

Effects of dimethylsulfoxide and pentoxifylline in the vitality of cutaneous flaps in rats

Efeitos do dimetilsulfóxido e da pentoxifilina na vitalidade de retalhos cutâneos em ratos

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A B S T R A C T

Objectives: to verify the influence of dimethylsulfoxide and pentoxifylline on the vitality of cutaneous flaps in rats and the tissue repair process. **Methods:** were studied 30 Wistar rats, submitting them to a 2cm wide by 8cm long dorsal cutaneous flap, of caudal base. We distributed the animals in three groups: Control Group (n=10) with application gauze moistened with 0.9% Saline in the flap bed for 30 seconds; Dimethylsulfoxide group (n=10), with administration of 1ml of 5% dimethylsulfoxide divided into five injections of 0.2ml in the transition of the flap segments; Pentoxifylline group (n=10), with administration of pentoxifylline 20mg/kg, diluted to 1ml and divided into five injections of 0.2ml in the transition of the flap segments. Drugs were administered intraoperatively, in a single dose and subcutaneously. We observed the skin flaps for changes in color and texture. On the 10th postoperative day, we checked the dimensions of viable and necrotic tissues, followed by excision of the specimen for histological analysis. **Results:** the measurements of length of the viable and necrotic tissues between groups showed no differences. Histological analysis showed that the Dimethylsulfoxide group presented neovascularization, inflammatory infiltrate with leukocytes and more structured conjunctival stroma. The Pentoxifylline group showed neovascularization and inflammatory infiltrate, with moderate to intense granulation. The control group evolved with a higher rate of necrosis in the distal segment. **Conclusion:** dimethylsulfoxide and pentoxifylline influenced the vitality of the flap and the tissue repair process. However, they did not prevent necrosis macroscopically.

Keywords: Wound Healing. Dimethyl Sulfoxide. Pentoxifylline. Rats. Surgical flaps.

INTRODUCTION

Cutaneous flaps are one of the pillars of reconstructions in the specialty of Plastic Surgery. A surgical resource widely used in situations of tissue loss of the integumentary system due to trauma, tumor resection and ischemia, it can present several complications, among them, hematoma, infection, dehiscence and necrosis. The cutaneous flap consists of elevation, detachment and repositioning in the bed, a procedure that can induce a series of events, among which vascular deficit, with reduced perfusion, for a variable and transient period¹. In addition to partial interruption of blood flow, the venous return in the dermal and subdermal plexus is impaired. Venous occlusion or congestion can be damaging to the vitality of the flap. Lym-

phatic vessels are also injured, resulting in interstitial edema, contributing to the reduction of capillary perfusion. The sensory and sympathetic nerves are injured with the flap elevation, which causes the release of catecholamines, inducing vasoconstriction and reduction of the local blood supply. It is important to note that the skin flap has to survive the events that occur during the period of reduced perfusion¹.

The ischemia of the cutaneous flap on the more distal area of vascular supply, especially in the distal segment, even if fleeting and for a short period, can generate reactive oxygen species. The mechanism involved in reperfusion, with the restoration of vascular supply and oxygen influx in the ischemic tissue, can culminate in cellular, inflammatory and metabolic changes mediated by free radicals, with structural and

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functional modifications in the cell, which may contribute to tissue necrosis²⁻⁷. This event requires organic adaptations and, where possible, the use of antioxidant drugs may reduce the toxic metabolites responsible for tissue damage.

Simultaneously with the events described with surgical trauma and elevation of the flap, an inflammatory process mediated by kinins, serotonin and histamine occurs, resulting in increased microcirculation permeability, with tissue edema, which impairs perfusion and contributes to decrease vascularization in the distal segment of the flap⁷. Post-trauma tissue edema may last from four to 24 hours⁸⁻¹⁰. Partial or total skin flap necrosis may require other surgical interventions, which is a challenging situation for the surgeon.

