Morphological study of rat skin flaps treated with subcutaneous dimethyl sulfoxide combined with hyperbaric oxygen therapy

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ABSTRACT. This study investigated the effects of hyperbaric oxygen therapy (HBOT) and dimethyl sulfoxide (DMSO) in tissue necrosis, genotoxicity, and cell apoptosis. Random skin flaps were made in 50 male Wistar rats, randomly

divided into the following groups. Control group (CT), wherein a rectangular skin section (2 x 8 cm) was dissected from the dorsal muscle layer, preserving the cranial vessels, lifted, and refixed to the bed; distilled water (DW) group, in which DW was injected into the distal half of the skin flap; DMSO group, wherein 5% DMSO was injected; HBOT group, comprising animals treated only with HBOT; and HBOT + DMSO group, comprising animals treated with 100% oxygen at 2.5 atmospheres absolute for 1 h, 2 h after the experiment, daily for 10 consecutive days. A skinflap specimen investigated by microscopy. The percentage of necrosis was not significantly different between groups. The cell viability index was significantly different between groups ($P < 0.001$): 87.40% (CT), 86.20% (DW), 84.60% (DMSO), 86.60% (DMSO + HBO), and 91% (HBO) ($P < 0.001$), as was the cell apoptosis index of 12.60 (CT), 12.00 (DW), 15.40 (DMSO), 9.00 (HBO), and 12.00 (DMSO + HBO) ($P < 0.001$). The genotoxicity test revealed the percentage of cells with DNA damage to be 22.80 (CT), 22.60 (DW), 26.00 (DMSO), 8.80 (DMSO + HBO), and 7.20 (HBO) ($P < 0.001$). Although the necrotic area was not different between groups, there was a significant reduction in the cellular DNA damage and apoptosis index in the HBOT group.

Key words: dimethyl sulfoxide; Hyperbaric oxygenation; Rats; Surgical flaps

INTRODUCTION

The repair of cutaneous lesions with substantial material loss due to trauma, tumor resection, infection, or tissue ischemia involves the use of skin flaps. The relationship between the intended area of the flap and the extent of the vascular pedicle that is required to maintain arterial blood supply and venous drainage remains a challenge for surgeons during the creation of so-called random skin flaps (Okamoto, 2001).

The amount of ischemia to which the flap is subjected mainly during the first few hours is a crucial determinant of the degree of its viability (Chem et al., 1982). Considering that random skin flaps are exposed to ischemic events that can lead to the production of reactive oxygen species shortly after their creation, the use of free-radical scavenger drugs would be extremely valuable in this phase (Kosmider et al., 2004). For these drugs to be effective, they should be available at the surgical site at pharmacologically efficacious levels and at the appropriate time (Liu et al., 2004).

Another desirable feature of such a drug would be single-dose application. A review of the literature on basic pharmacology published during the last two decades showed that dimethyl sulfoxide (DMSO) acts as a free-radical scavenger and has beneficial effects when applied before, during, or some hours after a cutaneous lesion (Jeffrey et al., 1987). DMSO has been shown to improve the viability of skin flaps in different experimental models, at different concentrations, and through various routes of administration (Mallory et al., 1993).

A drug can be administered subcutaneously to achieve an adequate concentration at the desired location, quickly and safely. This route also allows the administration of a single dose during or shortly after surgery (Almeida et al., 2004).

To increase its tissue-protectant effect, we chose to combine DMSO with hyperbaric oxygen therapy (HBOT), which has been used globally for over 50 years (Zamboni, 1996). Some studies have described its protective efficacy in axial (Zamboni et al., 1992) and random (McFarlane and Wermuth, 1966) ischemic flaps.

The use of random skin flaps has been tested in several experimental animal models of disease (Rocha et al., 2013). These studies have shown its protective efficacy in reducing ischemia and tissue necrosis. However, there has been no report in the literature of a combination of HBOT with intra-lesional DMSO (Oliveira, 2008).

The purpose of this study was to assess the protective effect of DMSO in combination with HBO in preventing necrosis in a rat random flap model, using a subcutaneous route of drug administration.

