The positive effect of Botulinum toxin type A on the viability of random flap in tobacco exposed in rats[1]

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DOI: http://dx.doi.org/10.1590/S0102-865020160110000003

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ABSTRACT

PURPOSE: To evaluate the effect of Botulinum Toxin A in different time of tobacco exposure.

METHODS: 60 male, Wistar rats were divided into two tobacco exposure groups: a 2- month or a 4-month regimen. After this period, these two groups were subdivided as two: saline solution(SS) or botulinum toxin A(Bonta), at the time of the surgery. Seven days before the SS or Bonta injection, the animals were submitted to a random flap (3x10cm). On the seventh postoperative day, all animals were assessed for total flap area, viable area, and the viable/ total area ratio.

RESULTS: This study showed a difference between groups 2-month saline vs. BontA injection (p=0.04); groups 4-month saline vs. BontA injection (p=0.001); groups 2-month saline vs. 4-month BontA (p=0.003), and, between groups 2- month BontA vs. 4-month saline(p=0.03).

CONCLUSIONS: Botulinum Toxin A increased random flap viability in tobacco-exposed rats. Two months of tobacco exposure had the same effect as exposure for four months.

Key words: Surgical Flaps. Botulinum Toxins, Type A. Survival. Tobacco. Rats.

Introduction

Tobacco consumption is a global health problem; it is considered a pandemic involving 1.3 billion people. In Brazil, the prevalence of tobacco consumption is 14.7%. Tobacco smoke was responsible for 6 million deaths per [1]

One of the deleterious effects of smoking is wound healing complications.

Tobacco-exposure increases three times the rate of complications in wound Healing when Compared to a health patient[2,3].

For this reason, several strategies were studied to decrease surgical

complications[4-6], one of the strategies was to use BontA as a vasodilator[7-9].

We examined the effect of BontA in healthy rats, diabetic rats and, in rats exposed to smoke for 28-days, for the viability of skin flaps. Although BontA showed a difference in health and diabetic animals, there was no BontA effect in the flap viability for the tobacco-exposed group[9]. This hypothesis was based on other studies[10,11]. These studies showed a paradoxical increase of bronchodilation by using botulinum toxin type A (BontA) in a short time exposure to tobacco. Considering our previous study and the controversial results In the literature, we decided to test the effect of BontA in random flap for different smoke exposure periods.

Methods

The protocol was approved by the Ethics Committee of the Medical School, Universidade de São Paulo (028/2014). All procedures strictly followed current regulations related to animal experimentation dictated by the Council for International Organization of Medical Sciences, ethical code for animal experimentation. All animals were submitted to 24h night/day cycles, water and chaw ad libitum.

Sixty Wistar rats were used, 2-month old, and weighing 250-300g. These animals were divided into two groups;

- 2-month smoke exposure regimes, Monday to Friday exposure, twice a day, 30 minutes/session;
- 4-month smoke exposure regimes, Monday to Friday exposure, twice a day, 30 minutes/session

Each group was subdivided as saline solution or BontA injection, seven days before the surgical procedure.

Tobacco exposure

The animals were exposed in a 28-L plastic box with three orifices: on the inlet for synthetic air (2 L/min); another for smoke; and, an outlet to ventilate the excessive smoke. The smoke inlet was connected to a Venturi system controlled by means of fluxometry (2.5 L/min),which was connected to a lit cigarette.

Carbon monoxide (CO) was monitored using a single gas detector (ToxiPro; Biosystems, USA) to maintain a CO concentration of 300–350 ppm (parts per million) inside the box.

At the end of the group exposure period, all animals were submitted to a random dorsal cutaneous flap (3 x 10cm).

