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The Effect of Hypoxic Mesenchymal Stem Cells on The Gene Expression of *Transforming Growth Factors-β* in Wistar Rats Excision Wound Model

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ABSTRACT

Background: Hypoxic Mesenchymal stem cell (MSC) therapy may accelerate the wound healing process through a paracrine mechanism by increasing the expression of transforming growth factor-β (TGF-β).

Objective: This study aims to investigate the effect of hypoxic MSC on TGF-β gene expression in excision wound models.

Methods: This is an experimental study with a post-test-only control group design. Sixty male Wistar rats were divided into 4 groups consisting of 5 each to represent group I (sham/normal control), group II (excision wound model), group III (excision wound model and normoxic MSC injection), group IV (excision wound model and hypoxic MSC injection) for observation of TGF-β gene expression on days 3, 6 and 9. Both MSC ($3x10^6$ cells) was injected subcutaneously after wound exision at five locations 0,5 cm from the wound edge. TGF-β gene expression was examined by qRT-PCR.

Results: The highest average TGF-β gene expression on the three observation days were shown by group II. TGF-β gene expression in groups III and IV was lower than in group II, while groups III and IV were relatively similar. In the normal wound healing process, TGF-β is highly expressed, and both MSC injection reduces TGF-β gene expression.

Conclusion: Hypoxic MSC injection accelerated the proliferative phase of the excisional wound healing process, but the acceleration effect was equivalent to that shown by Normoxic MSC.

Keywords: Mesenchymal stem cells, Hypoxic, Normoxic, TGF-β, Excision Wound.

INTRODUCTION

Wound management is one of the main pillars of patient care in all health care services[1,2]. Inadequate wound management will result in the wound becoming wider and causing prolonged inflammation, causing an increase in the burden for sufferers and for health services[3]. Recent research has shown that mesenchymal stem cell (MSC) therapy can accelerate the wound healing process through a paracrine mechanism by increasing the expression of transforming growth factor-beta (TGF-β) in the wound area[1,4]. MSC culture under hypoxic conditions was able to increase its viability, the ability to adhere to damaged tissue, and the ability to secrete anti-inflammatory and proliferative molecules[5].

Approximately 6 million people in the United States are affected by chronic wounds with a yearly wound management cost of about 25 billion dollars per year, while in Europe about 2% of the health budget is devoted to wound management[6]. The cost required for wound management in Singapore hospitals reaches 216 million US dollars, while in primary health services, it reaches 596 thousand US dollars[7]. The proportion of wounds in Indonesia according to the Riskesdas report (2018) for the type

of abrasions/bruises/bruises is 64.1%, while for the types of cuts/tearing/stab wounds it is 20.1% and having severed limbs is 0.5%[8]. It is important to find a more effective treatment so that the wound does not get worse and cause an increase in the cost of treatment. These efforts can be tried by utilizing the hypoxic MSC.

Hypoxic is one of the factors that can trigger the release of inflammatory and vasculogenic cytokines during tissue regeneration[9]. Several studies, both *in vitro* and *in vivo*, have demonstrated the role of hypoxic conditions in enhancing the therapeutic effect of MSC. *In vitro* test conducted by Jun *et al.* (2014) showed that MSC derived from hypoxic human amniotic fluid can improve wound healing by increasing paracrine factors through activation of the suppressor of mothers against decapentaplegic 2 (SMAD2) and phosphatidyl-inositol-3-kinase/protein kinase B (PI3K) pathways/AKT[10]. Another *in vitro* test conducted by Sapruddin *et al.* (2018) showed that hypoxic MSC can secrete higher growth factors and anti-inflammatory molecules[11]. Anti-inflammatory molecules play a role in stopping the inflammatory phase of the wound which then moves to the proliferative phase which is characterized by an increase in TGF-β[12].

