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Evaluation of secondary intention wound healing process after botulinum toxin-A injection in rat

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Abstract

Today, the increasing use of beauty products such as Botox has raised concerns about their secondary impact on the body. The healing of wounds created at the site of these injections and paralyzed areas in case of this medicine has been considered by many researchers and physicians. The aim of this study was to investigate the process of wound healing in an area that previously treated with Botox. This study was performed on 30 rats weighing 200 to 250 g. 10 days before surgery Animals in Botox group, was injected 5 units of Botox and in the control group, saline serum with a volume of 1 ml subcutaneously in an area of 20 × 20 mm. After 10 days, the animals were ready for surgery. The surgery was performed by removing the injected area of the skin in full thickness and then the animals were kept in separate cages. Each of the Botox and control groups was divided into three equal subgroups (n = 5). Each of the subgroups was examined macroscopically and microscopically on days 7, 14 and 21, respectively, after euthanasia. In macroscopic evaluation of wound size and wound closure percentage, no significant difference was observed between Botox and control groups; However at all animals the percentage of wound closure in the Botox group was higher than the control group. In microscopic evaluation of skin sections in the day of 7 after wounding, in the Botox group, a large number of fibroblasts and blood capillaries that contained abundant erythrocytes were seen. On the day of 14 in the Botox group, the fleshy bud tissue showed faster maturation and the fleshy bud tissue contained fewer fibroblasts and more collagen fibers were seen. In the Day of 21 In all mice of Botox group, most of the wounds were completely covered by proliferated keratinocytes and also in the dermis, compared to the control group, connective tissue with pink fibers and a small number of fibroblasts and blood capillaries were visible. Overall, this study showed that wound healing in areas where previously injected by Botox is not problematic but also performed with better speed and quality.

Key words: Botulinum toxin type A, Botox, rat, wound healing

Introduction

One of the most important and influential factors in the wound healing is a contraction that created on the edges of the wound during the healing process. The superficial junctions of the cutaneous muscles cause a constant stretch on the healing skin wound, which will eventually lead to the wound healing. Numerous published reports demonstrate the benefits of using botulinum toxin type A in the management of various facial wounds (Al-Qattan et al., 2013; Gassner and Sherris, 2003).

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Botulinum toxin is a potent neurotoxin that causes paralysis of the striated muscles which remains for 2 to 6 months. This toxin is extracted from the bacterium Clostridium botulinum and is present in different serotypes (A to F). For more than 20 years, the use of botulinum toxin type A has been proven to be a safe and effective treatment for many disorders, including blepharospasm, convulsive speech, and overactive facial muscle (Sherris et al., 2002; Carruthers et al., 1996).

Due to the importance of studying these effects, this toxin has attracted a lot of attention in recent years. In 2014 a study by Kucukkaya et al. examined the effect of botulinum toxin type A (BTX-A) on wound contraction and skin grafting. The results of this study showed that the injection of BTX-A reduces wound contraction, increases inflammatory cells, diminishes the number of fat cells and hair follicles and increasing the amount of collagen fibers (Kucukkaya et al., 2014).

Also in 2014, Park et al. examined the effect of botulinum toxin type A on the survival of the rectus abdominis flap in rats with a vertical midline scar. This study also showed that the injection of BTX-A improved blood flow to the rectus abdominis flap (Park et al., 2014).
Investigated the role of BTX-A in preventing peripheral vasoconstriction in the dermal flap and increasing flap longevity in rats. The skin flaps on the backs of the mice were removed and then returned. In BTX-A group there was a significant increase in survival and in the control group there was a significant decrease in blood flow immediately after flap removal. Blood flow was high in all neighborhoods in the BTX-A group during the week, and most arteries remained intact without constriction.

As a result of BTX-A pretreatment, increased flap retention was seen with increased blood flow (Kim et al., 2009).

Due to the fact that botulinum toxin (Botox®) is widely used today for cosmetic and other therapeutic applications, the present study aimed to investigate the healing process of wounds caused by car accidents, etc. in the area previously administered Botox.

Material and methods

Animals

The present study was performed on 30 rats with a weight range of 200 to 250 g. They were allowed free access to drinking water and standard laboratory food. The Institutional Animal Care and Use Committee approved all animal procedures.

