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The Administration of Botulinum Toxin-A Reduced Degree of Fibrosis but Did Not Reduce Scar Tissue Formation in Laceration Wounds in Male Wistar Rats

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ABSTRACT

A wound is a break in the continuity of the skin, mucous membranes or organs. The concept of anti-aging medicine in terms of improving immune function and decreasing collagen and elastin production along with the aging process is closely related to the end result of wound healing. One of the important things thataffect wound healing is the tension of the wound edges which can be reduced by injecting Botulinum Toxin A (BTA). This study tested the effectiveness of BTA doses of 5 IU, 10 IU, and 15 IU on the degree of fibrosis and scarring in male white rats of the Wistar strain. This experimental study used four groups of Wistar rats, with a random sample of seven rats per group, with the control group given 0.9% NaCl (P0), while the treatment groups was given an injection of BTA 5 IU (P1), 10 IU (P2), and 15 IU (P3) respectively in the same volume immediately after the incision was made. Evaluation of the Vancouver scar score (VSS) was carried out weekly for four weeks, and biopsies in the 4th week. The results showed that P0 had the highest fibrosis rate (mean 15.22% and SD 4.70%) and although 5 IU BTA was not effective in preventing fibrosis (p=0.098, mean 12.56% and SD 2.44%), doses of 10 IU and 15 IU were effective (average 4.18% and SD 1.04%). However, BTA 5 IU, 10 IU, 15 IU did not significantly prevent scar tissue formation at week 4. The weaknesses of this study were the short study period, the VSS assessment was carried out by only one researcher and the presence of infected wounds in mice was not considered to have an influence on the results of the study. The conclusion of the study was that the administration of 10 IU and 15 IU AFB reduced the degree of fibrosis but did not reduces the formation of scar tissue in the laceration wounds of male white Wistar rats.

Keywords:BTA, Degree of Fibrosis, Scar Tissue, Incisions

1. INTRODUCTION

Wounds are damage to the integrity of the skin, mucous membranes and bones or other organs. In the context of wound healing, anti-aging medicine can be used to speed up the wound healing process by increasing collagen and elastin production and enhancing immune function. Literature shows that wound healing slows down with age Extracellular matrix (ECM) components are also known to contribute to changes in wound healing with age. The skin of younger patients can mount a strong response by producing an ECM that can adapt to the mechanical conditions of the wound. Whereas older skin shows considerable atrophy, a prolonged healing response with increased inflammation, and differences in signal transduction, which result in lower ECM production (**Gould *et al.*, 2015**).Tissue healing involves regeneration (replacement of damaged cells by similar cells) and/or fibrosis. Fibrosis is a natural process to restore tissue function in healthy wounds. In pathological conditions, fibrosis can cause the formation of excessive connective tissue fibers in organs or tissues during the healing process. In terms of response to injury this process is called scarring, and if the fibrous cells are composed of single parallel cells, it is called a fibroma (**Baranoski and Ayello, 2004; El Ayadi*et al.*, 2020**).

One of the important things that affect wound healing and the cosmetic outcome of scars is the tension imposed on the wound edges during the healing process (**Al-Qattan*et al.*, 2013**). Excessive tension on the tissue must be avoided in the wound healing process because it can impair blood flow and increase the fibroblastic response. Various surgical techniques, such as aligning the scar with a relaxed skin tension line, using local flap techniques or intervening the wound edges, have been applied to reduce excessive tension from the incision. Botulinum Toxin A inhibits the release of acetylcholine at the neuromuscular synapses which causes temporary muscle paralysis, due to chemical immobilization (chemoimmobilization) of the wound which ultimately has a positive effect on the wound healing process. Paralysis or chemical fixation of the muscles under the wound can be achieved by BTA injections (**Lee *et al.*, 2017**). Several studies have been conducted to prove the effect of BTA in preventing esophageal strictures in rabbits and reducing surgical wound fibrosis in rats by administering a dose of 10 IU (**Lee *et al.*, 2009; Meng *et al.*, 2022**).

