

# The Relationship of The Amount of Caspase 3 Protein Expression and Macrophage Cells to Apoptosis of Squamous Epithel Cells in Ropivacaine Additional Area Around Operating Incisions (Laboratory Experimental Study on Wistar Strain Male White Mice)

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## Abstract Introduction

Surgery is sure to cause injury and cause pain which can interfere with wound healing if not handled adequately. In the process of wound healing, macrophages have a major role both as pro-inflammatory and anti-inflammatory agents, as well as the role of executors of cell death in regulating tissue hemostasis. Ropivacaine as a local anesthetic is believed to be able to help the wound healing process, apart from being an analgesic. Aim and Objective: This research was aimed to prove the effect of Ropivacaine on macrophages and caspase-3 which play a role in the wound healing process. Materials and Methods: This study is a quasi experimental prospective analysis with a randomized post-test-only control group design with white male Wistar rats as subjects. The samples were 24 subjects who met the inclusion and exclusion criteria which were divided into four groups, namely the treatment group on day 3 and 7 with 0.2% ropivacaine infiltration in the incision wound and the control group with 0.9% NaCl infiltration on day 3 and 7. Excisional biopsy procedures were done on third and seventh day. Examination under microscopy to assess expression caspase-3 and the amount of macrophages. Then analyzed by SPSS. Caspase-3 expression and the number of macrophage cells were lower than the control group ( $p < 0.05$ ). Results and Conclusion: There was a significant difference in the expression of caspase-3 protein in the control group on the 3rd day with the 3rd day treatment ( $p = 0.007$ ), the 7th day control group with the 7th day treatment ( $p = 0.008$ ), and the 7th day treatment group 3rd day with 7th day treatment ( $p = 0.026$ ), and the number of macrophage cells in all study groups ( $p = 0.0001$ ). Therefore, ropivacaine infiltration had an effect on decreasing the expression of caspase-3 protein and the number of macrophage cells on the 3rd and 7th day.

**Keywords:** Caspase-3, Infiltration of Ropivacaine, Macrophages, Wound Healing

## 1. Introduction

Wounds are defined as damage or disruption of the normal anatomical structure and function of the skin epithelial cells to the subcutaneous tissue, and injury to other structures such as tendons, muscles, blood vessels, nerves, parenchymal organs and even bones (Velnar et al., 2009).

In wounds, damage and cell death occur, which morphologically can be divided into two forms,

namely necrosis and apoptosis (Fawthrop et al., 1991). Apoptosis can occur via extrinsic pathways (triggered by ligand binding to Death Receptors) and intrinsic pathways (triggered in the mitochondria in response to various stressors or DNA damage) and is almost always associated with activation of caspase. Caspase is a cysteine protease, in extrinsic factor characterized by caspase-8 (activated by the death-inducing signaling complex) and intrinsic factor in the form of caspase-9 (activated by apoptomose) and caspase-2 (activated by p53 through injured DNA),

in the end the three initiator caspases activate the effectors namely caspase-3, caspase-6 and caspase-7 so that apoptosis occurs (Miller & Zachary, 2017). In injured tissue, an inflammatory response occurs which plays a role in the process of normal and pathological wound healing. The innate immune system was immediately activated when an injury occurs as a response to a local inflammatory process that attracts inflammatory cells from the circulatory system. One of them is macrophages around the area of injury which are activated by proinflammatory mediators in response to injury (Koh & DiPietro, 2011). Macrophages have many roles in the body, namely as a function of defense against pathogens such as microbes, playing a role in the process of homeostasis by removing residual material in the body and repairing tissue structures (Hirayama et al., 2017). Pain refers to a sensation and emotional experience caused by tissue damage, so that it can have a negative effect and delay the wound healing process, due to the increased stress it causes. Stress has an effect on psychology which can increase the hormone cortisol, thereby disrupting the function of inflammation and immunity (Upton & Solowiej, 2010). The process of wound healing that occurs physiologically and is organized consists of four main phases, namely homeostasis, inflammation, proliferation and remodeling (Wilkinson & Hardman, 2020). Local anesthetics also contributed in wound healing by providing anti-inflammatory effects, inhibiting the synthesis of mucopolysaccharides, reducing collagenization and reducing the number of mast cells in the wound area, which mainly occurs during the wound healing phase in the level of inflammation and proliferation (Huss et al., 2019). Ropivacaine is expected to be able to cut pain pathways and suppress endocrine responses to stress hormones, so that the process of apoptosis can be suppressed through inhibition of caspase-3 formation (Straub et al., 1998). In addition, Ropivacaine also reduces the number of inflammatory enzymes such as iNOS and COX-2 (L. Wu et al., 2019).