Operation

In this study, the animals were randomly divided into two equal groups of 15 Botox (B) and control (C).

The first stage of the study: Botox injection in the desired area

In the Botox group, 5 units (IU 5) of Botox and in the control group, saline serum with a volume of one ml was injected (Huang, 2018). Subcutaneous injections were performed in an area measuring 20×20 mm at 9 points with equal distance (Figure 1).

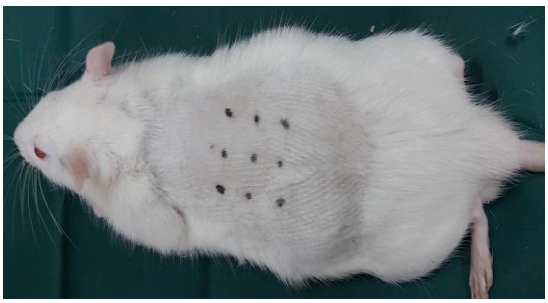

Figure 1: Injection sites of BTX-A and physiological serum into animals

Rest was given for 10 days to achieve the side effect of BTX-A, which is muscle relaxation.

The second stage of the study: wound formation

On the tenth day after injection, the animals were prepared for surgery. A 20×20

mm thick piece of skin was removed from the area previously treated with Botox using a scalpel (Figure 2).

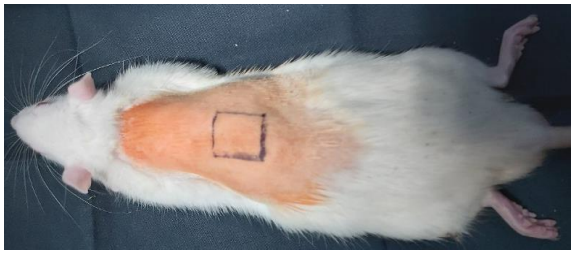

Figure 2: The area of removed skin

The skin sample taken was considered as the zero-day skin sample. This skin sample was placed in a sample container containing 10% formalin. After skin removal, the surgical site was compressed with a compression bandage, and to prevent wound infection, the oral antibiotic enrofloxacin was injected through their drinking water (100 mg / liter) for 10 days and the wounds were washed with normal saline. Also during this period, the animals were kept in separate cages to prevent any interference with each other's wounds.

Each of the Botox and control groups was randomly divided into three equal subgroups (nn = 5). Each of the subgroups was examined macroscopically and microscopically on days 7, 14 and 21, respectively.

Results

Macroscopic evaluation

For macroscopic evaluation at the day of 7, 14 and 21 after surgery, the wounds were photographed and the wound area was calculated using image J software and the wound closure percentage was computed using the formula from literature, stated as percentage of wound closure=[(Area on 1 day – Area of X days)/Area on 1 day]×100% (Zhou et al., 2017). All wounds in both control and Botox groups had a scab on the first day after surgery. This scab and the size of the wound had a decreasing trend during the study period. There is no significant difference between Botox and control groups at the time of evaluation; However, at all times, the percentage of wound closure in the Botox group was higher than the control and the difference in the percentage of wound closure between these two groups of Botox and the control on the 7th day was the highest (9.53%) (control 49.62% and Botox 59.15%) and gradually this difference has decreased so the difference on the 14th day was 1.46% and on the 21st day was 1.31%.

The form of the wound closure was different in Botox and control groups. In the control group, the closing of the square wound is almost equal in the direction of the longitudinal and transverse axis (square). While in the Botox group, wound closure is not equal and is rectangular. The healing of the transverse axis is faster than the longitudinal axis. This shape of healing made the shape of wound closure different in two groups and at different times (Figure 3, Table 1)

Table 1: Mean percentage of wound healing in control and Botox groups on evaluation days

Mean ± standard error of wound healing percentage in control and Botox groups		
Day	Groups	Percentage of wound healing (%)
7	Control	49.62 ± 5.93
	Botox	59.15 ± 4.72
14	Control	88.37 ± 0.77
	Botox	89.83 ± 0.44
21	Control	94.44 ± 0.82
	Botox	95.75 ± 0.67

