Transforming Growth Factor Beta Expression and The Amount of Fibroblast Cells on Ropivacaine Administration around the Surgical Incision (Laboratory Experimental Study on The White Male Wistar Rats)

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Abstract

Wounds can be defined as a pathological condition characterized by damage to the microstructure and function of normal anatomical tissue. Wound healing has a complex mechanism of cellular function involving the interaction of multiple cells, growth factors, and cytokines. TGF-β is a multifunctional cytokine that plays an important role in the regulation of several cellular processes and cell differentiation that are known to contribute to the wound healing process. Fibroblasts present in blood clots proliferate and secrete extracellular matrix (ECM) and also contribute to the formation of new granulation for proliferation phase. This study is a true experimental prospective analysis with a randomized post-test-only control group design with white male Wistar rats as subjects. The sampling technique used the probability sampling method, namely stratified random sampling. There 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. An anatomical pathologist then performed an immunohistochemical examination under microscopy to assess TGF β-expression and the amount of fibroblast cells.

Keywords: *Wound Healing, Ropivacaine Infiltration, TGF-β, Fibroblast.*

1. Introduction

Wounds can be defined as a pathological condition characterized by damage to the microstructure and function of normal anatomical tissue [1]. When an injury occurs, the body's natural physiological response to tissue injury begins the wound-healing process. Wound healing has a complex mechanism of cellular function involving the interaction of multiple cells, growth factors, and cytokines [2]. TGF-β is a multifunctional cytokine that plays an important role in the regulation of several cellular processes and cell differentiation that are known to contribute to the wound healing process [3]. The effects of TGF-β signaling are multipotent in each phase of wound healing directly in the function of monocytes, endothelial cells, fibroblasts, and keratinocytes [3]. Fibroblasts present in blood clots proliferate and secrete extracellular matrix (ECM) and also contribute to the formation of new granulation tissue and blood vessels to maintain the viability of new tissue [3,4] Improper pain management can impede wound healing. In pain, levels of beta (β) endorphins secreted by the pituitary gland will increase and suppress macrophages so that macrophage activity decreases resulting in cytokines released by macrophages such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1, IL -6, IL-8, TGF-β will also decrease [5]. Local anesthetics are drugs that are commonly used to relieve pain caused by trauma, and ropivacaine is one of them [6]. Local anesthetic infiltration can reduce pain intensity by inhibiting pain impulse transmission pathways, and it is also associated with a decrease in the inflammatory response associated with the wound healing process [7-9].

2. Materials and Methods

Sample

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 sampling technique used the probability sampling method, namely stratified random sampling. This study was conducted at the Department of Anatomical Pathology, Faculty of Medicine, Airlangga University, Surabaya. An ethical certificate is issued by the Ethics Committee of the Faculty of Medicine, Airlangga University, Surabaya No. 216/EC/KEPK/FKUA/2022.

The inclusion and exclusion criteria were determined by the veterinarian in charge of the unit. The inclusion criteria include: (1) pure descent and male category; (2) two to two and a half months old; (3) body weight 250-300 grams; (4) no anatomical abnormalities were observed. Exclusion criteria included: (1) illness during the 7-day adaptation period; (2) previous scars; (3) anatomical abnormalities in rats; (4) there is a 10% weight loss during the adaptation period. There were 24 subjects who met the inclusion and exclusion criteria which were divided into four groups, namely the treatment group on days 3 and 7 with 0.2% ropivacaine infiltration in the incision wound and the control group with 0.9% NaCl infiltration on days 3 and 7. An anatomical pathologist then performed an immunohistochemical examination under microscopy to assess TGF β-expression and the amount of fibroblast cells.

Research Implementation

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-xylazine 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 is done along the 2 cm with depth in subcutis tissue. Then subcutaneous tissue was given 1 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.

Statistic Test

Research results are recorded, collected and processed. Kolmogorov-Smirnov test was used to determine the normality of the data. Analysis for comparison purposes with parametric data scale used analysis of variant (ANOVA) and for correlation purposes used Spearman's rho correlation test. Analysis for comparison purposes with nonparametric data scale used Mann-Whitney.