Based on this background, researcher proved the effectiveness of BTA doses of 5 IU, 10 IU and 15 IU in the formation of the degree of fibrosis and scar tissue in the laceration wounds of male white Wistar rats.

2. MATERIALSANDMETHODS

2.1. STUDY DESIGN AND EXPERIMENTAL ANIMALS:

This research is experimental research with randomized post-test only control group design. The research was conducted at the Animal Unit Section of the Pharmacology Department, Faculty of Medicine, Udayana University, Denpasar, Bali. Histopathological examination of the tissue was carried out at the Histology Laboratory, Faculty of Veterinary Medicine, Udayana University, Denpasar, Bali. The sample needed in this experiment was 24 male Wistar rats (n=6), 120-150 days old, weighing 100-150 grams. To anticipate drop out, 10% of total sample were added, with the total amount to 28 rats divided into 4 groups: (n=7).

2.2. RESEARCH PROCEDURE:

A total of 28 male Wistar white rats were adapted for 7 days and given standard food and drink in moderation. After the adaptation period ended, the rats were given anesthesia with Ketamine. The rats were then shaved in the thigh area, in the longitudinal direction. Then, the skin and subcutaneous tissue are cut in straight lines with the size of 1 cm x 0.5 cm x 0.2 cm. Random sampling was carried out and the rats were divided into 4 groups: control group was given 0.5 mL 0.9% NaCl while treatment group 1, 2 and 3 were given 0.5 mL of botulinum toxin A of 5 U, 10 U, 15 U respectively. BTA was injected intramuscularly around the incision wound immediately after the incision was made.

Histological examination was carried out to see the degree of fibrosis and scar tissue formation. In the 4th week, the injured skin, including the epidermis, dermis and loose subcutaneous tissue with normal tissue around it, was removed. The removed tissue was then fixed using formalin, processed and embedded in paraffin. The paraffin block was cut into slices of 4 – 6 µm thickness and then stained with HE staining and read using the ImageJ technique for histological examination. Staining was assessed by setting a threshold using a thresholding tool. The HE painting image was opened in the app and then the following steps were applied. (1) Select “Image–Adjust–Threshold.” Auto setting could be selected or the slider could be moved manually until all stained areas were selected. A histogram was shown to provide assistance. (2) Choose "Dark background" for fluorescence. (3) Click "Set" to set the drawing threshold. (4) Select “Analyze–Set Measurements” and select the parameters to be measured. Make sure all gray level measurements were selected. The "Limit to Threshold" option should also be selected, otherwise the entire image would be measured, not the selected area. (5) Select “Analyze–Measure”. A table of results would appear, which could then be saved. The percentage of fibrosis was obtained from the ratio of blue pixels to the number of red and blue pixels.

To measure scar tissue, the Vancouver Scar Score (VSS) was used with interpretation as such: a score of 0 was normal skin, a score of 1-3 was a characteristic of mild scarring, a score of 4-6 was a characteristic of moderate scarring, 7-13 was a characteristic of severe scarring. To assess the degree of vascularization, a transparent object was used to suppress blood vessels. and the color of the capillary refill was assessed after the skin had blanched from pressure. This transparent object could also be used to determine the degree of pigmentation by eliminating the effect of vascularization on the skin during pressure and assessing the degree of pigmentation. The degree of flexibility was assessed by feeling the scar tissue between the thumb and forefinger and assessing whether it was normal, flexible, flexible, tight, stiff, contracture. To assess the elevation, the top of the scar was measured against the surrounding skin and recorded in millimeters (mm).