MATERIAL AND METHODS

Approval

This research project was approved by the Ethics Committee on the Use of Animals of Universidade Federal de Mato Grosso do Sul (UFMS), protocol No. 317/2011. This study was conducted in accordance with the ethical principles adopted by the National Board of Animal Experimentation Control, current legislation, and other provisions on the ethics of research directly involving animals.

Sample

This research involved 50 male Wistar rats (*Rattus norvegicus albinus*), with an average weight of 300 g, provided by the UFMS vivarium, at an average age of 3 months. The animals were kept in polypropylene cages (14 x 40 x 60 cm), provided with feed specific for the species, and maintained in a light- and temperature-controlled environment.

Group distribution

The control group (CT, N = 10) had a dorsal flap with a 2-cm proximal base and 8-cm length elevated from its vascular bed and repositioned. Rats in the distilled water group (DW, N = 10) underwent the same procedure as the CT group and received a subcutaneous injection of 0.8 mL distilled water in the distal half of the flap. Rats in the DMSO group (DMSO, N = 10) underwent the same procedure as the CT group, but received an injection of 0.8 mL 5% DMSO in the distal half of the flap.

The animals in the DMSO combined with HBO group (DMSO + HBO, N = 10) were subjected to the same procedure as the CT group and were administered a subcutaneous injection of 5% DMSO followed by treatment with HBO at 2.5 atmospheres absolute (ATA), once daily for 90 min (15 min to pressurize, 60 min with 100% oxygen, and 15 min to depressurize) on 10 consecutive days.

Rats in the HBO group (HBO, N = 10) underwent the same procedure as the CT group and were treated with HBO for 10 consecutive days, once a day, for 90 min (15 min to pressurize, 60 min with 100% oxygen, and 15 min to depressurize).

Procedures

Surgical procedure

After anesthesia, the rats were weighed and placed in ventral recumbency, and subjected to trichotomy of the dorsal region, after which they were fixed in the prone position. After demarcating an area of 16 cm^2, i.e., a 2.0-cm cranial base and 8.0 cm in length following the method of McFarlen et al. (1966), an incision was performed with a scalpel (No. 15 blade) and the flap, including the epidermis and dermis up to the muscle plane, was desiccated. The flap was then lifted from the bed and reattached to its original position with a 4.0 polyamide yarn, interrupted every 2.0 cm to minimize the risk of dehiscence of the suture, in all directions.

Treatment administration procedure

In the DW and DMSO groups, 0.8 mL distilled water or 0.5% DMSO, respectively, was injected into every square centimeter of the distal half of the flap using a 1.0-mL syringe and a 13 x 3.8 insulin needle. The DMSO + HBO group underwent the same procedure as the DMSO group, followed by HBO treatment.

HBOT procedure

The rats in the DMSO + HBO and HBO groups were placed in an acrylic box 2 h after the experiment. The box had oxygen input and output controlled by a flowmeter and was placed inside the hyperbaric chamber, where oxygen saturation was recorded directly from the control panel. The box was adapted to be placed in a multiplace chamber, where it was pressurized for 15 min up to 2.5 ATA, and then subjected to 100% oxygen for 60 min at the same pressure, for 10 consecutive days.

Sample collection

On day 10 of the experiments, the rats were anesthetized again, weighed, photographed, and a specimen of tissue encompassing the entire thickness of the flap was collected by incisional biopsy. This tissue fragment measured 0.5 x 5.0 cm and was located in the central region of the distal third of the area without necrosis.

Euthanasia

The animals were anesthetized and euthanized in a carbon dioxide chamber after sample collection.

Macroscopic study

Evaluation of percentage of necrotic area

After anesthesia and immobilization (on day 10), the rats were photographed with a SONY

12.1 Mega pixel digital camera with 4-fold optical zoom, 30-mm lens, at a focal distance of 30 cm. The photos were scanned at a resolution of 640 pixels horizontally and 480 pixels vertically in 24-bit color (16 million colors). The area of necrosis was demarcated and the percentage was calculated using the ImageJ software.

Microscopic study

Histological procedure

Tissue samples were placed in 0.9% saline solution for the study of genotoxicity, apoptosis, and cellular necrosis.