3. Results

Calculation of TGF-β expression on immunohistochemical staining using TGF-β specific antibodies.Cells that expressing TGF-β appear brown in the cytoplasm as shown in the figure. The calculation of this examination was conducted using the Allred Scoring Guideline which is the sum of the proportion scores and intensity scores of cells expressing TGF-β. While the amount of fibroblast cells in hematoxylin eosin staining is colored by purplish blue and oval in shape as shown in Figure 1, the calculation is carried out by adding up all fibroblast cells in micorscopic fields of view with 400x magnification. There are 3 sites to be examined, namely the two edges of the wound and the middle of the wound.

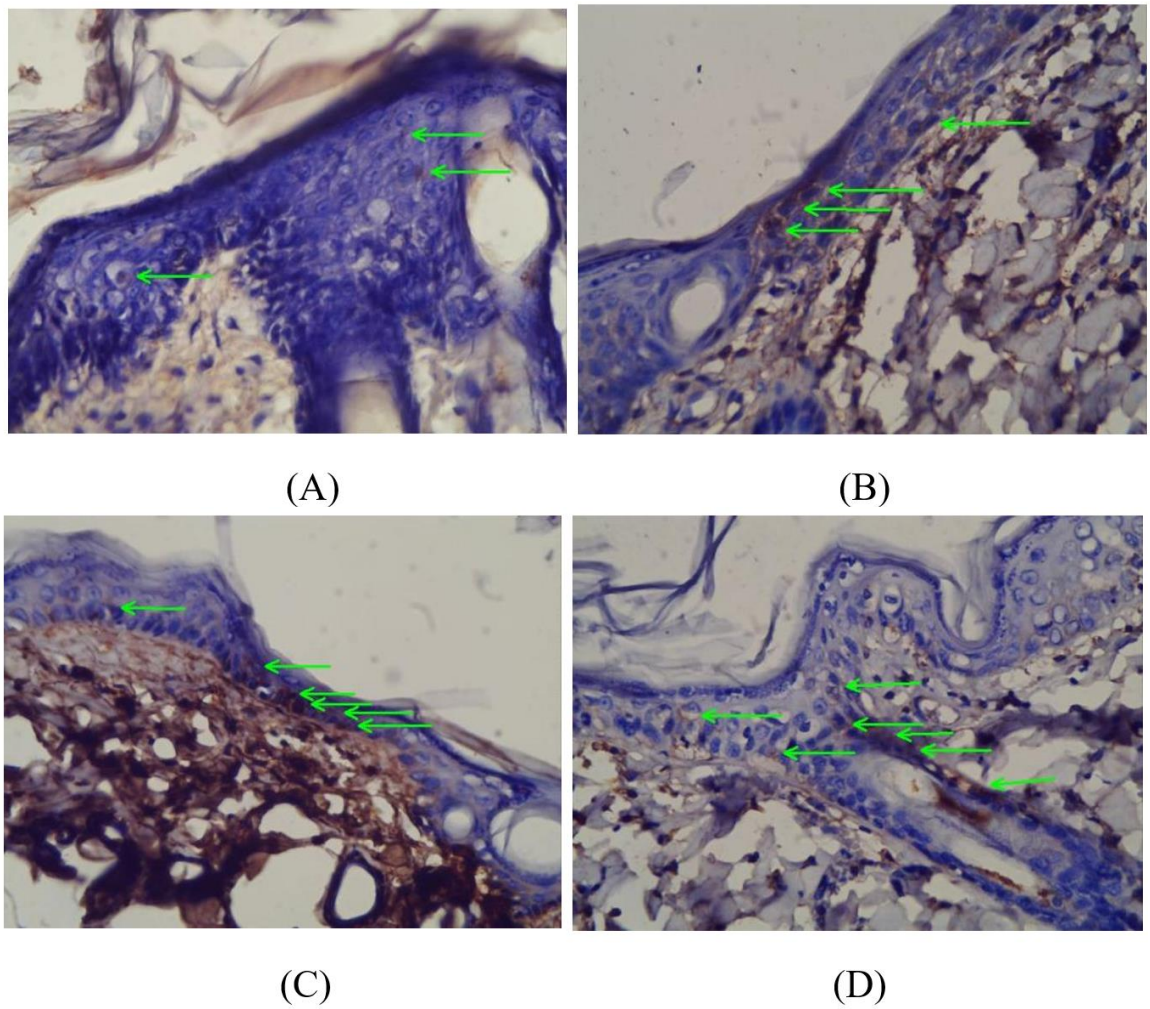


Figure 1. TGF-β expression (green arrow) in control group (A) on day 3; (B) control group on day 7; (C) treatment group on day 3 ; and (D) treatment group on day 7

On the third day observation of the control group showed relatively less TGF-β expression compared to the seventh day. In the treatment group, TGF-β expression was more than the control group. And the amount of TGF-β expression in the treatment group on the seventh day was more than on the third day.