BontA/ saline solution injection

Seven days before the surgical procedure, all animals were anesthetized with intraperitoneal ketamine (100mg/Kg) and xylazine (5mg/Kg). The torso was trichotomized and we designed a 3x10cm flap. According to the subgroup, we injected (intradermal) 0.02ml of BoNTA/puncture, a total of 20u/rat (Botox® 100u, Allergan. Irvine. CA. USA) or the same volume of saline solution. The puncture scheme was 2 rows of 10 puncture points (1cm apart).

Surgical procedure

Antisepsis was performed using 0.5% chlorhexidine. Under inhaled anesthesia (20% isoflurane; 150–200 ml/min) the 3×10cm cranial flap based on the scapulae was elevated. The flap was then returned to its original position and sutured using separate stitches of 4-0 mononylon. Seven days after surgery, the rats were euthanized by anesthetic overdose (ketamine 200 mg/kg).

Flap area analysis

Immediately before euthanasia the flaps were photographed (Olympus 3.5mm digital camera; Olympus Stylus®, Japan) with a ruler along the length of the flap. The total area of the flap and the necrosis area were measured (squared millimeters) using the ImageJ® software. The viable skin was defined as pink, warm, and soft to touch. Necrotic areas were defined as brown-to-black, cold, and hard to touch. Then we calculated the percentage of healthy skin area (Survival area).

Carboxyhemoglobin analysis

At the end of the experiment, all animals were euthanized by anesthetic overdose (ketamine 200 mg/kg).

Immediately after euthanize, we punctuated the heart of all animals for blood collection. We measured carboxyhemoglobin by ABL radiometer (Radiometer Medical Aps, Bronshoj-Dinamarca)

Statistical analysis

Performed descriptive statistics for data analysis with mean and standard deviation. We used the Kruskal- Wallis test and the Dunnet post hoc, for non- parametric data. We considered a p-value < 0.005 and 80%power. We used STATA (StataCorp.2015.Stata Statistical Software: Release 14. College Station, TX:StataCorp LP)for all statistics analysis.

Results

Nine animals did not survive: five (two in group1, and three in group 4) died due to anesthesia; and four animals due to diarrhea (one in group 3 and three in group 2) (Table 1).

TABLE 1 - Comparison among total area, viable area, and ratio (viable area/total area) mm^2.

Group	Total area	Viable area	Ratio
1	2484.26± 220.71	1133.31 ±462.00	0.44
2	2277.205 ±196.43	1389.96 ±320.37	0.61
3	2124.50± 317.68	1428.73± 359.10	0.59
4	2401.32± 387.69	1644.94±393.80	0.77

The Kruskal –Wallis test showed difference among the groups (p= 0.07) and Dunn test showed a difference between group 1 and 2 (2-month saline versus BontA injection) p=0.04; group3 and 4 (4-month saline versus BontA injection) p= 0.001; group 1 and 4(2-month saline versus 4-month BontA) p=0.003, and between group 2 and 3 (2-month BontA versus 4-month saline) p=0.03 (Figure 1).

On the other hand, there was no difference between group 1 and 3 (2-month saline versus 4-month saline) p= 0.43 and, group 2 and 4 (2-month BontA versus 4-month BontA) p=0.16.

The overall carboxyhemoglobin seric concentration was 14.1% for all animals.

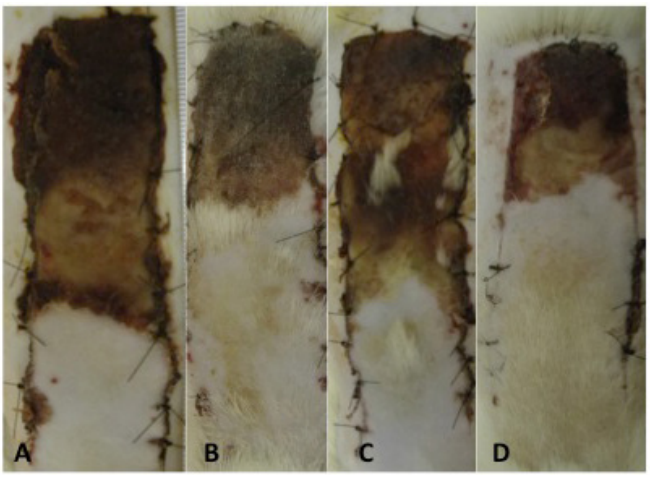

FIGURE 1 - Dorsal cutaneous flaps of rats at the day of euthanasia. **A**, 2-month tobacco exposure+saline; **B**, 2-month tobacco exposure +BoNTA; **C**, 4-month tobacco exposure+saline; **D**, 4-month tobacco exposure+BoNTA.