2.3. DATA ANALYSIS:

The normality test was done with the Shapiro-Wilk Test, to compare the data studied with normally distributed data based on the mean and standard deviation, with the data being normally distributed when $p > 0.05$. The homogeneity test was done using the Levene's T test, with the variances of the two data groups were the same or homogeneous when $p>0.05$. Bivariate analysis was performed using one-way ANOVA test, to determine differences in data between the degree of fibrosis and scar tissue formation. Then, it was followed by a post hoc analysis test to identify significant two-group comparisons. If the distribution of data was not normal ($p <0.05$), a Kruskal Walis analysis was performed. The data is processed using the SPSS Version 25 for Windows

3. RESULTANDDISCUSSION

Normality test on degree of fibrosis and scar tissue formation was done usingSaphiro-Wilk test, presented on Table 1 and Table 2.

Table 1-Normality Test on Degree of Fibrosis

Subject Group (Degree of Fibrosis)	n	p	Description
Control	7	0.219	Normal
Treatment 1	7	0.079	Normal
Treatment 2	7	0.628	Normal
Treatment 3	7	0.127	Normal

Table 2-Normality Test on Scar Tissue Formation with VSS

Subject Group (Scar Tissue Formation)	n	p	Description
Week 1			
Control	7	0.810	Normal
Treatment 1	7	0.055	Normal
Treatment 2	7	0.376	Normal
Treatment 3	7	0.236	Normal
Week 2			
Control	7	0.000	Non-Normal
Treatment 1	7	0.119	Normal
Treatment 2	7	0.153	Normal
Treatment 3	7	0.599	Normal
Week 3			
Control	7	-	-
Treatment 1	7	0.001	Non-Normal
Treatment 2	7	0.129	Normal
Treatment 3	7	0.030	Non-Normal
Week 4			
Control	7	0.000	Non-Normal
Treatment 1	7	0.000	Non-Normal
Treatment 2	7	0.000	Non-Normal
Treatment 3	7	0.001	Non-Normal

Because some of the data was found to be non-normal, the next step was to transform the data using square roots. The results of the data transformation were still not normally distributed ($p<0.05$) and are presented in Table 3.

Table 3-Normality Test on Scar Tissue Formation with VSS Assessment After Data Transformation

Subject Group (Scar Tissue Formation)	n	p	Descripstion
Week 2			
Control	7	0.001	Non-Normal
Week 3			
Treatment 1	7	0.001	Non-Normal
Treatment 2	7	0.030	Non-Normal
Week 4			
Control	7	0.001	Non-Normal
Treatment 1	7	0.001	Non-Normal
Treatment 2	7	0.001	Non-Normal
Treatment 3	7	0.001	Non-Normal

Homogeneity test on degree of fibrosis and scar tissue formation was done using Levene's test. The results showed that the data on the degree of fibrosis and the formation of scar tissue at weeks 3 and 4 were not homogeneous ($p<0.05$), while the formation of scar tissue at weeks 1 and 2 was homogeneous ($p>0.05$), presented Table 4.

Table 4-Homogeneity test on Degree of Fibrosis and Scar Tissue Formation with VSS

Variables	F	P	Description
Degree of Fibrosis	3.995	0.019	Not Homogeny
Scar Tissue Formation Week 1	1.720	0.190	Homogeny
Scar Tissue Formation Week 2	1.286	0.302	Homogeny
Scar Tissue Formation Week 3	9.432	0.001	Not Homogeny
Scar Tissue Formation Week 4	4.665	0.010	Not Homogeny

Comparability test on degree of fibrosis after BTA injection was done using One Way Anova and is presented in Table 5, with $p<0.001$, meaning that the mean degree of fibrosis in the four groups after treatment was significantly different ($p<0.05$).