Comet assay

Assessment of DNA damage by electrophoresis of isolated cells

The comet assay is a rapid, simple, and sensitive method of quantifying DNA damage in a small number of cells. This assay is particularly important for detecting intercellular differences in DNA damage and repair, in virtually any eukaryotic cell where it is possible to obtain a cell suspension, even from a small sample. Moreover, the results can be obtained in one day, the cost is relatively low, and the sensitivity of cell damage detection is comparable to other methods that assess DNA damage in a population of cells (Olive et al., 1992). The comet assay has been employed in the evaluation of DNA damage caused by physical and chemical agents, and in clinical settings (Anderson and Plewa, 1998). The association between cytotoxicity and genotoxicity is well established, since DNA damage is considered an important mechanism in the induction of apoptosis and necrosis. On the other hand, other substances or compounds may induce damage in the genetic material of cells without necessarily inducing cell death, which is why we chose this method to evaluate the damage induced by DMSO and HBO. The comet assay is considered one of the best methods for quantifying DNA damage because of its high sensitivity (Pereira, 2009). The data from this assay were collected using computer image analysis systems, which can evaluate different parameters. In this study, we performed a visual analysis as described by Jalonszysnski et al. (1997). This process evaluated only the extent of the formed tail, and the nucleoids were ranked from 0 to 4, according to the tail length. Nucleoids in classes 1 and 2 had moderate fragmentation, while those in classes 3 and 4 had intense fragmentation.

The comet assay was performed as describe by Singh et al. (1988) with some modifications. A fragment of skin (0.5 cm^3) was placed in a cryotube containing 100 µL saline solution (0.9% NaCl). The fragment was cut finely with a pair of scissors until completely homogenized. The fragment was then dissociated with the aid of a micropipette. A 40-µL sample of the cell suspension was mixed with 120 µL low-melting-point agarose (0.5%). This solution was placed on slides pre-coated with normal agarose (5%). The biological material was then covered with a glass coverslip and the slides were cooled to 4°C for 20 min. The coverslips were then removed and the slides were immersed in freshly prepared lysis solution (89.0 mL stock lysis solution, 2.5 M NaCl, 100.0 mM EDTA, 10.0 mM Tris, pH 10.0, adjusted with solid NaOH, 1.0 mL Triton X-100, and 10.0 mL DMSO). Lysis occurred for 1 h at 4°C, under protection from light. The slides were placed in the electrophoresis tank in a pH > 13 buffer (300.0 mM NaOH and 1.0 mM EDTA, prepared from a

stock solution of 10.0 N NaOH and 200.0 mM EDTA, pH 10.0) for a period of 20 min at 4°C in order to denature the DNA. Thereafter, electrophoresis was performed at 25.0 V and 300.0 mA (1.25 V/cm) for 20 min. Then, slides were neutralized with pH 7.5 buffer (0.4 M Tris-HCl) for three cycles of 5 min each, air-dried, and fixed in absolute ethyl alcohol for 10 min. The material was subsequently stained (100.0 µL ethidium bromide-20 x 10^{-3} mg/mL) and analyzed by florescent microscopy (Bioval® Brazil), at 40X magnification, with 420-490-nm excitation filter and a 520-nm barrier filter, as described by Kobayashi et al. (1995). The total score was calculated by adding the values obtained from multiplying the total number of cells observed in each designated class of cell-damage level by the value of the class.

Data generated by the comet assay can be visually analyzed (Kobayashi, 1995) by observing staining with a fluorescent intercalating agent (ethidium bromide) under a fluorescent microscope. The comets may be classified as follows: Class 0-undamaged nucleoids with no tail; Class 1-nucleoid with a tail smaller than the diameter of nucleoid; Class 2-nucleoid with tail 1-2 times the diameter of the nucleoid; Class 3-nucleoid with tail >2 times larger than the diameter of the nucleoid. Nucleoids of apoptotic cells that are fully fragmented are not usually counted (Speit and Hartmann, 1999).

The slides were visualized using a LEICA microscope, model DM500, dual head, coupled with a LEICA ICC 50 HD digital camera, with LAS software version 4.0.0, 2011, connected to a Dell XPS/M13 GHz Notebook (3582 MB RAM, and Microsoft Windows Vista® Ultimate operating system).