Several experimental studies have used drugs to attenuate the formation of reactive oxygen species and tissue edema, and to stimulate tissue repair⁷, such as hyaluronidase⁸, dimethylsulfoxide^{9,11-13}, sildenafil¹⁰, pentoxifylline^{6,14-16}, streptokinase and allopurinol¹⁷, L-arginine¹⁸, Kaurenoic acid¹⁹ and latex sap of *Hevea brasiliensis*²⁰. Dimethylsulfoxide has an analgesic and anti-inflammatory effect, acts on coagulation, mainly in the cascade of arachidonic acid, is an inhibitor of platelet aggregation and acts as a subdermal vasodilator, antioxidant, free radical scavenger, protecting from the ischemia-reperfusion phenomenon^{2,5,9,11-13,19}. Pentoxifylline has hemorheological properties, increases erythrocyte deformability and acts on the coagulation cascade, with thromboxane release and increased prostacyclin synthesis. As an important vasodilator, it acts on the morphological and biochemical immunomodulation mechanism and contributes to decrease reactive oxygen species^{4,6,14-16,19}.

The present study aims to evaluate the effects of dimethylsulfoxide (DMSO) and pentoxifylline (PTFL) on the vitality of skin flaps in rats.

METHODS

We studied 30 male, non-isogenic Wistar rats (*Rattus norvegicus*, Rodentia Mammalia), with a mean age of three months and a mean weight of 302 grams,

acquired by the Reproduction Biology Center (CBR) of the Federal University of Juiz de Fora - MG (UFJF-MG). We followed the ethical principles in animal experimentation according to the Brazilian College of Animal Experimentation (COBEA). All protocols and procedures are in accordance with Federal Law No. 11.734 of 10/08/2008²¹⁻²³ and the project was approved by the Ethics in Research Committee of the Federal University of Minas Gerais, according to protocol 251/2013.

We divided the sample into three groups, distributed as follows: Control group (n=10): gauze moistened with 0.9% saline solution in the flap bed for 30 seconds; DMSO group (n=10): injection of 1ml of 5% dimethylsulfoxide divided into five injections of 0.2ml in the transition of the flap segments; PTFL group (n=10): injection of pentoxifylline 20mg/kg, diluted with bidistilled water to complete the volume of 1ml and divided into five injections of 0.2ml in the transition of the flap segments.

We anesthetized the animals with 90 mg/kg of 5% ketamine hydrochloride, associated with 10mg/kg of 2% xylazine intraperitoneally²⁰. We marked the flap on the back of the animals with a blue pilot overhead and a millimeter ruler, of 2cm x 8cm (16 cm²), according to the McFarlane's rectangular flap, with caudal base vascular supply^{3,8,11}. We divided the flaps in every 2.6cm into proximal, intermediate and distal segments, and performed flap dissection up to the fleshy panniculus, including it. We submitted all the animals to the procedure with the same technique and, after elevation of the flap, we administered the drugs. We intraoperatively injected the flap borders in a single, subcutaneous dose, with 1-ml syringe, 3-mm needle, as follows: two injections of 0.2ml on the right side and two injections on the left side, at the transition of the proximal and intermediate segment and at the transition of the intermediate and distal segment, and one injection of 0.2ml at the center of the distal segment (Figure 1). Then, we repositioned the flap in the bed and sutured it with single, separated stitches of 4.0 monofilament nylon, 0.5cm distant from each other. We used no antibiotic and made no dressings.

We observed the animals daily, evaluating the behavior, weight and aspect of the flap. We measured the total and viable tissue lengths with the anes-

thetized animal prior to the procedure. Total size and necrotic tissue size were measured with the anesthetized animal on the day of euthanasia. The same observer performed all the measurements, with a millimeter ruler. We considered a macroscopic aspect of viable tissue to be one with normal coloration and texture or with few changes in the superficial skin layer, and necrosis, when the skin had a hard texture, dark color and crust²⁴.

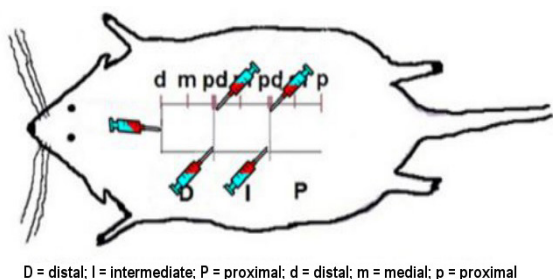


Figure 1. Drug Administration Scheme.