Table 5- Differences in Mean Degree of Fibrosis Between Groups After Botulinum Toxin-A Injections

Variables	Group	n	Mean	SD	p
Degree of Fibrosis (%)	Control	7	15.22	4.70	<0.001
	Treatment 1	7	12.56	2.44	
	Treatment 2	7	9.67	2.04	
	Treatment 3	7	4.18	1.04	

To find out which groups were different from the control group, further test was carried out with the Least Significant Difference test (LSD). The test results are presented in Figure 1.P2 and P3 were significantly different from the control group (P0), while P1 was not significantly different from P0. The P3 group was found to be significantly different from P1 and P2. P2 did not differ from P1. Of the three treatment groups, the mean degree of fibrosis in the P3 group was the lowest.

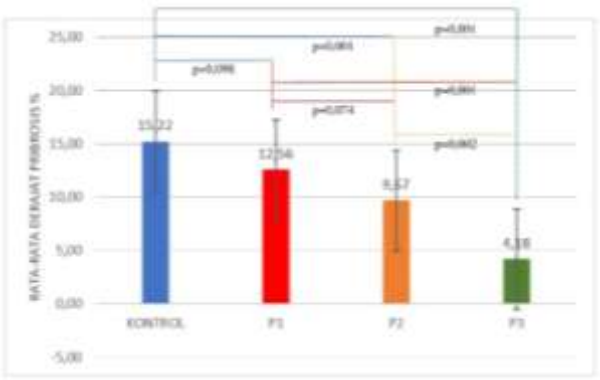

Fig.1-Comparison of Degrees of Fibrosis Between Groups

In this study, the injection of Botulinum Toxin A 10 IU and 15 IU resulted in a significant decrease in the degree of fibrosis compared to the control group (P0). Compared to P1, there was a decrease in the degree of fibrosis in Botulinum Toxin A 5 IU but not significantly. Administration of Botulinum Toxin A 15 IU injection had the lowest degree of fibrosis. Histological picture of the sample can be seen in Figure 2.

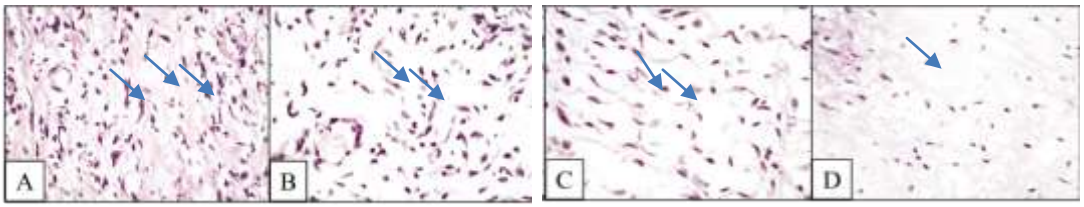

Fig.2-(a) Control group; (b) Treatment 1; (c) Treatment 2; (d) Treatment 3

The results of the analysis of significance with the One Way Anova and Kruskal Wallis tests are presented in Table 6. Analysis of significance usingOne Way ANOVA test at week 1 showed p = 0.045, meaning the average scar tissue formation in the four groups at week 1 after being given treatment was significantly different (p<0.05). Meanwhile, the significance analysis using the Kruskal Walis test at weeks 2, 3, and 4 showed a p>0.05, meaning the average scar tissue formation in the four groups at weeks 2, 3, and 4 after the treatment was not significantly different. In this study it was shown that the treatment given had a significant initial effect in reducing scar tissue formation, but this effect was not sustainable.

Table 6-Differences in Average Scar Formation Between Groups After Botulinum Toxin-A Injections

Week	Groups	n	Mean	SD	p
Week 1[a]	Control	7	6,14	3,53	0.045
	Treatment 1	7	2,71	1,60	
	Treatment 2	7	2,28	1,97	
	Treatment 3	7	3,28	2,87	
Week 2[b]	Control	7	1,14	1,95	0.200
	Treatment 1	7	1,71	1,70	
	Treatment 2	7	2,85	2,96	
	Treatment 3	7	3,57	2,14	
Week 3[b]	Control	7	0,00	0,00	0.124
	Treatment 1	7	1,28	2,36	
	Treatment 2	7	2,42	2,57	
	Treatment 3	7	1,00	1,00	
Week 4[b]	Control	7	0,28	0,75	0.543
	Treatment 1	7	0,28	0,48	
	Treatment 2	7	0,57	1,13	
	Treatment 3	7	1,14	2,03	

a: Repeated ANOVA test; b: Kruskal Walis test

To find out which groups were different from the control group, further tests were done: the Least Significant Difference – test (LSD) in the previous One Way ANOVA test; Mann Whitney test in the previous Kruskal Wallis test. The test results are presented in Figures3–6.