Apoptosis and necrosis

Criteria for classification of apoptotic, necrotic, or viable cells

The quantification of cells undergoing apoptosis and necrosis was performed by determining the color parameters and morphological appearance of the nuclei, in addition to condensation and fragmentation of chromatin. This distinction is possible because acridine orange penetrates living and dead cells, while ethidium bromide (EB) only penetrates cells with alterations in their membrane (late apoptosis or necrosis), and emits orange florescence when it intercalates into the DNA. Thus, according to Takahashi et al. (2004) and Kosmider et al. (2004), different cell patterns can be observed and classified as follows: 1) Living cells have uniform green nuclei; 2) cells in the early stages of apoptosis have intact membranes and therefore have green nuclei, but are not uniformly colored. These cells undergo chromatin condensation, DNA cleavage, and/or nuclear fragmentation. 3) Cells in late stages of apoptosis show chromatin condensation and orange areas in the nuclei, since in the final stages of the process, they lose membrane integrity and EB predominates over acridine orange. Apoptotic cells can also display apoptotic bodies (Liu et al., 2004); 4) necrotic cells lose membrane integrity and therefore exhibit uniform orange nuclei.

Cell viability assay

The detection of cell viability, apoptosis, and necrosis was achieved by differential staining with acridine orange and EB, visualized by fluorescent microscopy. Slides were prepared using a 50.0-µL aliquot of cell suspension obtained in the micronucleus test for the cell line CHO-xrs5 and

in the comet assay for the CHO-K1 cell line, previously described, and 2.0 µL dye (100.0 µg/mL acridine orange and 100.0 µg/mL EB, both diluted in PBS). A total of 200 cells were analyzed using a Nikon microscope at 60X magnification, with fluorescent filters (515-560 nm).

The cell classification was performed according to the following description: I) living cells with functional membrane have uniform green coloration in their nuclei; II) cells in the initial apoptotic stage with a functional membrane, but with DNA fragmentation, show a green stain in the nuclei and cytoplasm that is visible as a marginalization of their nuclear content; III) late-stage apoptotic cells exhibit orange colored areas in both the cytoplasm and in the sites were the chromatin is condensed in the nuclei, which distinguishes them from necrotic cells; and IV) necrotic cells have uniform orange color in the nuclei.

The cell viability index was calculated by determining the relationship between the number of normal cells x 100 and the number of cells analyzed. For the apoptosis index, we calculated the ratio of the sum of cells in early and late apoptosis x 100 and the total number of cells analyzed. For the necrosis rate, we calculated the relationship between the number of necrotic cells x 100 and the number of cells analyzed (Waters et al., 1990).

Statistical analysis

The comparison between the experimental groups in terms of weight gain, percentage of necrosis, cell injury, injury reduction, apoptosis, and necrotic cells, as well as the index of apoptosis, necrosis, and cell viability was performed using one-way ANOVA followed by the Tukey post-hoc test. Statistical analysis was performed using the SigmaPlot software, version 12.5, and the statistical significance level was defined as 5%.

RESULTS

The variation in weight and extent of the necrotic area between groups was not statistically significant, with P values of 0.266 and 0.249, respectively; however, there was a slight reduction in the necrotic area in the group treated with HBO alone, with the percentage of the necrotic area being 38.30% (Table 1).

Table 1. Difference in weight gain and percentage (%) of necrosis in each experimental group at the end of the experiment.

Variable	Experimental group					P value
	CT	HBO	DW	DMSO	DMSO + HBO	
Weight gain (g)	-16.10 ± 5.60	-30.89 ± 3.11	-14.50 ± 3.27	-27.80 ± 8.87	-25.63 ± 8.71	0.266
Necrosis (%)	43.83 ± 4.04	38.30 ± 3.27	46.79 ± 3.62	47.19 ± 3.75	45.94 ± 5.02	0.249

The results are reported as means ± SD. P value determined by one-way ANOVA. CT = control, HBO = hyperbaric oxygen therapy, DW = distilled water, DMSO = dimethyl sulfoxide, DMSO + HBO = DMSO combined with HBO.