On the 10th postoperative day, we euthanized the animals under anesthesia with 180mg/kg 5% ketamine and 20mg/kg 2% xylazine²³. We resected the flap specimen, which was adhered in a filter paper mold and placed in 10% neutral buffered formaldehyde solution for 48 hours for fixation. The specimen was duly identified and sent for histological analysis, which was performed in 4- μ m thickness sections stained with hematoxylin and eosin. The steps were carried out at the Pathology Laboratory of the Holy Home of Mercy of Juiz de Fora, MG, and in the Department of Morphology of the Biological Sciences Institute of the Federal University of Juiz de Fora, MG, by an observer unknown to the group of the sample studied. An Olympus BX 51 microscope was used. The histological study of the flap was done in order to evaluate the evolution of the tissue repair process and the effect of dimethylsulfoxide and pentoxifylline on their vitality^{7,10,25,26}. In the qualitative analysis of each sample of proximal, intermediate and distal segment, we observed the epidermis, layers of the dermis and the layers of the fleshy panniculus. The presence of neoformed vessels and leukocyte inflammatory infiltrate was considered granulation, and necrosis was the presence of devitalised elements.

The categorical variables were: granulation, granulation with leukocytes, granulation and/or necrosis, analyzed in the central portion of the proximal, intermediate and distal segments, respectively^{10,25,26}. We divided the variables into six categories, assigning the respective scores and descriptions of each, according to the histological findings: a) ABSENT (score= 0) - absence of vessels and cellular elements of granulation; b) DISCRETE (score= 1) - granulation with few vessels, collagen fibers and sparse leucocytes in the optical field; c) MODERATE (score= 2) - granulation with greater frequency of dispersed vessels in the field and of collagen fibers, and leukocytes forming aggregates, with areas free of infiltration; d) INTENSE (score= 3) - granulation with vessels arranged in the whole field, collagen fibers and leukocytes with dense aggregate, without areas free of infiltrate; e) INTENSE + NECROSIS (score= 4) - granulation with vessels arranged throughout the field, collagen fibers and leukocytes forming a dense aggregate, without an area free of infiltrate, with areas of necrosis, intercalated or close to the granulation tissue, called granulation and necrosis foci; f) NECROSIS (score= 5) - fields full of devitalized elements, frequently evidenced, called necrosis.

The image capture of the slides was performed at the Genetics Laboratory of UFJF-MG, with the Cybernetics® Image-Pro Plus 4.5 software, coupled to the Olympus BX 51 microscope, in 25x, 40x and 100x magnifications. The photographic documentation of the animals was performed in all stages of the experiment on the same days, D1, D3, D5, D7 and D10, in all groups, by the same observer, with a professional digital camera Canon EF 100 mm, 1:2:8 USM - Tokyo - Japan.

We calculated the sample size with significance level between 0.01 and 0.05, coefficient of variation of 15 to 20% for differences between the treated and control groups¹⁷. We performed the statistical study of the variables of the three groups with the software Statistical Package for Social Sciences (SPSS)¹⁸, and analyzed the categorical variables of the histological analysis with the software R Core version 3.0.1 (2013: 05.16), both with the contribution of the Biostatistical Support Nucleus of the Faculty of Medicine of UFMG. We analyzed the pairs of means different from the categorical

variables studied with the ANOVA and Fisher's exact tests. The associations between the control, DMSO and PTFL groups were interpreted and clarified. The adjusted residue was equal to or greater than 1.96, when there was a significant association between the group variable and the category and p value <0.05 .