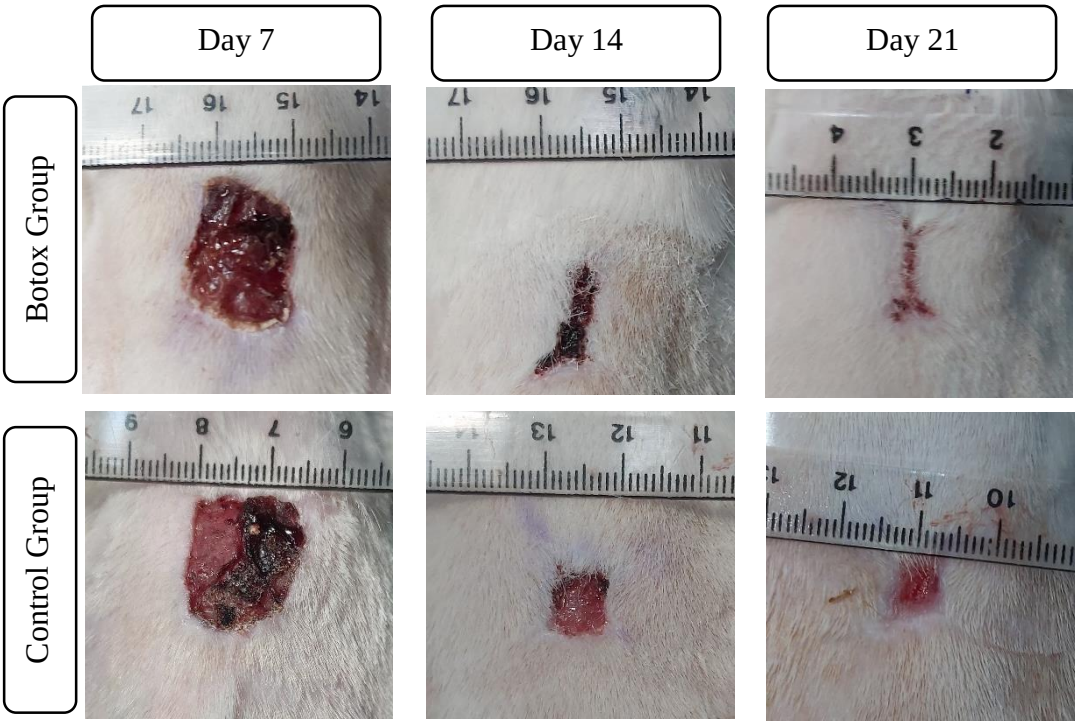

Figure 3: Wound size on assessment days

Microscopic evaluation

In the examination of the skin sections 7 days after wound formation, scabs were seen on the wounds and keratinocytes had begun to proliferate on both sides. Flesh bud tissue was formed on both sides of the wound but did not cover the entire wound site. Inflammatory cells were still visible in the center of the wound. The fleshy bud tissue was composed of a large number of fibroblasts and new blood vessels. Comparison of control and Botox groups showed a significant difference in the numbers of new fibroblasts and blood vessels. In the Botox group, a large number of fibroblasts were seen. Also, many blood capillaries containing abundant erythrocytes were visible in all parts of the flesh bud tissue in the control group (Figure 4).

Histopathologic examination of skin 14 days after wounding revealed complete formation of fleshy bud tissue in all parts of the wound in both groups. Proliferated keratinocytes also migrated to the wound but did not yet completely cover the wound surface. Fewer inflammatory cells were visible in the superficial parts of the wound. In the Botox group, the fleshy bud tissue showed faster maturation compared to the control group. In the Botox group, fleshy bud tissue contained fewer fibroblasts and more collagen fibers were seen compared to the control group (Figure 5).