Discussion

One of our previous studies about the effect of BontA in diabetic, healthy and tobacco exposure on the cutaneous flap showed no vasodilation effect in 28 days of tobacco exposure for the BontA treatment group. This negative result guided us to reflect about this model being adequate for flap survival studies.

While searching for data in the literature, we identified studies about the paradoxical effect of tobacco in bronchiolar physiopathology. In a short period of tobacco-exposure the bronchia dilated but in a long-term exposure (more than 30days), the bronchia contracted. This piece of information suggested that we should test two- and four-month of tobacco exposure effect on the viability of cutaneous flap[10,11].

Moreover, there was no difference in terms of flap viability between health (no exposure) and smoke-exposure animals in this previous study[9]. We hypothesized that the 3x10cm flap would cause a very severe vascular insufficiency resulting in necrosis, and that no additional deleterious environmental factor (tobacco-exposure) would increase necrosis. For this reason, we decided to inject a vasodilator (BontA), to evaluate the effect of two different time exposure regimens in flap viability.

According to our results, we showed that 2- and 4-month exposure regimens were adequate to produce vascular alterations that could benefit from vasodilator use (ratio = 0.61, p=0.04 and ratio= 0.71, p=0.01, respectively).

Additionally, there was no difference in flap viability for BontA groups related to exposure time (subgroups comparing 2 and 4months, p=0.16).

Comparing to literature data, most studies injected nicotine to evaluate strategies to improve flap viability. However, to reproduce a clinical condition, we believe that nicotine injection was inadequate. The cigarette had more than one thousand of substances that can interfere with flap survival, and for this reason we adopted this model[12].

Few exposure rat models were used in experimental studies. Nolan *et al*.[14], showed a mainstream exposure model. These authors exposed rats during a 28-day period and evaluated flap viability. The flap dimensions were 2.5x10cm and after 28 days of tobacco exposure, the necrosis was 78.2%. Necrosis found in our study was different for several reasons; one reason was the flap dimensions (2.5x10cm versus 3x10cm). The other difference was the mainstream exposure regimen. Our model promotes an intermediate state of exposure between mainstream and side stream regimen. Carboxyhemoglobin measurement was also different, in Nolan *et al*.[14] the carboxyhemoglobin level was 25%, while in our project the carboxyhemoglobin level was 14.1%. This last measure represented a moderate smoker patient[15].

Moreover, it was difficult to reproduce the equipment used in Nolan *et al.*[14], thus, we adopted the model of Biselli et al because it was a feasible and reproducible equipment[12,14].

We consider these results methodological steps to define this tobacco exposure model as feasible and reproducible. Our study had some limitations; we did not assess the pathophysiology of tobacco smoke exposure. It would be important for next projects to study this outcome. Random cutaneous flap is routinely used in plastic surgery, but new studies must perform axial and composite flaps. More studies about pathophysiology and other therapeutic strategies should be done.

Conclusions

Botulinum Toxin A increased random flap viability in tobacco-exposed rats. Two months of tobacco exposure had the same effect as four months of exposure.

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Received: July 12, 2016
Review: Sep 14, 2016
Accepted: Oct 13, 2016
Conflict of interest: none
Financial source: none

[1]Research performed at Laboratory of Microsurgery and Plastic Surgery (LIM-04), Department of Surgery, Universidade de São Paulo (USP), Brazil.