RESULTS

Four animals died in the immediate postoperative period, three in the PTFL group and one in the control group. On the 10th postoperative day, one animal of the DMSO group had clear fluid and trabeculae in the flap bed, suggestive of infection. We replaced these five animals. At

clinical observation until the 10th day, the animals in the experiment showed no signs of suffering, such as bent posture, behavioral changes, ocular or nasal secretion, hair bruising and diarrhea. They accepted food and water routinely. The aspect of the cutaneous flap varied from pallor, cyanosis to necrosis. The texture in the distal segment was gradually modified in all groups, becoming hardened, with crusts, with subtle differences between the DMSO and PTFL groups. We observed the delimitation of tissue necrosis on the seventh postoperative day. We submitted the measurements of the total flap size, viable tissue size and necrotic tissue size of each animal, as well as the equivalent percentages among the three groups, to the ANOVA test, which did not significant differences, with ($p>0.05$ - Table 1).

Table 1. Means and standard deviations (SD) of the flap dimensions and p -value.

Dimension of the skin flap	Control	DMSO	PTFL	p
	Mean (SD)	Mean (SD)	Mean (SD)	
Overall dimension (cm)	7.16 (± 0.00)	(6.98 ± 0.36)	6.87 (± 0.30)	0.240
Viable tissue (cm)	3.81 (± 0.53)	3.85 (± 0.75)	(3.69 ± 0.51)	0.831
Necrotic tissue (cm)	3.35 (± 0.47)	(3.13 ± 0.63)	3.18 (± 0.47)	0.630
Viable tissue (%)	53.17 (± 6.40)	53.98 (± 10.03)	53.69 (± 6.87)	0.973
Necrotic tissue (%)	46.37 (± 6.40)	45.37 (± 9.24)	45.41 (± 6.96)	0.888

The frequency in absolute numbers of the variables granulation and granulation/leukocytes in the proximal and intermediate segments was not significant ($p>0.05$). The control group presented the variable granulation/necrosis, with three cases of intense granulation (score= 3), one with intense granulation and necrotic foci (score= 4) and six with necrosis alone (score= 5). The DMSO group presented, in the variable granulation/necrosis, three cases of intense granulation (score= 3) and seven with intense granulation and necrotic foci (score= 4). The PTFL group presented, in the variable granulation/necrosis, three cases with moderate granulation (score= 2), five with intense granulation (score= 3) and only two with intense granulation with necrotic foci (score= 4). The frequency in absolute numbers of the variable granulation/necrosis in the distal segment in the control, DMSO and PTFL groups were significant ($p=0.0004791$). No spe-

cimen from the DMSO or PTFL groups displayed necrosis (score= 5) when compared with the control group, which had six cases of necrosis ($p=0.0004791$). These results suggest effects of the drugs on tissue repair (Table 2).

Figure 2 shows photomicrographs of the histological section of the distal segment of the control group, where there is inflammatory infiltrate and predominance of vacuoles, edema and necrosis. Figure 3 shows a histological section photomicrograph of the distal segment of the DMSO group with elements with intense inflammatory infiltrate with necrotic foci and neovascularization, structured connective tissue stroma. Figure 4 depicts a histological section photomicrograph of the distal segment of a PTFL group specimen, showing moderate inflammatory infiltrate, neovascularization and absence of necrosis. All specimens were analyzed with a 100x magnification and hematoxylin and eosin staining.

Table 2. Scores assigned in histological evaluation.

Variable/Group	Score (category)	Control	DMSO	PTFL	<i>p</i>
Granulation	Absent (0)	5	9	5	0.124
	Discreet	5	1	5	
Granulation/ Leukocytes	Discrete (1)	5	7	4	0.643
	Moderate (2)	5	3	5	
	Intense (3)	0	0	1	
	Moderate (2)	0	0	3 *	
	Intense (3)	3	3	5 *	
Granulation/ Necrosis*	Intense/necrosis (4)	1	7 *	2	0.0004791
	Necrosis (5)	6 *	0	0	

* statistical significance

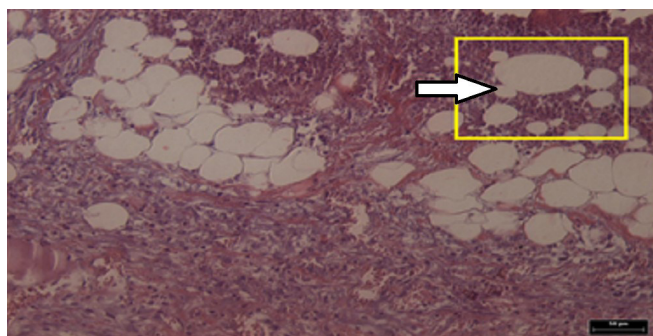


Figure 2. Photomicrography of control group specimen: (i) inflammatory infiltrate, edema, necrosis, HE-100x.