The comet assay indicated that there were no differences (P > 0.05) between the DW, DMSO, and CT groups. The DMSO group showed a slight increase in the number of damaged cells (-0.88% *vs* CT). When the DMSO + HBO and HBO groups where compared to the CT group, the percentage of reduction in damage was 2.59- and 3.17-fold, respectively (Table 2).

Table 2. Mean values ± standard deviation of the frequency of damaged cells, distribution into damage class, and genotoxicity score of each experimental group.

Variable	Experimental group					P value
	CT	AD	DMSO	HBO	DMSO + HBO	
Damaged cells	22.80 ± 0.80[a]	22.60 ± 2.98[a]	26.00 ± 1.41[a]	7.20 ± 1.79[b]	8.80 ± 4.025[b]	< 0.001
Damage classe						
0	77.60 ± 1.17	77.40 ± 2.98	74.00 ± 1.41	92.80 ± 0.80	91.20 ± 1.80	0
1	16.40 ± 1.50	16.60 ± 1.50	23.00 ± 0.84	6.80 ± 0.73	6.00 ± 1.34	
2	5.80 ± 1.65	3.60 ± 1.03	3.00 ± 0.71	0.40 ± 0.24	2.40 ± 0.24	
3	0.60 ± 0.24	2.40 ± 1.36	0.00 ± 0.00	0.00 ± 0.00	0.40 ± 0.24	
Score	29.80 ± 2.67[a]	31.00 ± 5.90[a]	29.00 ± 2.07[a]	7.60 ± 0.93[b]	12.00 ± 2.50[b]	< 0.001
Damage reduction	-	1.00	-0.88	3.17	2.59	

CT = control, HBO = hyperbaric oxygen therapy, DW = distilled water, DMSO = dimethyl sulfoxide, DMSO + HBO = DMSO combined with HBO. Different letters indicate statistically significant differences (P < 0.05, ANOVA/Tukey's post-hoc test).

Percentage of damage reduction

The percent damage reduction (%DR), was calculated according to Waters et al. (1990) as per the following formula:

$$\%DR = \left[\frac{\text{Mean of positive control} - \text{Mean of associated group}}{\text{Mean of positive control} - \text{Mean of negative control}} \right] \times 100$$

The index of cell viability between groups, which is the ratio between the number of normal cells divided by the number of cells analyzed, multiplied by 100, was greater in the HBO group than in the CT group, i.e., 91.00 *vs* 87.40, respectively. Moreover, the combination of DMSO and HBO did not change the cell viability index when compared with the CT, DMSO, and DW groups (P > 0.05; Table 3).

Table 3. Means ± SD of the frequency of apoptotic and necrotic cells in rats from each experimental group.

Variable	Experimental group					P value
	CT	DW	DMSO	HBO	DMSO + HBO	
Cell type						
Normal	87.40 ± 0.40[b]	86.20 ± 0.74[b]	84.60 ± 0.98[b]	91.00 ± 0.63[a]	86.6 ± 0.75[b]	<0.001
Initial apoptosis	8.00 ± 0.55[b]	9.80 ± 0.37[a]	9.80 ± 0.37[a]	6.80 ± 0.38[c]	7.20 ± 0.37[b]	<0.001
Late apoptosis	4.60 ± 0.24[a]	2.20 ± 0.49[b]	5.60 ± 0.81[a]	2.20 ± 0.38[b]	4.80 ± 0.49[a]	0.002
Necrosis	0.00 ± 0.00[b]	1.80 ± 0.58[a]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	1.40 ± 0.25[a]	<0.001
Cell viability index	87.40[b]	86.20[b]	84.60[b]	91.00[a]	86.60[b]	<0.001
Apoptosis index	12.60[b]	12.00[b]	15.40[a]	9.00[c]	12.00[b]	<0.001
Necrosis index	0.00[b]	1.80[a]	0.00[b]	0.00[b]	1.40[a]	<0.001

CT = control, HBO = hyperbaric oxygen therapy, DW = distilled water, DMSO = dimethyl sulfoxide, DMSO + HBO = DMSO combined with HBO. Different letters indicate statistically significant differences (P < 0.05, ANOVA/Tukey's post-hoc test).