## 2. Research Method

### Study Design

This type of research was a prospective in vivo quasi experimental analytic using Wistar male white rats as an object with a "randomized post test only control group design". The treatment given was infiltration of the local anesthetic Ropivacaine with evaluation of caspase-3 protein expression and the number of macrophage cells. Preparations were made by staining HE and caspase-3 monoclonal antibody and then examined under a microscope.

### Study Sample

The inclusion and exclusion criteria were determined by the veterinarian in charge of the unit. The inclusion criteria include: (1) Thoroughbred; (2) Age two to two and a half months; (3) Weight 250-300 grams; (4) Male

sex; (5) No abnormalities in the skin anatomy of the rats were seen, after being evaluated by a UPHP veterinarian. Exclusion criteria included: (1) Sick during the 7-day adaptation period, which was examined by a UPHP veterinarian; (2) Mice behaved aggressively and attacked other rats, in observation during the treatment process. Drop out criteria included: (1) Dead before the treatment process; (2) Infection on the skin before the treatment process, which was evaluated by a UPHP veterinarian. A total of 24 rats were divided into 2 control groups and 2 treatment groups, each group consisted of 6 rats and both treatment groups were given Ropivacaine infiltration, then terminated on the 3rd and 7th day. Histological evaluation was performed to evaluate the expression of caspase-3 protein and macrophage cells around the wound tissue.

### Study Procedure

The white male Wistar rats were grouped by simple random method, grouped into 4 experimental groups. Each group consisted of 6 rats, divided into 4 cages with the size of each cage 30x20x7 cm. The white male Wistar rats underwent wound incision along 2 cm on their backs after being shaved and sterilized, and administered 0.2% ropivacaine infiltration, then evaluated on days 3 and 7 for control groups and treatment groups. All treatment groups were anesthetized using ketamine-xylozine at a dose of 75-100 mg/kg + 5-10 mg/kg intraperitoneally with duration of 10- 30 minutes. In the treatment group 1, after anesthetized, the hairs around the back were shaved, then disinfected using povidone iodine. Subsequently, incision was done along the 1 cm with depth in subcutis tissue. Afterwards, subcutaneous tissue was given 0,5 ml of 0.2% ropivacaine infiltration approximately 0.5 cm around the wound, then covered with aseptic sterile plaster. Histology preparations, hematoxylin-eosin staining, and immunohistochemical preparations procedures were carried out using standard methods. The research data results were recorded, collected, and processed. Saphiro-Wilk test was used to determine the normality the data. Analysis for comparison with parametric data scale used analysis of variant (ANOVA) and Post Hoc test LSD. Analysis for comparison purposes with nonparametric data scale used Mann-Whitney and for correlation purposes used Kruskal-wallis test.

## 3. Results and discussion

### Effect of Ropivacaine Infiltration around the Wound on Caspase-3 Protein Expression in Tissues in the Wound Healing Process between the Control Group and Treatment Group in Day 3 and Day 7

In the process of maintaining the stability of squamous epithelial cells in the skin both under normal and pathological conditions, protein caspase-3 played a role in stimulating cell apoptosis, which also contributed in the healing process of incision wounds.

The process of identifying caspase-3 protein expression in this study was by carrying out immunohistochemical examination by identifying

and localizing antigens in squamous epithelial tissue samples examined using caspase-3 specific monoclonal antibodies. Squamous epithelial cells

expressing the Caspase 3 protein has various forms of differentiation and maturation and will be colored (silver) brown as shown in Figure 1.