Table 1. TGF-β expression

Variable	Control (n=6)		Treatment (n=6)		P value
	Mean	SD	Mean	SD	
Day 3	4.67	0.516	6.0	0.632	0.009*
Day 7	5.0	0.632	7.0	0.632	0.002*

* Mann-Whitney test, significant if p<0,05

Table 2. Expression of TGF-β treatment group

Variabel	Day 3 (n=6)		Day 7(n=6)		P value
	Mean	SD	Mean	SD	
TGF-β	4.67	0.516	5.0	0.632	0.485*

* Mann-Whitney test, significant if p<0,05

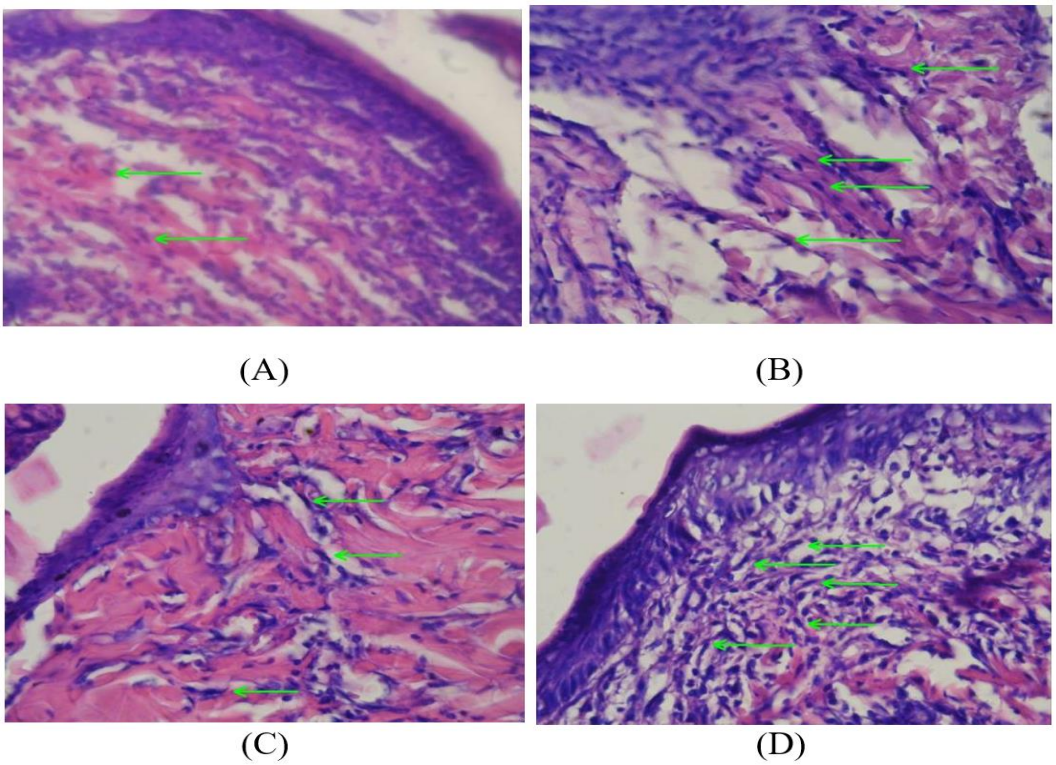
Table 3. Expression of TGF-β treatment group

Variabel	Day 3 (n=6)		Day 7(n=6)		P value
	Mean	SD	Mean	SD	
TGF-β	6.0	0.632	7.0	0.632	0.041*

* Mann-Whitney test, significant if p<0,05

Table 1 shows a significant difference in TGF-β expression between the treatment group and the control group was examined on the third day ($p = 0.009$; $p < 0.05$). This also happened on the seventh day where TGF-β expression increased and there was a significant difference between the two groups ($p = 0.002$; $p < 0.05$). The treatment group on the third day to the seventh day showed no significant difference for table 2 ($p = 0,485$; $p > 0.05$).