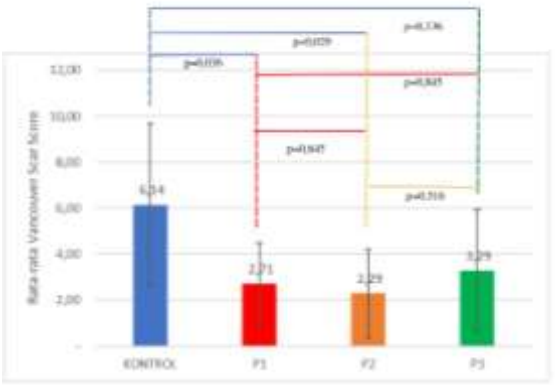

Fig.3-Comparison of Vancouver Scar Scores between Groups at Week 1

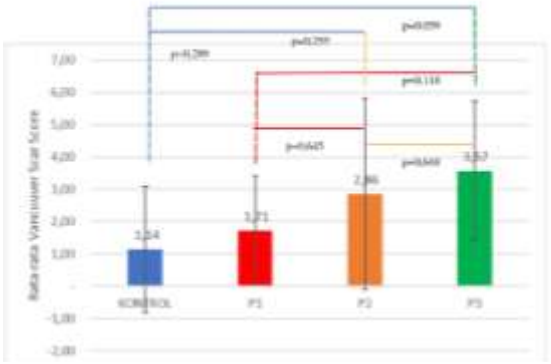

Fig.4-Comparison of Vancouver Scar Scores between Groups at Week 2

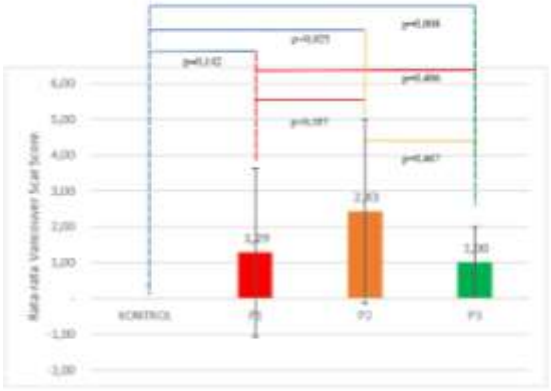

Fig.5-Comparison of Vancouver Scar Scores between Groups at Week 3

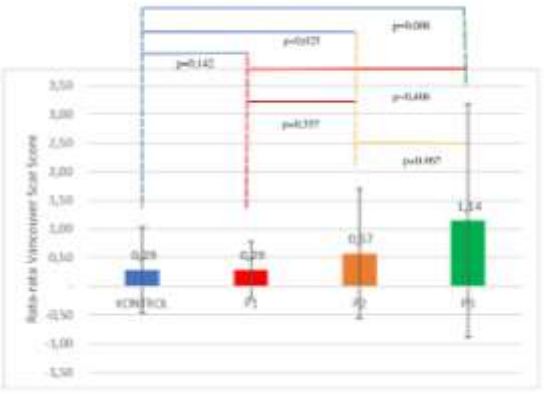

Fig.6-Comparison of Vancouver Scar Scores between Groups at Week 4

Effect of AFB on Reducing the Degree of Fibrosis in Laceration Wounds in Wistar Male White Rats