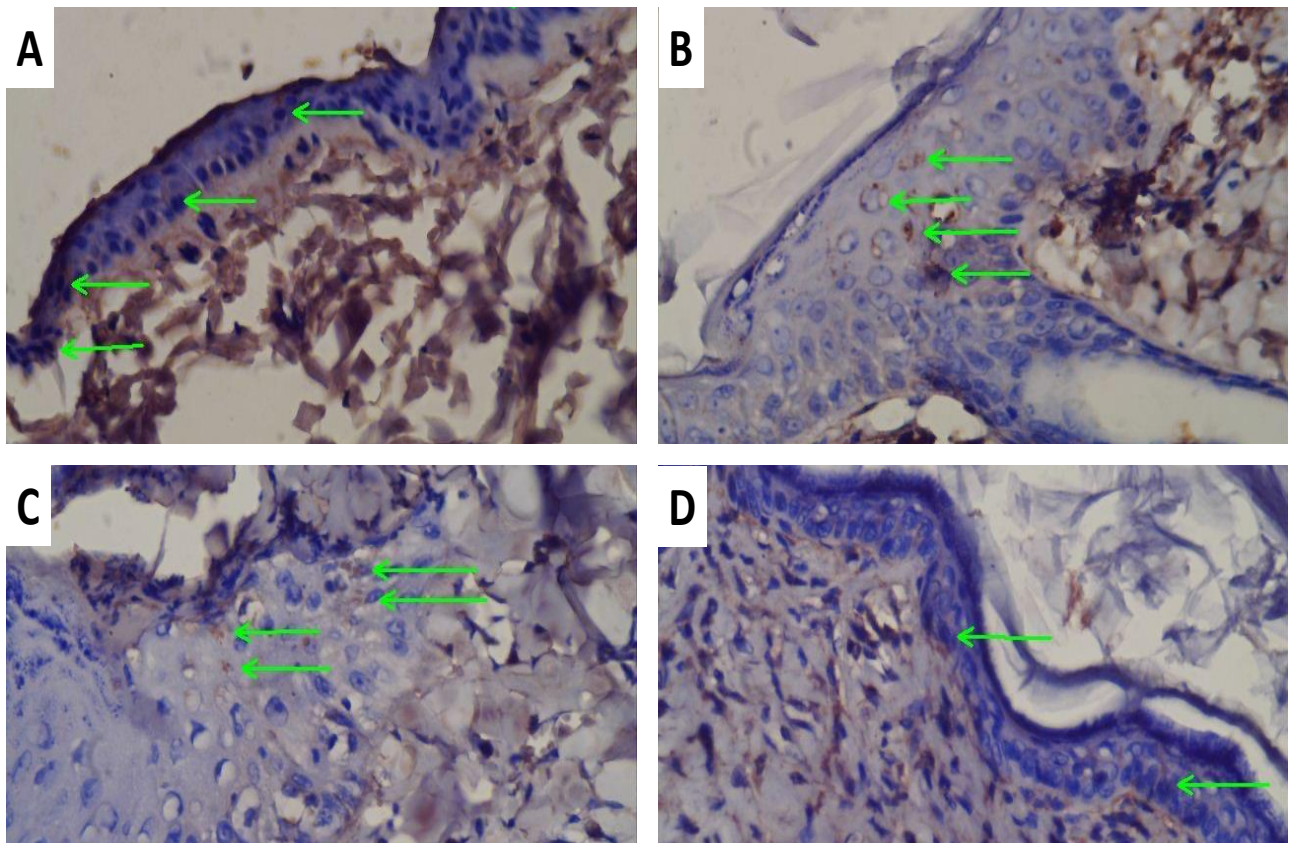


Figure 1: Expression of Caspase-3 Protein (green arrow) in Wound Preparations with Immunohistochemistry Staining (Magnification 400x). (A) Control group on day 3, (B) Control group on day 7, (C) Ropivacaine injection group on day 3, and (D) Ropivacaine injection group on day 7

On the 3rd day of observation (Figure 1), it was seen that the expression of protein caspase-3 was found more in the control group (C1) than in the treatment group (T1), as well as on the 7th day (Figure 1) it was seen that the expression of protein caspase-3 found more in the control group (C2) than in the treatment group (T2). In the control group on day 3 (C1), the highest value of protein caspase-3 expression was seen, while the lowest value was found in the observation of the treatment group on day 7 (T2) as shown in Figure 2.