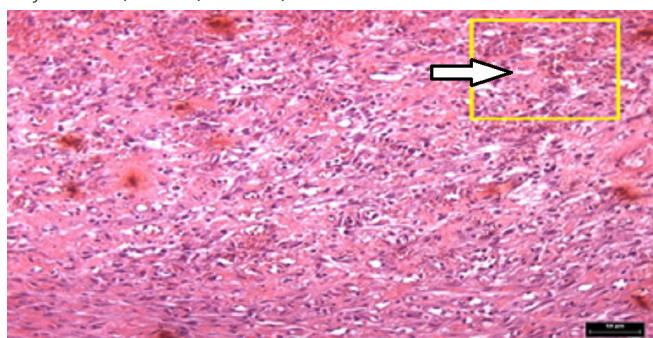


Figure 3. Photomicrography of DMSO group specimen: neoformed vessels, inflammatory infiltrate, H.E-100x.

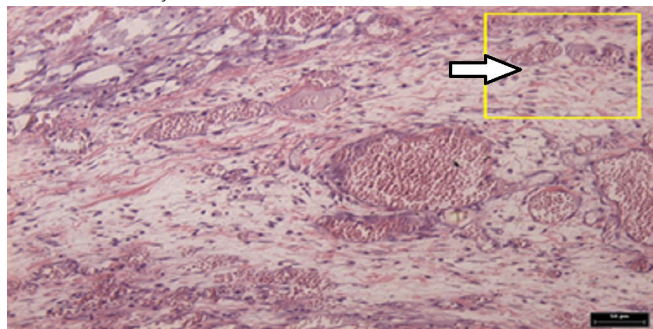


Figure 4. Photomicrography of PTFL group specimen: neoformed vessels, inflammatory infiltrate, H.E-100x.

DISCUSSION

The choice of the rat for the experiment in this research was due to the ease of animal acquisition, simple handling, low cost and feasible conditions for research. Moreover, this animal is widely used as a model in research^{1,4,8,9,11,14,16}. The McFarlane skin flap was standardized with a caudal base vascular supply and we created a difficult condition of venous return, favoring tissue edema, increased capillary pressure and consequent increase in necrosis, thus allowing investigation of the influence of the possible effects of DMSO and PTFL on tissue repair and on the vitality of the flap in face of such conditions^{3,8,11,24,27-29}.

Studies indicate that the tissue dimensions with cutaneous necrosis get delimited on the seventh postoperative day²⁴. In the present work we decided to maintain the clinical observation of the animals for ten days, considering that Almeida *et al.*⁹, in 2004, reported that the period required for the reorganization of the vascular supply of the flap was nine days.

Drug administration was performed with a 3-mm needle to reduce local trauma, and the volume of 1ml, not to cause edema and to avoid the reduction of perfusion. We chose the subcutaneous route because of the better dispersion of the substance, since it is easily accessible, capable of obtaining an adequate level of concentration in the tissues adjacent to the flap, with little systemic absorption, thus increasing admi-

nistration safety⁹. We did not find in the literature a study with PTFL administration, but only the Almeida⁹ study, with subcutaneous DMSO.