DISCUSSION

There are literature reports on several models of ischemic flaps, with different proportions between the size of the flap and the pedicle. In our study, we used the model described by McFarlane

et al. (1966), which is already fully established and has been used in several studies (Almeida et al., 2004). The dimensions of the flap were 8.0 x 2.0 cm, with a 4:1 proportion. The subcutaneous route of administration was selected as the most viable for dispersion of the injected test drug. Intradermal administration was not selected, since it can increase ischemia by compressing the intradermal vascular complex of the flap.

DMSO is a promising drug because of its recognized antioxidant properties, which have been described in other studies dealing with ischemic skin flaps (Okamoto, 2001). In designing the study, we chose a concentration of 5% DMSO and a total volume of 0.8 mL (corresponding to a total dose of 4 mg), based on the doses recommended in the literature for intradermal and transcutaneous administration.

In the literature, only the study by Almeida et al. (2008) describes the use of DMSO, administered subcutaneously through a single application, in random skin flaps in rats. The results of that study prompted our present study, which combined the administration of DMSO with HBO (Arturson and Khanna, 1970). The use of an experimental hyperbaric chamber for small and medium animals has been described in several studies; however, we found few studies (Oliveira, 2008) describing the use of a multiplace chamber (for medical treatment), with a campanula system with input and output of 100% oxygen, and an intermittent stream for 60 min, for use with mice and rabbits. These studies did not evaluate the oxygen saturation inside the enclosure in real time (Rocha et al., 2011). Hence, a commercial company (TOTALMAT-SP/BR) specially created an experimental box to assess oxygen saturation. This box has an output connection to the analyzer box in the control panel, to facilitate assessment of the exact concentration of oxygen in the interior of the box.

The biometric data of the rats showed an apparent reduction in weight, but this was not statistically significant. These data suggested that administration of DMSO or HBOT had no toxic effects, since all the rats were treated the same way during the creation of the skin flap.

The DW group had a higher percentage of necrotic skin area. This suggested that the administered DW may have acted as a barrier to vascularization, similar to what occurs with edema. This happened despite choosing an injection volume that would minimize this effect, based on the knowledge that edema and hematoma formation (physical barriers) could hamper the vitality of random skin flaps (Acevedo-Bogado et al., 2002). This was also corroborated by Almeida et al. (2008), who used saline solution (NaCl 0.9%) in a larger volume (0.1 mL/cm^2). These authors also reported that trauma caused by the insulin needle used for administration of the test drug or its vehicle may be an important factor for increase in the size of the necrotic area, similar to what was observed with the DW group in our study.

Almeida et al. (2004) observed that the administration of DMSO causes a reduction in the necrotic area in random skin flaps. These authors demonstrated that the CT group had a necrotic area of 47.99%, which was decreased to 41.57% after application of DMSO. Their results are in fact different from those described in this study, where we showed that the DMSO group had a necrotic area of 51.72%, which demonstrates a lack of protection.

When DMSO was combined with HBOT, there was a small decrease from 46.79 to 45.94%, as compared to the DW group, and an increase from 43.83 to 47.19%, as compared to the CT group, demonstrating the absence of protection in terms of the size of the necrotic areas. However, studies performed on random flaps by Arturson and Khanna (1970) using DMSO administered intraperitoneal 25 min after surgery, with or without hyperbaric treatment, at 2 and 3 ATA for 4 and 2 h, respectively, showed that these two treatments were capable of reducing the area of necrosis.

The difference in results between these studies can be explained by the possibility that the physical barrier created by DMSO, or any other liquid that simulates the formation of edema, may interfere with the protective effects of HBOT on the tissues. It can also be assumed that the increase in ATA and the exposure time in the hyperbaric chamber may be important factors in reducing the physical barrier, and may therefore increase the efficiency of the oxygen therapy, since a larger infusion would occur with greater protection from ischemia.

The HBO group showed the best results, i.e., the smallest area of necrosis. However, this reduction was not statistically significant. Nonetheless, it represents a trend with important biological significance.