Figure 2 The amount of fibroblast cells (green arrow) in control group (A) on day 3;



(B) control group on day 7; (C) treatment group on day 3; and (D) treatment group on day 7

On the third day observation of the control group showed a lower number of fibroblast cells compared to the seventh day. In the treatment group the amount of fibroblast cells was more than the control group. And the amount of fibroblast cells in the seventh day treatment group was more than the third day.

Table 4. The Amount of Fibroblast Cells

Group	Day 3 (n=6)		Day 7(n=6)		P value
	Mean	SD	Mean	SD	
Control	210.67	3.67	222.67	3.14	0.001*
Treatment	224.5	2.739	248.17	6.01	0.001*
P value		0.001*		0.001*	

P* One Way Anova & Post Hoc LSD

Table 4 shows a significant difference in the amount of fibroblast cells between the treatment group and the control group that was examined on the third day ($p = 0.001$; $p < 0.05$). This also happened on the seventh day where the amount of fibroblast cells increased and there was a significant difference between the two groups ($p = 0.001$; $p < 0.05$). The control group on the third day to the seventh day showed a significant difference ($p = 0.001$; $p < 0.05$). This also happened in the treatment group where there was a significant difference on the third day to the seventh day ($p = 0.001$; $p < 0.05$).

Tabel 5. Correlation of TGF-β expression with the amount of fibroblast cells

Variable	P value	Correlation coefficient
Day 3	0,01*	0,707
Day 7	0,001*	0,825

**Spearman's rho* correlation test; significant if p<0,05

The correlation between TGF-β and the number of fibroblast cells is quite significant. If we look at the third day and the seventh day, there is a significant correlation. Table 5 shows a significant correlation between TGF-β expression and the number of fibroblast cells on the third day between the treatment and the control groups ($p = 0.01$; $p < 0.05$). The table also shows a significant correlation between TGF-β expression and the number of fibroblast cells on the seventh day between the treatment and control groups ($p = 0.001$; $p < 0.05$).

4. Discussion

In this study, the effect of ropivacaine infiltration on TGF-β expression was clearly significantly different compared to the control group. This can be seen on the third day where the expression of TGF-β was more in the treatment group. In accordance with the explanation of the existing theory that ropivacaine is not only a local anesthetic but also has a direct influence on the occurrence of the inflammatory process. Ropivacaine infiltration can increase the expression of TGF-β thereby accelerating the inflammatory process and proliferative processes immediately occur. This increase is expected to accelerate the wound healing. This is in line with study which explains that administration of 0.5% ropivacaine infiltration can increase the amount of TGF-β with

immunohistochemical antibodies on the third day compared to the control group who received 0.9% saline infiltration [10]. Administration of local anesthetic infiltration in the surgical incision area can reduce postoperative pain and cell death. This can be explained by the reduced production of cytokines due to adminstration of ropivacaine which inhibits pain transmission pathways. Ropivacaine also has other cellular function related to inflammation, namely anti-inflammatory effects [11]. Administration of local anesthetics can inhibit leukocyte adhesion to blood vessel walls, induce prostacyclin release, and cause release of leukocytes that were previously firmly attched to blood vessel endothelium [10].

Likewise on the seventh day where in this phase, it has become a proliferative phase. And in this study showed that TGF-β expression increased in the treatment group compared to the control group. This suggests that TGF-β plays an important role in the regulation of the wound healing phase, both in the inflammatory and proliferative phases. Ropivacaine infiltration in sugical wounds is one of the factors that can help better wound healing. The role of ropivacaine infiltration can occure due to good control of the inflammatory phase, so the role of TGF-β in the proliferative phase can immediately function according to the influence of TGF-β on the wound healing process such as ECM formation and fibroblast activation. As in Pakyari's study (2013) showed that the amount of TGF-β can determine the transition from inflammation to the proliferative phase during the wound healing process which will produce more TGF-β on the seventh day compared to the first day after skin damage [12]. In humans, TGF-β expression will increase on the seventh day in regulating scar tissue contraction and modulating fibroblasts to become myofibroblast [13].