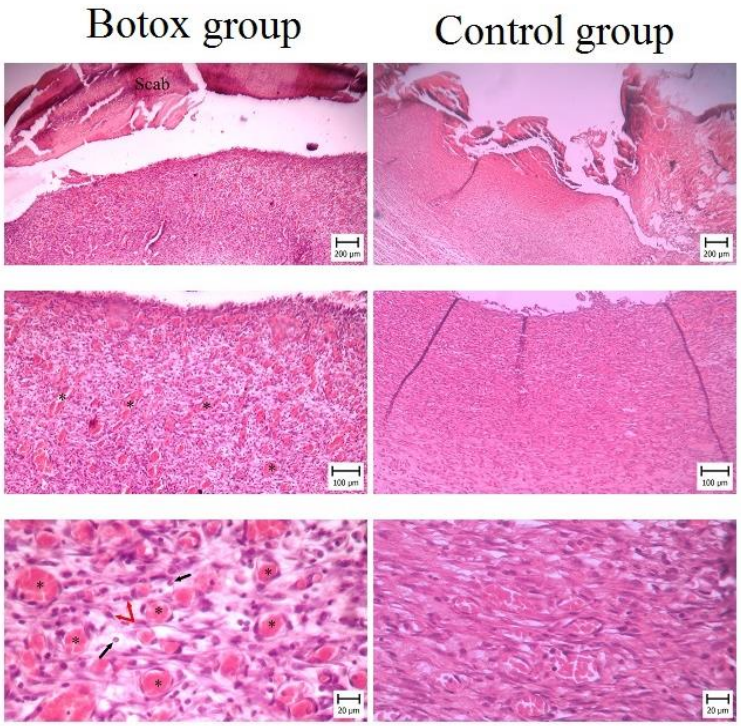

Figure 4: Day 7. In the Botox group, a large number of newly formed capillaries (star) and fibroblasts (red arrow) are seen compared to the control group. Inflammatory cells were also fewer (staining of hematoxylin and eosin).

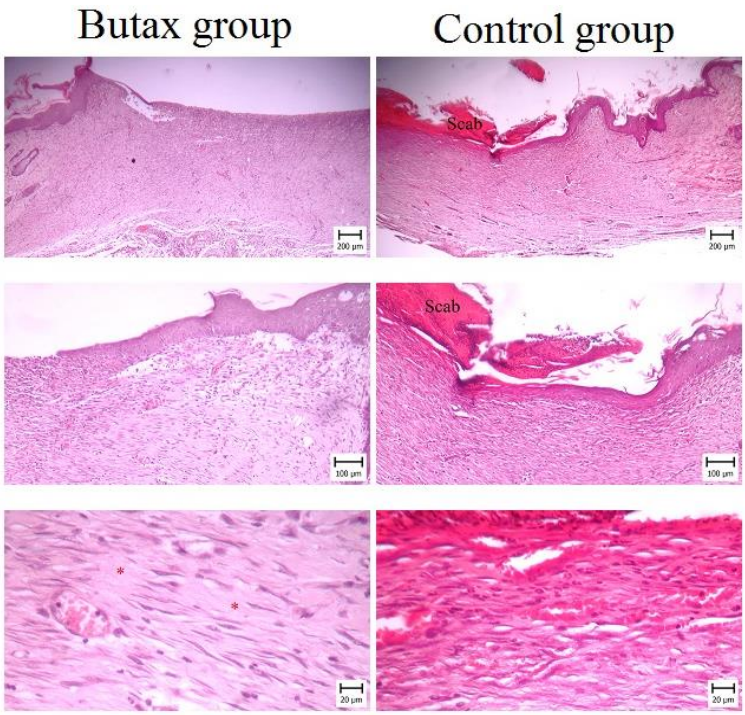

Figure 5: Day 14. In the Botox group, the fleshy bud tissue was more mature. This meant that more collagen fibers (red star) were formed and the number of fibroblast cells was reduced (hematoxylin and eosin staining).

Microscopic examination of the skin 21 days after surgery showed that the wound was further covered by proliferated keratinocytes. This stage completely done in all rats in Botox group(4-4). While in two animals in the control group, parts were not still covered totally. In the dermis, connective tissue with pink fibers and a small number of fibroblasts and blood capillaries were visible in all rats of Botox group. While in two rats in control group, a large number of fibroblasts and blood capillaries with inflammatory cells were still observed (Figure 6).