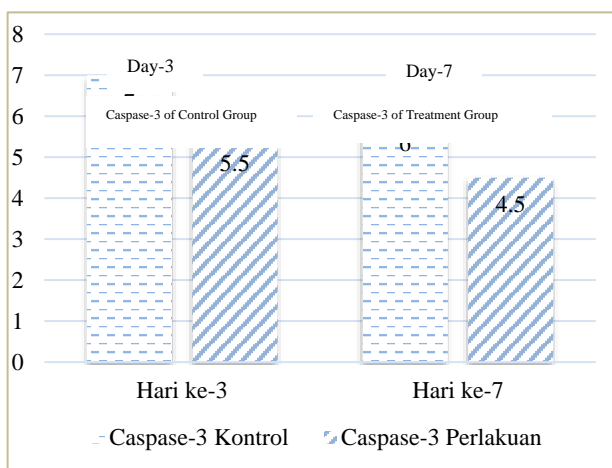


Figure 2: Expression of Caspase-3 Protein Day 3 and 7

Table 1 showed the expression of protein caspase-3 in the control and treatment groups with the Allred

Scoring System in each group. It was found that there was a significant effect of infiltrating Ropivacaine around the wound on the expression of caspase-3 protein in the tissue during the wound healing process ( $p < 0.05$ ). These results were described in more detail in Table 1, Table 2, and Table 3.

Day	n	Control		Treatment		p value
		Median	Min-Max	Median	Min-Max	
D-3	6	7	6-8	5,5	5-6	0,001 <sup>a</sup>
D-7	6	6,16	5-7	4,5	4-5	

a. Kruskal-Wallis test

There was a significant difference in the expression of caspase-3 in all groups, except between groups C1 and C2, namely the control group on day 3 and day 7 (see Table 2).

Group	n	Caspase-3		p value
		Median	Min-Max	
C1	6	7,0	6-8	0,081 <sup>a</sup>
C2	6	6,16	5-7	

a. Mann-Whitney U test

Meanwhile, there was a decrease in caspase-3 expression in the control group on day 3 (C1) compared to the treatment group on day 3 (T1) (see Table 3).

**Table 3: Differences in Caspase-3 Protein Expression in the Control Group on Day 3 and the Treatment Group on Day 3**

Group	n	Caspase-3		p value
		Median	Min-Max	
C1	6	7,0	6-8	0,007 <sup>a</sup>
T1	6	5,5	5-6	

a. Mann-Whitney U test

There was a decrease in caspase-3 expression in the control group on day 7 (C2) compared to the treatment group on day 7 (T2) (see Table 4).

**Table 4: Differences in Caspase-3 Protein Expression in the Control Group on Day 7 and the Treatment Group on Day 7**

Group	n	Caspase-3		p value
		Median	Min-Max	
C2	6	6,16	5-7	0,008 <sup>a</sup>
T2	6	4,5	4-5	

b. Mann-Whitney U test

The Effect of Ropivacaine Infiltration Around the Wound on the Expression of Caspase-3 Protein in Tissues in the Process of Wound Healing Day 3 to 7

There was a significant decrease in caspase-3 expression in the treatment group on day 3 (T1)

compared to the treatment group on day 7 (T2) with  $p=0.0026$  (Table 5). Hence, it can be concluded that the infiltration of Ropivacaine can affect the decrease in the expression of protein caspase-3 around the wound.