Scar formation is part of the physiological healing process, but hypertrophic and keloid scars are still a challenge, not only because they cause functional impairment but because they can have cosmetic effects that can interfere with social interaction and quality of life (**Tebble*et al.*, 2004**). However, due to incomplete understanding of the mechanism of scarring, each treatment has limitations and comparisons, so studies are needed to treatand prevent hypertrophic and keloid scarring (**Wang *et al.*, 2019**). Several studies have shown that injecting BTA around the wound can reduce formation

of hyperplastic scar tissue (**Xiao *et al*., 2009, Xiao *et al*., 2011**). Study done by Park *et al*. confirmed that BTA decreased TGF-β1 expression levels in hypertrophic scar tissue cultures along with proliferating fibroblasts. CTGF is a central mediator of tissue remodeling and fibrosis, and its inhibition can reverse the process of fibrosis. Previous studies found significant differences in CTGF protein levels between smear-treated and untreated control groups. The significant downregulation of CTGF expression by BTA has a direct effect on the ability of TGF-β1 to promote ECM production and other mechanisms involved in this process (**Park *et al*., 2019**). Olaeanolic acid induces apoptosis through the mitochondrial death pathway by triggering p38 and JNK signaling (**Chen *et al*., 2014**). Park *et al*., found that JNK was activated by BTA treatment, whereas the level of phosphorylation of p38 MAPK kinase and ERK1/2 was not affected by BTA. Inhibition of the JNK pathway shows the effect of BTA inhibition on the proliferation of cultured cells and decreased production of pro-fibrotic factors through downregulation of p-JNK. These results indicate that BTA suppressive effect is closely related to the activation of the JNK pathway (**Park *et al*., 2019**).

Administration of BTA can reduce the formation of scar tissue in iris wounds of Wistar male white rats

BTA has been used for medical purposes since the 1980s and has been widely used in treating pathological muscle contractions and autonomic hyperactivity (**Matak&Lacković, 2015; Shaarawy*et al*., 2015**). In recent years, many doctors have chosen intralesional BTA as the therapy of choice for the prevention and treatment of hypertrophic scars and keloids. Its use is used as monotherapy or in combination therapy (**Carrero*et al*., 2018**). Preventively, BTA has been used to prevent scar formation after surgery, based on the mechanism of action which involves muscle relaxation and reduced pressure on the newly recovered tissue. Thus, the healing process can proceed more evenly, reducing the risk of visible scar formation (**Ziade*et al*., 2013**). A study conducted by Lee *et al*., found early postoperative BTA injection to be safe and effective in improving scar quality (**Lee *et al*., 2017**). A prospective blind randomized placebo-controlled study of 42 patients with forehead lacerations or surgical incisions was published by Gassner*et al*. in 2006. In the experimental group given 15 IU smear injection postoperatively, scars were found to have a much better cosmetic appearance compared to the control group after 6 months (**Gassner*et al*., 2006**).

In the studies conducted by Chang *et al*. in 2014, 60 patients underwent revision surgery for cleft lip scars and 30 patients in the treatment group received 15 IU of botulinum injections. At 6 months follow-up, patients who received BTA injections had significantly better scars than the control group (**Chang *et al*., 2014**). Kim *et al*. also conducted a trial on post-thyroidectomy patients where the results showed that patients who received botulinum injections had a different scar appearance (**Kim *et al*., 2014**). Randomized study conducted by Ziade*et al*. also reported that at follow-up 1 year after treatment, patients who received botulinum injections showed better scarring (**Ziade*et al*., 2013**). In a study conducted by Hu *et al*., at a 6-month follow-up, it was found that the facial side of the patients who received BTA injections had a significantly better appearance and narrower scars compared to the control side (**Hu *et al*., 2018**).

CONCLUSION

Administration of BTA at doses of 10 IU and 15 IU reduced the degree of fibrosis but did not show a significant difference in reducing scar tissue formation in the laceration wounds of Wistar male white rats.

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