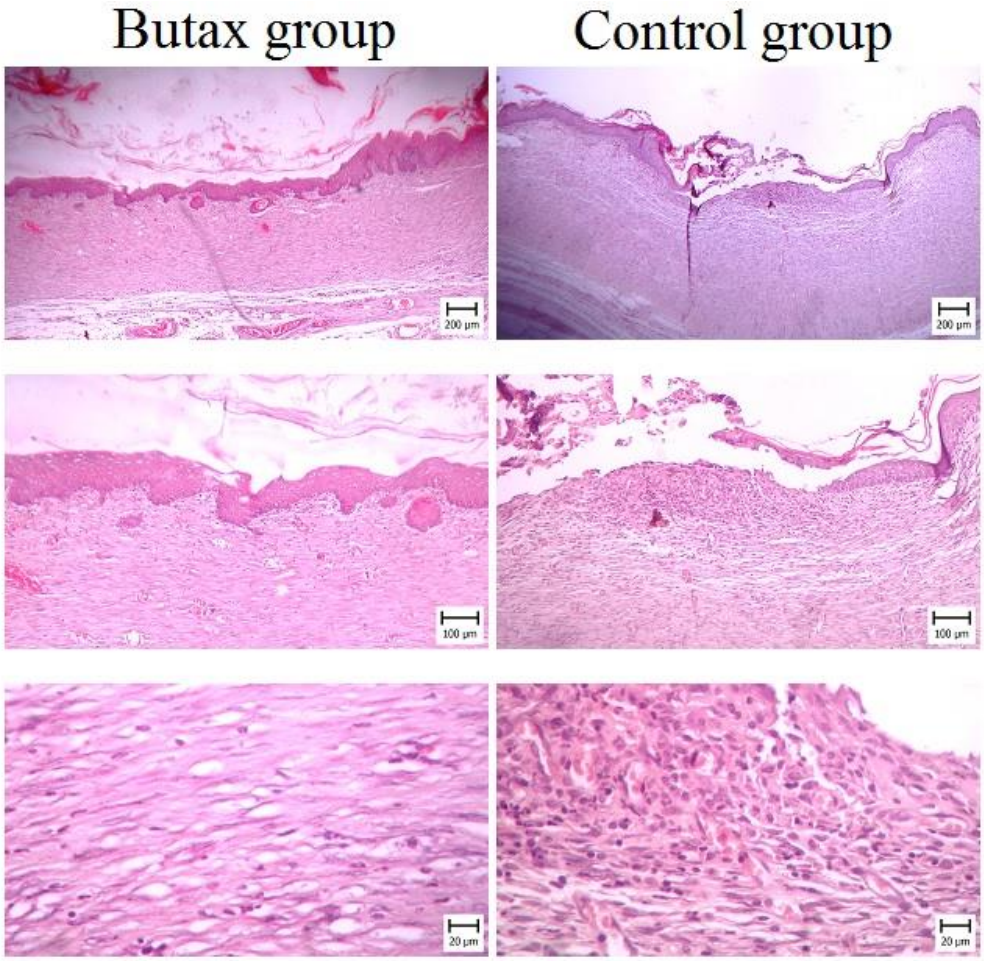

Figure 6: Day 21. In Botox group, the wound surface is covered by proliferated keratinocytes and in the dermis, connective tissue containing pink fibers and a small number of fibroblasts and capillaries can be seen. In the control group, fleshy bud tissue containing numerous fibroblasts, blood capillaries and inflammatory cells was also seen. (hematoxylin and eosin staining)

Discussion

Macroscopic evaluation

Today, one of the biggest challenges facing surgeons is closing wounds that are large in size and cannot be repaired with sutures and must be repaired secondarily. In addition, the tendency to use some medicines such as Botox for cosmetic work has increased significantly. So the evaluation of the effects of these medicines in the healing process of skin wounds has been considered. In most studies Botox injections have been done at the time of the lesion creation or immediately after surgery

(Oryan and Alamzadeh, 2020; Lee et al., 2009; Kcukkaya et al., 2014). Then the aim of this study was to create a model for traumatic wounds in areas that have previously been injected with Botox for cosmetic purposes.

In fact, the process of secondary wound healing in areas that have been injected with Botox few days ago has been evaluated in this study.

