows no selectivity in inhibiting COX isoenzymes. It inhibits both COX1 and COX2 enzymes.[15] COX-2 mRNA is expressed in the low dose (5 and 50 µg) of metamizole treated fibroblast. We suggested that metamizole which is having a weak anti-inflammatory effect, inhibits COX-1 stronger in low dose thus causes fibroblast damage and increase COX-2 expression. Meanwhile in higher concentration, metamizole might inhibits COX-2 stronger than COX-1.

Ketorolac is non-selective NSAID. However, Ketorolac in a high dose tends to block COX-1 and has been associated with gastrointestinal toxicity.[16] Since COX-1 has a protective role, we suggest that the COX-1 inhibition will stimulates injury than causes inflammation, including increase the expression of COX-2. The increase of COX-2 expression is followed by the increase of VEGF-A as shown in this study.

CONCLUSION

The effect of various kinds of NSAIDs towards VEGF-A and COX-2 mRNA expression of colon fibroblast is different. It might because of their different inhibition selectivity towards COX-1 and COX2.

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