The reperfusion of the ischemic area can lead to the formation of reactive oxygen species that cause tissue damage and culminate with necrosis⁴. Oxidative stress, a result of this process, starts from the first minutes after trauma and can be one of the factors responsible for cell death, with tissue loss, mainly in the flap's distal segment. We believe that surgical trauma, such as the flap confection, causes an endogenous imbalance in the formation of reactive oxygen species from the first postoperative minutes. We rely on the premise that oxidative stress occurs early in the surgical trauma, and has repercussions throughout the flap healing, which is why we decided to perform the injections of drugs intraoperatively in a single dose^{1,2,4,9,10}. In previous research, it was found that antioxidant substances²¹, as well as DMSO and PTFL, can act favorably and early in the operative wound^{1,4,5,6,9,11-16,19}.

The intermediate segments of the flap are those called the "transition zone", since they are between the proximal portion, where the survival of the flaps is expected due to the proximity of the vascular supply, and the distal portion, where necrosis can occur due to the distance from the blood supply. These areas submitted to a period of ischemia and subsequent reperfusion are those in which the pharmacological intervention can modify the flap's evolution. The purpose is to improve the vitality of the flap through

the vasodilation in the microcirculation and increase the perfusion pressure with consequent benefit to the tissues¹⁰. Since DMSO and PTFL have antioxidant properties, the research proposal was to administer them subcutaneously and to evaluate the effects on the flap's tissue repair. We observed histological features of granulation, such as exuberant neovascularization, inflammatory infiltrate with leukocytes in the PTFL group, and more structured conjunctival stroma with neovascularization in the DMSO group, in addition to the absence of necrotic specimens in the group treated with PTFL^{25,26}. We observed necrosis in 60% of the specimens in the distal segment of the control group animals.

This research envisaged a horizon for the use of DMSO and PTFL to expand effective strategies to improve the vitality of cutaneous flaps, reducing or even avoiding necrosis. These drugs are affordable and available for therapeutic use in skin flaps. It is necessary to dedicate new researches to promoting knowledge about the safety and the tissue modifications under the influence of these drugs and, thus, to extrapolate their use for medical practice.

Our study demonstrated that DMSO and PTFL influenced the vitality of the skin flap and the tissue repair process in rats. The animals treated with these drugs had neovascularization and infiltrate with leukocytes statistically superior to those observed in the control group animals, which expressed devitalized tissues. However, they did not prevent macroscopic necrosis.

R E S U M O

Objetivos: verificar a influência do dimetilsulfóxido e da pentoxifilina na vitalidade e no processo de reparo tecidual de retalhos cutâneos em ratos. **Método:** foram estudados 30 ratos Wistar, nos quais foi confeccionado retalho cutâneo dorsal de 2cm de largura por 8cm de comprimento, de base caudal, e distribuídos em três grupos: Grupo Controle (n=10) com aplicação de gaze umedecida com solução salina a 0,9%, no leito do retalho, por 30 segundos; Grupo dimetilsulfóxido (n=10) com injeção de 1ml de dimetilsulfóxido a 5% divididos em cinco injeções de 0,2ml na transição dos segmentos do retalho; Grupo pentoxifilina (n=10) com injeção de 1ml pentoxifilina 20mg/kg, divididos em cinco injeções de 0,2ml na transição dos segmentos do retalho. Os fármacos foram administrados no transoperatório, em dose única e por via subcutânea. Os retalhos cutâneos foram observados quanto às alterações de cor e textura. No décimo dia de pós-operatório aferiu-se a dimensão do tecido viável e de necrose, seguido da exérese da peça para análise histológica. **Resultados:** a medida da dimensão de tecido viável e de necrose dos grupos não apresentou diferenças. A análise histológica mostrou que o grupo dimetilsulfóxido apresentou neovascularização, infiltrado inflamatório com leucócitos e estroma conjuntivo mais estruturado. O grupo pentoxifilina, mostrou neovascularização e infiltrado inflamatório com granulação moderada e intensa. O grupo controle evoluiu com maior índice de necrose no segmento distal. **Conclusão:** dimetilsulfóxido e pentoxifilina influenciaram na vitalidade do retalho e no processo de reparo tecidual. Entretanto, não evitaram a necrose macroscopicamente.

Descritores: Cicatrização. Dimetil Sulfóxido. Pentoxifilina. Ratos. Retalhos Cirúrgicos.

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