In this study, the percentage of wound closure in the two groups of Botox and control was not significantly different. However, at all sampling times the percentage of wound closure in the Botox group was higher than the control. A similar conclusion was reached in the Oryan and Alamzadeh study in 2020. This can be due to reduced wound traction, decreased release of proinflammatory cytokines such as IL-IB, and suppression of chronic inflammation, all of which can improve the quality of healing (Oryan and Alamzadeh, 2020). Contradictory results were obtained with the results of the present study in the study of Lee et al. In 2009. They stated that the size of the wounds in which Botox was injected was larger than the control group during the healing process and this difference was significant. They attributed this to a decrease in wound contractility due to a reduction in the tensile strength of the underlying muscles with Botox. They also considered the direct effect of Botox on inflammatory cells and inhibition of inflammatory mediators by a direct mechanism impaired the healing process. Same results were obtained in a study by Kucukkaya et al. in 2014.

Another important point was the appearance of the wounds in the control and Botox groups at different times. In the control group, the repair of the skin defect in all dimensions was done almost uniformly, while in the Botox group, the wound closure was rectangular. This is probably due to the looseness and laxity in the muscle fibers of the subcutaneous muscles due to Botax injection, especially the muscle of Paniculus carnosus, which plays a major role in wound contraction (Naldaiz - Gastesi et al., 2018). Thus, it can be inferred that the muscle fibers of Paniculus carnosus muscle, which are located longitudinally and parallel to the longitudinal axis of the body are relaxed due to Botox injection, and therefore the contraction of the wounds in the Botox group in the longitudinal axis has delayed.

Microscopic evaluation

In the Botox group on day 7, the number of inflammatory cells was significantly lower than in the control group, which was also observed in the study of Lee et al. In 2009 and Oryan and Alamzadeh in 2020. Also in the present study, as in the study of Lee et al. and the study of Oryan and Alamzadeh in 2020, the formation of new blood vessels in the Botox group was more than the control on day 7 and the amount of fibroblasts in the Botox group was significantly increased. All of the above factors can be the reason for the higher percentage of wound closure in the early days of wound formation in the Botox group compared to the control group.

On histopathological examination, on the 14th and 21st day, the inflammatory cells in the two groups gradually decreased, which was normal due to the wound healing process (Oryan and Alamzadeh, 2020; Lee et al., 2009). Also in the Botox group, the number of fibroblast cells on days 14 and 21 was significantly lower than the control group. This is due to the muscle relaxation caused by Botox and the reduction of muscle tension in the wound and the change in the functional status of fibroblasts and slowing down their population growth, which ultimately improves the quality of wound scars (Oryan and Alamzadeh, 2020). The reason for the decrease in fibroblasts in the Botox group, as stated in a study by Lee et al. In 2009, could be due to a decrease in the secretion of various cytokines such as platelet growth factors, TGF, epidermal growth factor, fibroblast growth factor and

insulin-like factor which it is a consequence of reducing the Inflammatory response and shortening of the inflammatory phase following Botox injections.

In the present study, the amount of collagen on days 14 and 21 in the Botox group was significantly higher than the control group, which contradicted the study of Oryan and Alamzadeh. They stated that Botox seems to reduce collagen and glycosaminoglycan deposition in wounds by preventing wound muscle contraction, which reduces scar formation (Oryan and Alamzadeh, 2020; Chatchai Pruksapong et al., 2017; Lebeda and Et al., 2012). In the present study, a significant increase in collagen on days 14 and 21 in the Botox group compared to the control and also a greater degree of maturity in this group was consistent with the study of Lee et al. He stated that the reason for this difference with the control group was the increase in the maturity of collagen fibers following a decrease in the inflammatory response and a shortening of the inflammatory phase. In fact, with the shortening of the inflammatory phase of the wound, the maturation of collagen fibers occurs more rapidly (Lee et al., 2009). In a study carried out by Xiao and Qu in 2012, the thickness of hypertrophic scars in the Botox treatment group was clearly less than the control group. In the control group, collagen fibers were thicker and the collagen fibers were irregular. Also in a study by Roh et al. in 2013, they stated that the expression of type I and III collagen was increased by Botox administration. Also, in the study by Kucukkaya et al. in 2014, on histological examination, there was a significant increase in the amount of collagen as well as connective tissue cells in the Botox injection groups. In this study novascularization was significant in Botox group. Also in the Botox groups the capillary diameter was larger. Regarding neovascularization, a statistically significant increase was observed in Botox groups. Fibroblast density increased in Botox groups and the difference between the groups was statistically significant.