TGF-β is a cytokine produced by all white blood cell lineages and is a multifunctional cytokine that plays a role in accelerating the inflammatory phase, stimulating the proliferation phase, and forming collagen so that it can accelerate the wound healing process[14]. The aforementioned research evidence prompted researchers to investigate the effect of hypoxic MSC on TGF-β gene expression in excision wound Wistar rats, considering that so far these studies have not been studied. The use of an excisional wound model is considered to represent an acute wound condition where the wound healing process can occur physiologically or normally. Excision wound is a wound condition where there is a release of skin tissue in the epidermis, dermis and fascia layers.

MATERIAL AND METHODS

Research subject

Wistar rats aged 2-3 months, weighing 200-250 grams without anatomical abnormalities, have never been used in previous studies, were selected as research subjects, but if they died or had an infection during the study, they were excluded. All rats were adapted for seven days, then randomly divided into 4 groups, each group consisting of 5 rats : (1) the KI group was the healthy rats group; (2) the KII group was a negative control group, an excision wound model, was made and was given standard feed ad libitum + water; (3) KIII group was treatment group 1 which made an excision wound model and was injected with normoxic MSC at a dose of $3x10^6$ cells; (4) KIV group was the treatment group 2 which made an excision wound model and injected hypoxic MSC at a dose of $3x10^6$ cells. The research has complied with the principles of handling experimental animals and has been approved by the Commission on Bioethics for Medical and Health Research, Faculty of Medicine, Universitas Islam Sultan Agung, Semarang with Number: 313/IX/2021/Commission on Bioethics.

MSCs Isolation and Hypoxic pre-condition

MSC was obtained from the isolation of the umbilical cord of 19-day pregnant Wistar rats cultured in Dulbecco's modified eagle medium (DMEM) added with fetal bovine serum (FBS), antifungals, and antibiotics and put into a hypoxic chamber to then be incubated under hypoxic conditions with low levels of 5% oxygen for 24 hours at 37ºC.

Making Excision Wound Model

The modeling of the excision wound was initiated by anesthetizing the rats using ketamine and xylazine in a ratio of 3:1 with a dose of 0.1 mL/100g body weight. After the rat was unconscious, the

hair was cut on the back using an automatic razor with a size of 5x5 cm until it was clean. The surface of the skin that has been cleaned is given povidone iodine to avoid infection during wound making. Wounds were made using a punch biopsy with a size of 0,6 mm to the dermis.

TGF-β Gene Expression Observation by qPCR

TGF-β gene expression was observed by the quantitative Polymerase Chain Reaction (q-PCR) method using Illumina's Eco Real-Time PCR and expressed in multiples (fold). Observations were made on tissue samples taken at the site of the excision wound after giving various treatments for 3, 6, and 9 days.

Data analysis

The TGF-β gene expression data were then described in terms of the mean values presented in graphical form, and the differences were analyzed using the Kruskal Wallis test because the distribution of the data in several groups was not normally distributed and not homogeneous. The results of the Kruskal Wallis test with $p<0.05$ followed by the Mann Whitney test. Analysis of the results was performed with SPSS for Windows version 25.0.

RESULTS

Hypoxic MSC regulated TGF-β gene expression Observation

The results of observations and comparative analysis of TGF-β gene expression at each time of observation are shown in Figure 1. On the day 3 of observation of TGF-β expression, group II (excision wound model) showed the highest TGF-β gene expression, while the lowest was indicated by group IV. Based on the results of the comparison of TGF-β gene expression with the Kruskal Wallis test, p-value = 0.439 indicated that there was no difference in TGF-β gene expression between the four groups. These results indicate that normoxic or hypoxic MSC injection did not significantly affect TGF-β gene expression.

On day 6 observation of TGF-β gene expression on day 6, group II (excision wound model) also showed the highest TGF-β gene expression, while the lowest was shown by group IV. Based on the results of the comparison of TGF-β gene expression with the Kruskal Wallis test, p-value = 0.030 indicates that there are differences in TGF-β gene expression between the four groups. Significant differences in TGF-β gene expression on day 6 were shown in group I and group II, as well as between group II and group III, and group IV. On day 6, Normoxic or hypoxic MSC injection reduced TGF-β gene expression in excision wound model rats. The decreasing effect of both types of MSC on TGF-β gene expression in excision wound rat model was relatively similar.