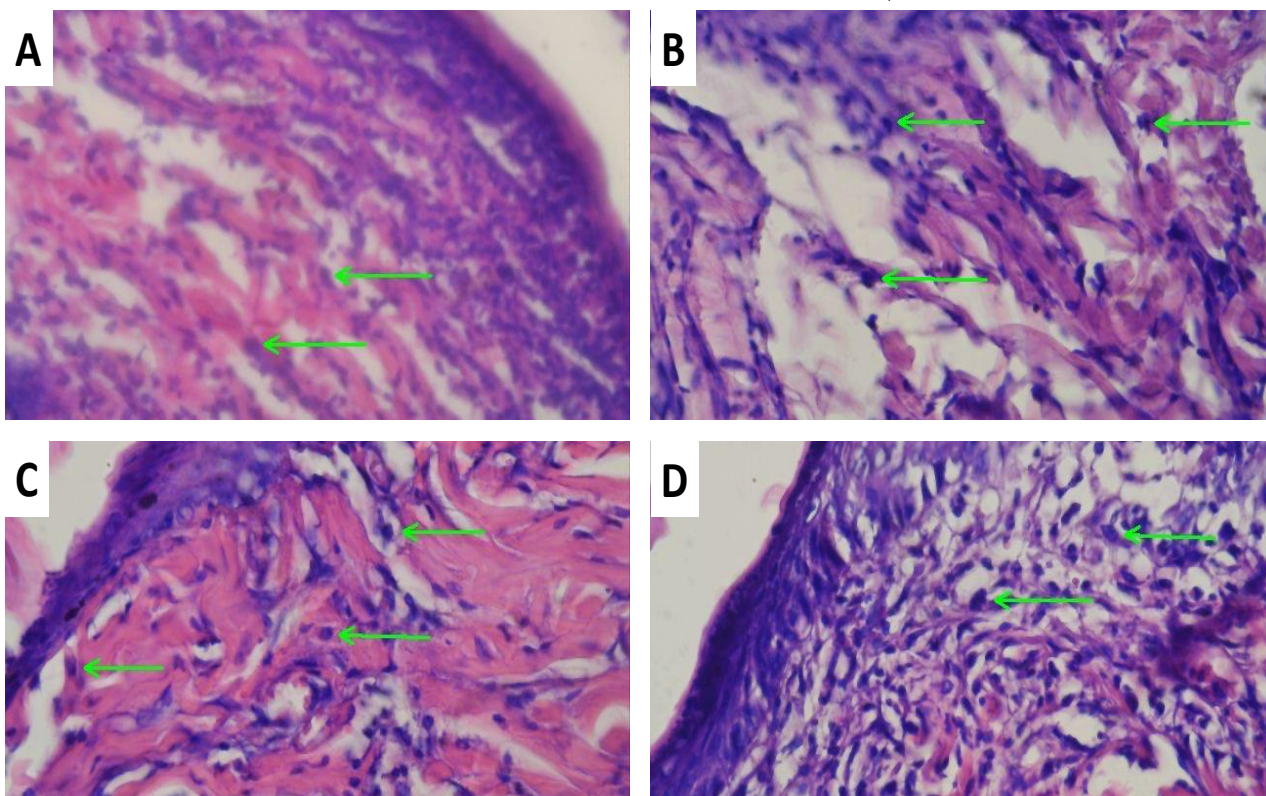
**Table 5: Differences in Caspase-3 Protein Expression on Days 3 and 7 After Ropivacaine Infiltration Around the Wound (Treatment Group)**

Group	n	Caspase-3		p value
		Median	Min-Max	
T1	6	5,5	5-6	0,026 <sup>a</sup>
T2	6	4,5	4-5	

a. Mann-Whitney U test

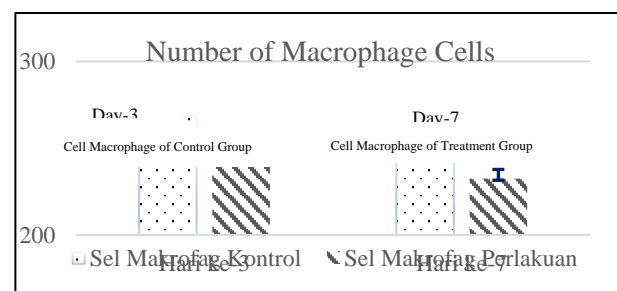
Effect of Ropivacaine Infiltration around the Wound on the Number of Macrophage Cells in Tissues in the Wound Healing Process between the Control Group and Treatment Group in Day 3 and Day 7

Identification of macrophage cells in skin squamous epithelial cells was taken from the research sample, which was then stained with Hematoxylin Eosin (HE). Macrophage cells were characterized by dark purple round and oval shapes (see Figure 3).



**Figure 3: Number of Macrophage Cells (green arrows) in Wound Preparations with Hematoxylin and Eosin Staining (400x Magnification). (A) 3rd day control group, (B) 7th day control group, (C) 3rd day Ropivacaine injection group, and (D) 7th day Ropivacaine injection group**

On the 3rd day of observation (Figure 3), especially in the control group (C1) it was seen that the number of macrophage cells was higher than in the treatment group (T1). Meanwhile, on the 7th day it was seen that the control group had more macrophage cells than the treatment group (T2). In the control group on day 3 (C1), the highest number of macrophage cells was seen, while the lowest number was found in the observation on day 7 (T2) as seen in Figure 4.