TGF-β expression on the seventh day also incresed compared to the third day in the treatment group and in the control group, but there was no significant difference. This does not affect the results of the study because with an increase in TGF-β expression, it has been proven that TGF-β will increase in each phase because of its function. TGF-β has a role in the inflammatory phase which was shown on the third day of examination, while in the proliferative phase, it was shown on the seventh day of examination. The role of TGF-β in the proliferative phase includes many processes such as epithelialization, ECM production, angiogenesis, and myofibroblast activation. The role of this function may cause an increase in TGF-β expression on the seventh day. In normal incision wounds, TGF-β will reach a peak around seventh day to fourteenth day which directly increases ECM production and causes fibroblasts to synthesize type 1 collagen [14,15].

The amount of fibroblast cells indication the proliferative process also increased in the treatment group on the third day compared to the control group and there was a significant difference. This shows that the effect of ropivacaine in accelerating the inflammatory process can affect the acceleration of the proliferative process. As previously explained, the estimation of the proliferative process occured from fourth day to twenty-first day, but this study has shown that the effect of ropivacaine can increase the amount of fibroblast cells on third day. Previous study also explained that fibroblasts which are responsible for collagen production, especially type 1 collagen, will increase on third day in the group of mice that given 0.5% ropivacaine infiltration [10]. Granulation tissue is a combination of

cellular elements including fibroblasts and inflammatory cells together with the formation of collagen tissue. Fibroblasts are the main elements that produce large amounts of collagen which is the main element of the extracellular matrix which is useful for torming strength in scar tissue. In Pramono's study (2016), who assessed collagen on fifth day, it showed a significant increase in the group that received 2.52 mg of ropivacaine infiltration compared to the control group with 0.9% NaCl infiltration using pure strain Wistar rats [16].

The amount of fibroblast cells on the seventh day also increased in the treatment group compared to the control group. This shows that ropivacaine infiltration can affect the amount of fibroblast cells quite a lot in the proliferative phase. Benefits like this are expected so that wound healing can be better. Fibroblasts are actibe and highly proliferative within 2 – 4 days after injury where they increase three and a half times and are maintained in the long term period. Within 3 – 7 days, fibroblasts will differentiate and then will secrete ECM proteins [17]. The increase in fibroblasts in murine wounds begins on the third day and reaches a peak on the seventh day after injury where it produces ECM including type I and III collagen [18]. The amount of fibroblast cells on the seventh day increased compared to the third day in the treatment group and in the control group and there was a significant difference. This is in accordance with the explanation from the literature review that fibroblasts are indicators of proliferation. Good wound healing will result in an increase in the amount of fibroblast cells in the proliferative phase.

This study shows that the increase in TGF-β expression is significantly correlated with the amount of fibroblast cells. The longer the variable is examined, the stronger the correlation between TGF-β expression and the amount of fibroblast cells will show. This shows that the function of TGF-β in stimulating chemotaxis of fibroblast cells is proven in this study. Fibroblasts are the main components affected by TGF-β. TGF-β and fibroblasts are the basis for the proliferative and maturation phases of wound healing. As also explained in previous studies, fibroblasts that are responsible for collagen production, especially type 1 collagen, increased on the third day in the group with 0.5% ropivacaine infiltration in proportion to the increase in the amount of TGF-β [10]. TGF-β which plays a role in increasing the extracellular matrix (ECM) and increasing collagenization which is the result of fibroblast cells secretion after being stimulated by TGF-β. This is consistent with the normal process of wound healing where the amount of growth factors will increase until the maturation phase, so that the density of collagen will increase [19,20].

This study still has limitations because it only assessed the TGF-β on the third day and the seventh day and the amount of fibroblast cells which only represented the inflammatory and proliferative phases. This study also did not assess the role of TGF-β and fibroblast cells in the hemostasis phase and the maturation and remodeling phases which are part of the wound healing process.

5. Conclusion

There is a significant effect of giving ropivacaine infiltration around the surgical incision wound. TGF-β expression will increase on the third and seventh day in the group with ropivacaine infiltration. The amount of fibroblast cells also increased on the third and

seventh day in the group with ropivacaine infiltration. There is a significant correlation between TGF-β expression and the number of fibroblast cells where an increasing in TGF-β expression will also increase the amount of fibroblast cells.

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Conflict of Interest

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

Author contribution

All authors contributed equally in conducting the study as well as writing and revising the manuscript.

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