Overall, this study showed that wound healing in areas that have already been injected with Botox is not only not a problem but also performed with better speed and quality; However, the time between Botox injection and wound formation and also the frequency of long-term injections can affect the anatomy and histology of the tissue and ultimately affect the healing process of wounds in these areas.

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Conflict of interest

The authors declare no conflict of interest related to this report.

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ارزیابی هیستوپاتولوژی روند ترمیم زخم پوستی در نواحی دریافت‌کننده‌ی بوتولینوم توکسین نوع A در موش صحرایی

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چکیده

امروزه استفاده‌ی روز افزون از محصولات زیبایی از جمله بوتاکس نگرانی‌هایی را در تأثیر ثانویه‌ی آن‌ها بر بدن به وجود آورده است. ترمیم زخم‌های ایجاد شده در محل این تزریقات و محل‌های فلج شده از این دارو مورد نظر بسیاری از محققین و پزشکان قرار گرفته است. مطالعه‌ی حاضر با هدف بررسی روند ترمیم زخم ناشی از اتفاقات در ناحیه‌ای که پیشتر تحت تجویز بوتاکس قرار گرفته است، انجام شده است. این مطالعه در ۳۰ سر موش صحرایی در محدوده‌ی وزنی ۲۰۰ تا ۲۵۰ گرم انجام گرفت. حیوانات ۱۰ روز پیش از انجام جراحی در گروه بوتاکس، به میزان ۵ واحد بوتاکس و در گروه شاهد، سرم سالین با حجم یک میلی‌لیتر در فضای زیر جلدی در ناحیه‌ای به ابعاد ۲۰×۲۰ میلی‌متر تزریق شد. پس از گذشت ۱۰ روز، جراحی با برداشت ناحیه‌ی تزریق شده از پوست به صورت تمام ضخامت انجام شد. هر کدام از گروه‌های بوتاکس و شاهد به سه زیرگروه مساوی (n=۵) تقسیم شد که هر یک از زیر گروه‌ها به ترتیب در روزهای ۷، ۱۴ و ۲۱ و پس از آسان‌کشی حیوانات از نظر ماکروسکوپی و میکروسکوپی بررسی گردیدند. در ارزیابی ماکروسکوپی اندازه‌ی زخم و درصد بسته شدن زخم، تفاوت معنی‌داری بین دو گروه بوتاکس و کنترل مشاهده نگردید؛ اگرچه در تمامی زمان‌ها درصد بسته شدن زخم در گروه بوتاکس از کنترل بیشتر بود. در ارزیابی میکروسکوپی در مقاطع پوست ۷ روز پس از ایجاد زخم، در گروه بوتاکس تعداد زیادی فیبروبلاست و مویرگ‌های خونی که حاوی اریتروسیت‌های فراوان بودند. در روز ۱۴ در گروه بوتاکس، بافت جوانه‌ی گوشتی بلوغ سریع‌تری را نشان داد و بافت جوانه‌ی گوشتی، حاوی تعداد کمتری فیبروبلاست بود و میزان رشته‌های کلاژن بیشتری روئیت گردید. روز ۲۱ در همه‌ی موش‌های گروه بوتاکس بیشتر زخم توسط کراتینوسیت‌های تکثیر یافته به طور کامل پوشانده شده بود و در قسمت درم نیز به نسبت گروه شاهد بافت همبندی با رشته‌های صورتی رنگ و تعداد کمی فیبروبلاست و مویرگ خونی قابل مشاهده بود. به طور کلی، این مطالعه نشان داد که ترمیم زخم در مناطقی که قبلا بوتاکس تزریق شده است مشکل ساز نیست، بلکه با سرعت و کیفیت بهتری انجام می‌شود.

کلمات کلیدی: سم بوتولینیوم نوع A، ترمیم زخم، موش صحرایی، بوتاکس

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