**Figure 4: Number of Macrophage Cells on Day 3 and 7**

Table 6 showed the difference in the number of macrophage cells in all study groups. There was a significant difference between the four study groups ( $p < 0.05$ ).

Group	Number of Macrophage Cells (Mean ± SD)	p value
C1	268,83±2,92	0,0001 <sup>a</sup>
C2	255,33±4,32	
T1	243,5±4,03	
T2	232,5±5,24	

a. one-way ANOVA

Table 7 shows the differences in each research group. There was a significant difference between the control group on day 3 and day 7, namely the number of macrophages in the control group on day 3 was 13.50 ( $p = 0.0001$ ) higher. There was a significant difference between the control group on day 3 and the treatment group on day 3, namely the number of macrophages in the control group was 25.333 higher ( $p = 0.0001$ ). In addition, there was a significant difference between the control group on day 7 and the treatment group on day 7, namely the number of macrophages in the control group was 22.833 higher ( $p = 0.0001$ ). Therefore, it can be concluded that there was a decrease in the number of macrophages due to infiltration of Ropivacaine around the wound, both on day 3 and day 7. This was supported by Figure 3 which showed the pathological anatomical preparations stained with hematoxylin & eosin, and 400x magnification.

Group	Mean Difference	CI (95%)	Sig. (p value)
C1 - C2	13,500	6,81 - 20,18	0,0001 <sup>a</sup>
C1 - T1	25,333	18,99 - 31,67	
C2 - T2	22,833	14,29 - 31,37	
T1 - T2	11,000	2,63 - 19,36	

a. LSD Post Hoc test ( $p < 0.05$  mean data is significantly different)

### The Effect of Ropivacaine Infiltration Around the Wound on the Number of Macrophage Cells in Tissues in the 3rd to 7th Days of Wound Healing Process

There was a significant difference between the treatment group on day 3 (T1) and the group on day 7 (T2), namely the number of macrophages in the treatment group on day 3 was 11,000 higher ( $p = 0.0001$ ) as shown in Table 7. As such, it can be concluded that the infiltration of Ropivacaine could affect the decrease in the number of macrophages around the wound.

## Discussion

Wound healing is a gradual and complex process that is influenced by many factors, such as cellular and paracellular pathways (Eming et al., 2014; Gurtner et al., 2008; Janis & Harrison, 2014; Rousselle et al., 2019). Wound healing consists of four stages, namely hemostasis, inflammation,

proliferative, and remodeling stages. During the healing process, the expression of caspase-3 protein and the number of macrophages play an important role, especially during the inflammatory phase. Meanwhile, Ropivacaine was the safest and most frequently used long-term local anesthetic because of its low toxicity to the cardiovascular system and central nervous system (B.-L. Li et al., 2020; Scherb et al., 2009; Wang et al., 2020).

In this study, there was a decrease in caspase-3 expression in the control group on day 3 compared to the treatment group on day 3. In addition, there was a decrease in caspase-3 expression in the control group on day 7 compared to the treatment group on day 7. This proves that there was a significant effect of Ropivacaine infiltration around the wound on the expression of caspase-3 protein in the tissue in the process of wound healing ( $p < 0.05$ ). This was supported by previous research, which found a decrease in the expression of caspase-3 protein (Abdurrahman et al., 2022). Due to limited research regarding caspase-3 expression and clinical benefits in wound healing, the role of caspase-3 still needs to be proven. Previous research has examined in vitro caspase-3 increases in scars and keloids compared to normal wounds (Akasaka et al., 2000; Slomiany et al., 1998).