On the 9th day of observation of TGF-β1 gene expression, group II (excision wound model) also showed the highest TGF-β gene expression, while the lowest was indicated by group IV. Based on the results of the comparison of TGF-β gene expression with the Kruskal Wallis test, the value of p=0.003 indicated that there were differences in TGF-β gene expression between the four groups. Significant differences in TGF-β gene expression on day 9 between the two groups were shown by group I and groups II and IV and between groups II and groups III and IV. On day 9, both normoxic and hypoxic MSC injection affected reducing TGF-β gene expression in excision wound rat model, the effect of decreasing TGF-β gene expression between normoxic and hypoxic MSC was relatively similar.

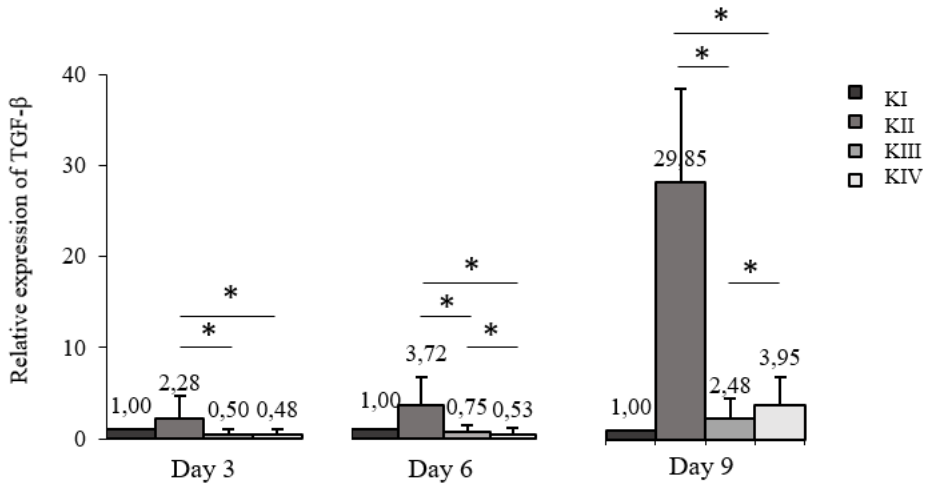

Figure 1. Relative gene expression of TGF-β in each group on day 3, 6, and 9 after treatments. *) p<0,05.

DISCUSSION

Injection of Hypoxic MSC affects reducing TGF-β gene expression in excision wound model Wistar rats on day 6 and day 9, while on day 3 there is no visible effect. The third-day post-injury is the inflammatory phase where the main cells involved in this process are neutrophils, macrophages, and lymphocytes. The role of TGF-β has not been shown in this phase[15]. The effect of MSC injection on TGF-β gene expression on day 3 has not been seen because on day 3, the role of MSC is to secrete proinflammatory cytokines belonging to neutrophils (such as IL-6, IL-8, IL-17, and CSF2). and M1 macrophages (CSF2, CCL2-4) as well as anti-inflammatory cytokines belonging to neutrophils (IL-10 and CXCL2) and part of M2 macrophages (IL-4, PGE2, and IDO)[16].

The 6th-day observation showed the highest TGF-β gene expression in group II. On the 6th day, it has entered the proliferative phase, and in this phase, keratinocytes and fibroblasts begin to proliferate and migrate to the wound edges to stimulate the formation of new tissue[17]. In the migration process, TGF-β shows its role as a mitogenic and chemotactic stimulator to attract keratinocytes to migrate to the wound and initiate epithelialization so that at this stage the gene expression of TGF-β in the normal wound healing process is high[1].