A reduction in the area of necrosis by HBO, has previously been demonstrated by Arturson and Khanna (1970). These authors demonstrated that the increase in pressure from 2 to 3 ATA, even with a decrease in the time of exposure to 100% oxygen, improves the response. Therefore, these authors inferred that increasing the atmospheric pressure is more important than increasing the number of sessions or the time of exposure to oxygen, even in the presence of a physical barrier that corresponds to trauma-related edema. Increased atmospheric pressure, reduced physical barrier, and neoangiogenesis in the margin of the injury may also facilitate the process of reducing the area of necrosis (Arturson and Khanna, 1970).

In the genotoxicity tests in which the comet assay was employed, the average frequency of damaged cells distributed among the damage classes increased slightly but not significantly (CT *vs* DW). In the group treated with DMSO, there was an increase of genotoxicity in the subcutaneous plane, but when DMSO was combined with hyperbaric treatment, this effect decreased markedly from 22.60% (DW) to 8.89% (DMSO + HBO). This further decreased when the DMSO + HBO group was compared with the HBOT group alone, reaching statistical significance, with a damage reduction of 2.59 and 3, respectively. The cell viability (apoptosis and necrosis) test used differential staining with acridine orange and EB for determination of viable and non-viable cells. In this study, we obtain data from different protocols for induction of DNA damage and chemoprotection, through the analysis of the frequency of micronuclei, for two supposedly protective agents. The apoptosis index observed in the cell viability test was similar to that determined by the comet assay (Waters et al., 1990), i.e., a slight increase with DMSO alone and a significant decrease when combined with HBOT, and an even further decrease when HBOT was used alone. According to Fenech et al. (1999), apoptosis eliminates micronucleated cells, thereby reducing the frequency of cells with clastogenic damage. Vukicevic et al. (2004) had two distinct considerations in this regard; firstly, the authors stated that a high frequency of cells undergoing apoptosis may be related to other genetic damage that would not result in the formation of micronuclei, and so would not further raise its frequency. Previous studies confirmed our results suggesting that oxygen acts as a flag for a series of events that promote healing. HBO-treated tissues show a reduction in hypoxia-inducible factor 1-alpha (HIF-1a), which is an important gene expression regulator involved in the regulation of tissue oxygenation and which increases in the presence of hypoxia (Bertoletto et al., 2007). The protein levels of BNip3 (a pro-apoptotic protein located in the mitochondria) was markedly elevated from day 10 in ischemic tissues. High oxygen levels induced by HBO reduced and attenuated the expression of BNip3, thereby modulating apoptosis despite the persistently high level of lactic acid. Thus, HBO increases anti-apoptotic activity and decreases pro-apoptotic activity, diminishing the release of pro-apoptotic molecules and mitochondria, as well as attenuating apoptosis (Zamboni, 1996). The molecular mechanisms of protection provided by HBO in the healing of ischemic wounds may involve modulation of HIF-1a. Wounds treated with HBO show decreased HIF-1a levels and

related gene expression, with consequently decreased apoptosis and reduced inflammation. Kranke et al. (2004) in an immunohistochemical study of cell apoptosis performed in random skin flaps, while evaluating apoptosis with various extracts, noticed the protective effects of HBOT in the mid and distal portions of the flap, representing a possible reduction in expression of caspase 3 in the outer skin of the mouse. This suggested that hyperoxygenation in healthy tissues is similar to that occurring during the surgical stimulus (Rocha, et al., 2013). These facts corroborated our findings, which showed that treatment with oxygen therapy was efficient in reducing cell genotoxicity and cytotoxicity. This can be attributed to the increase in hyperbaric oxygenation of the tissues during the initial ischemia phase.

CONCLUSION

In the morphological study, computerized macroscopic observation of the necrotic area of the flaps in the groups studied revealed a reduction in the extent of necrosis when DMSO was combined with HBO; this effect was even more pronounced in the group treated with HBO alone. However, the differences between groups were not statistically significant.

While evaluating the protective effect of DMSO and HBO on tissues, we found a significant effect in the group treated with HBO alone in terms of the reduction in cell damage and apoptosis.

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