Another study found that the expression of caspase-3 protein represents the process of apoptosis due to stress on cells in the process of wound healing (Riwaldt et al., 2021). The decrease in caspase-3 protein may represent a decrease in cell apoptosis. Caspase-3 activation was controlled by caspase-8 (Siegmund et al., 2017; Yang, 2015). Caspase-8 is an extrinsic pathway for caspase-3 signaling to apoptosis. Caspase-3 along with caspase-6 and caspase-7 carry out the process of breaking down proteins which become intracellular type I keratin and intermediate filaments which were observed at the beginning of the wound healing process (Oshima, 2002; Riwaldt et al., 2021). Apoptosis is an important process in the early phase of wound healing. High apoptosis may cause obstacles in the wound healing process. Research has shown that in stimulated conditions such as in outer space due to gravity disturbance can cause increased apoptosis and inhibit the wound healing process (Neutelings et al., 2015; Riwaldt et al., 2021). The process includes apoptosis of lymphocytes and endothelial cells, as well as activation of signaling pathways that support apoptosis (Liu & Wang, 2008).

In wound healing, there was an inflammatory process followed by the migration of neutrophils and macrophages into the wound tissue (Tidball, 2005). Macrophage cells also played a role in tissue destruction, elimination of necrotic tissue, and inhibition of satellite cells (Novak et al., 2014). Satellite cells played a positive role in the wound healing process. Neutrophils infiltrate the wound tissue and then differentiate into macrophages. Macrophages could trigger inflammatory effects on surrounding tissues and cells (Brand, 2009).

Macrophages contributed in the stages of inflammation, proliferative, and remodeling. Currently, the known macrophages were the M1 and M2 macrophages. The difference was that M1 macrophages were called pro-inflammatory and M2 macrophages called anti-inflammatory (Ferrante & Leibovich, 2012; Mosser & Edwards, 2008). Both played an important role in achieving balance and speed of wound healing. Previous study showed that M1 macrophages were detected on day 5 whereas on day 10, these macrophages were expected to decrease (Mirza & Koh, 2011).

Ropivacaine is a long acting local anesthetic and has a low toxicity effect, so it is more often used in postoperative wounds (B.-L. Li et al., 2020; K. Li et al., 2020; Wang et al., 2020). However, previous studies have shown that a 0.75% concentration of Ropivacaine can cause a decrease in the wound healing process. Other studies have shown no negative effect on the wound healing process even up to two weeks postoperatively using Ropivacaine 0.5% (X. Wu et al., 2022). This study used Ropivacaine 0.2% and showed a positive effect on wound healing which was represented by decreasing the expression of caspase-3 protein, as an anti-apoptotic agent, and the number of macrophage cells. Previous studies have also shown that 0.2% Ropivacaine infiltration can increase collagen synthesis in the wound healing process (Pramono et al., 2016). Ropivacaine 0.2% has a positive effect on the wound healing process through two mechanisms. First, Ropivacaine could reduce postoperative pain and reduce the production of proinflammatory cytokines (Cha et al., 2012; O'Neill et al., 2012). Ropivacaine also has anti-inflammatory effects (Cassuto et al., 2006). High levels of inflammation played a negative role in the wound healing process so that the anti-inflammatory effect of Ropivacaine plays an important role in the wound healing process (Deegan et al., 2010; Hu et al., 2010). These anti-inflammatory and analgesic effects has a positive impact on postoperative outcomes, such as shorter hospital stays or recovery rates (Sun et al., 2021).

#### 4. Conclusion

There is a significant effect of Ropivacaine infiltration around the wound on the expression of caspase-3 protein and the number of macrophage cells in the tissue in the process of wound healing. There is a significant effect of Ropivacaine infiltration around the wound on the expression of caspase-3 protein and the number of macrophage cells in the tissue during the 3rd to 7th day wound healing process.

#### Conflict of Interest:

The authors hereby declare that there is no conflict of interest in this study.

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#### Author Contribution

A) Dana Wandrianbaraseta -contributed in designing the study, execution of the project, statistical analysis, manuscript drafting.

B) Prananda Surya Airlangga -contributed in designing the study, execution of the project, statistical analysis, manuscript drafting.

C) Imam Susilo -contributed in designing the study, execution of the project, statistical analysis, manuscript drafting.

D) Prihatma Kriswidyatomo -contributed in designing the study, statistical analysis, manuscript drafting.

E) Hamzah -contributed in study design, guiding the research work, proofreading and manuscript correction.

F) Mahmudah -contributed in study design, guiding the research work, proofreading and manuscript correction.

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