The injection of KIII and KIV in wound excision rat model on the 6th day of observation showed a significantly lower TGF-β gene expression compared to group II. These results indicate that MSC injection can accelerate the proliferative phase. The acceleration of the proliferative phase in the wound healing process is obtained from the content of various important cytokines and chemokines including TGF-β itself[18]. In this proliferative phase, the TGF-β gene which is expressed in wounds plays a role in shifting the differentiation of M1 into M2 which have the function of increasing Th2 responses as immunomodulators, matrix deposition, and tissue remodelling[4]. The role of MSC related to TGF-β gene expression in the wound healing process lies in the phase of tissue turnover and remodeling[16,19].

Hypoxic MSC injection on the day 6 of observation had a similar effect to normoxic MSC injection on TGF-β gene expression. This result was since on the day 6 the group of rats injected with MSC had entered the wound closure phase while the control group without MSC injection still showed a normal wound healing process as reported in previous studies[10,20,21]. The TGF-β gene expression on the day 9 of observation also showed a similar pattern to the day 6. Both of normoxic or hypoxic MSC

injection resulted in a significant decrease in TGF-β gene expression when compared to TGF-β gene expression in the control group. Both normoxic and hypoxic MSC showed similar effects.

The effect of hypoxic MSC injection on accelerating the wound healing process was also demonstrated by Hamra *et al.* (2021) who stated that a hypoxic MSC dose of $3x10^6$ accelerated wound closure by controlling the expression of α-SMA expressed by myofibroblasts where TGF-β1 is the activator[22]. The effect of hypoxic MSC injection on the inhibition of TGF-β gene expression in this study is also relevant to the previous studies that hypoxic MSC in a dose of $3x10^6$ weakens peritoneal attachment through inhibition of TGF-β gene expression[23,24].

However, the inhibitory effect between hypoxic and normoxic MSC injections in this study was relatively similar. These results differ from those shown in the previous studies who reported that MSC derived from hypoxic-conditioned amniotic fluid enhances the healing effect of excision wounds in ICR mice by increasing fibronectin-stimulated dermal fibroblast cell migration and the TGF-β/SMAD2 and PI3K/AKT signaling pathways[10,25]. The difference in the results is because this study only identified TGF-β gene expression while the mechanism for accelerating wound healing in hypoxic MSC also involved SMAD2 and PI3K/AKT. This reason is also a limitation of this study because it did not evaluate the TGF-β/SMAD2 and PI3K/AKT signaling pathways. Another limitation of this study is not evaluating the specific type of TGF-β that is expressed in the wound healing process, considering that a balance between TGF-β1 and TGF-β3 is needed to achieve skin regeneration.

Mesenchymal stem cells affected the expression of TGF-β in excision wound model Wistar rats on the day 6 and 9 after injection. Injection of hypoxic MSC $3x10^6$ cells decreased TGF-β levels in excisional wound Wistar rats, but the effect was relatively similar to Normoxic MSC. The decrease in TGF-β gene expression produced by normoxic or hypoxic MSC injection on 6 day was equivalent to TGF-β gene expression in the Sham group, but on day 9 hypoxic MSC injection resulted in higher TGF-β gene expression than in the sham group. In future studies, it is necessary to examine the effect of hypoxic MSC on TGF-β/SMAD2 and PI3K/AKT signaling pathways in excisional wound model Wistar rats, as well as the effect of hypoxic mesenchymal stem cells on the balance between TGF-β1 and TGF-β3 in excisional wound model Wistar rats.

CONCLUSION

In summary, Injection of Hypoxic and Normoxic MSC regulates TGF- β1 expression which leads to an accelerated proliferative phase of the excisional wound healing process.

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AUTHORS' CONTRIBUTIONS

D.A.F.I. conducted the majority of the experiments, analyzed data, and prepared the manuscript. D.D. conducted data analysis and provide suggestions for the project. A.P. supervised the project, conceived the experiments, and wrote most of the manuscript.

COMPETING INTERESTS

The authors declare that there was no conflict of interest.

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