PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(12):1018-1028, December 2020
DOI: 10.1590/1678-5150-PVB-6543

Original Article
Small Animal Diseases

Effects of canine adipose-derived mesenchymal stem cells on the epithelialization of rabbits' skin autograft (*Oryctolagus cuniculus*)[1]

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ABSTRACT.- Alvarez Gómez J.L., Pazzini J.M., Barros M.A., Morais B.P., Firmo B., Matsui A., Ampuero R. & De Nardi A.B. 2020. **Effects of canine adipose-derived mesenchymal stem cells on the epithelialization of rabbits' skin autograft (*Oryctolagus cuniculus*)**. *Pesquisa Veterinária Brasileira 40(12):1018-1028*. Departamento de Clínica e Cirurgia Veterinária, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Júlio de Mesquita Filho", Via de Acesso Prof. Paulo Donatto Castellane s/n, Jaboticabal, SP 14884-9900, Brazil. E-mail: jorgeluisalgo@gmail.com

The present study aimed to evaluate the effects of mesenchymal stem cells derived from canine adipose tissue in the healing process of full-thickness mesh skin grafts in rabbits. The stem cells were collected from young dogs; and, after characterization, remained in cryopreservation, in independent doses containing 2 x 10^6 cells. The mesh distal limb graft technique was performed in 60 rabbits, divided into three groups, CG (Control Group), GT1 (Intralesional Stem Cell Treated Group), and GT2 (Intravenous Stem Cell Treated Group), containing 20 animals each. After grafting, each group was randomly divided into four subgroups according to euthanasia time 3, 7, 14, and 30 days, containing five animals in each group. Animals of GT1_14, GT1_30, and GT2_14, GT2_30 subgroups received a second dose of xenogeneic cells on the seventh day. Meanwhile, animals from GT1_30 and GT2_30 received the third dose of xenogeneic cells on day 14. The groups treated with xenogeneic stem cells positively affected type III collagen re-epithelialization and deposition, and possibly GT1 had a controlled inflammatory response. However, no effect on angiogenesis. Thus, it was possible to demonstrate tolerance and therapeutic action of mesenchymal stem cells from canine adipose tissue in skin grafts in rabbits.

INDEX TERMS: Canine, stem cells, epithelialization, rabbits, skin autograft, *Oryctolagus cuniculus* xenogens, mesh grafts, healing, revascularization, angiogenesis.

RESUMO.- [**Efeitos das células-tronco mesenquimais derivadas do tecido adiposo de cães na epitelização de autoenxertos de pele em coelhos (*Oryctolagus cuniculus*).**] O presente estudo teve como principal objetivo avaliar os efeitos das células-tronco mesenquimais derivadas do tecido adiposo de cães no processo de cicatrização de autoenxertos de pele de espessura total em malha em coelhos. As células-tronco foram coletadas de cães jovens, após a caracterização estas permaneceram em criopreservação, em doses individuais contendo 2 x 10^6 células. A técnica de enxerto em malha na região distal do membro foi realizada em 60 coelhos, divididos em três grupos, GC (Grupo Controle), GT1 (Grupo tratado com células-tronco intralesional) e GT2 (Grupo tratado com células-tronco via endovenosa), contendo 20 animais cada. Imediatamente após a enxertia, cada grupo foi dividido aleatoriamente em quatro subgrupos, de acordo com o tempo de eutanásia 3, 7, 14 e 30 dias contendo cinco animais cada. Animais dos subgrupos GT1_14, GT1_30 e GT2_14, GT2_30 receberam uma segunda dose de células xenógenas no sétimo dia. Ademais, animais do GT1_30 e do

[1] Received on June 21, 2020.
Accepted for publication on September 10, 2020.
Master's Research with support from CAPES.

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GT2_30 receberam a terceira dose de células xenógenas no dia 14. Os grupos tratados com células-tronco xenógenas tiveram um efeito positivo na reepitelização e deposição de colágeno tipo III, e possivelmente, o GT1 teve uma resposta inflamatória controlada, entretanto o efeito na angiogênese não foi observado. Dessa forma, foi possível demonstrar que houve tolerância e ação terapêutica das células-tronco mesenquimais derivadas do tecido adiposo de cães em enxertos de pele em coelhos.

TERMOS DE INDEXAÇÃO: Células-tronco, cães, autoenxertos, pele, coelhos, *Oryctolagus cuniculus*, xenógenas, enxertos em malha, cicatrização, revascularização, angiogênese.

INTRODUCTION

Full-meshed skin autografts are a cutaneous segment that includes the epidermis and the entire dermis. This grafting technique is commonly used to cover large defects after excision of neoplasms, trauma, or burns, mainly when the wounds are located in the limbs' distal region or on flexor surfaces (Bohling & Swaim 2012, MacPhail 2013). Traditionally, a recipient site with granulation tissue is recommended for the technique's success; however, this recommendation does not seem to influence graft survival (Tong & Simpson 2012, Riggs et al. 2015). Severe inflammation, the presence of clots, or the formation of seroma between the graft and recipient site site during the first week of grafting interfere negatively in phases of adherence, inosculation, and revascularization, causing delay of the regenerative process, failure of technique, and death of the graft (Andreassi et al. 2005, Bohling & Swaim 2012, MacPhail 2013). To improve the fixation and adherence of skin graft in the recipient site, and consequently to stimulate epithelialization and angiogenesis, some studies have evaluated negative vacuum pressure therapy (Nolff & Meyer-Lindenberg 2015, Or et al. 2017), platelet-rich plasma sponge (Pazzini et al. 2018) and laser therapy (Reis Filho et al. 2017), showing promising results.

In the field of regenerative medicine, it has been shown that mesenchymal adipose-derived stem cell (ADSCs) accelerates the stages of the skin wound healing process (Kim et al. 2007), including ischemic wounds caused by diabetes (Kim et al. 2011) and wounds caused by burns (Franck et al. 2019), with increased collagen secretion and deposition (Kim et al. 2007, Franck et al. 2019). Autologous ADSCs have also been shown to improve skin autografts' survival in diabetic rats (Zografou et al. 2013). Studies on ischemia and reperfusion models of axial skin flaps have reported a favorable effect of ADSCs, decreasing necrosis and associated complications (Suartz et al. 2014, Pu et al. 2017).

These facts corroborate that autogenous or allogeneic mesenchymal stem cells have a reparative effect, either by direct transdifferentiation compatible with the cells of the injured tissue or, indirectly by a paracrine effect (Rehman et al. 2004) by several growth factors with antifibrotic or anti-apoptotic action (Rehman et al. 2004), angiogenic action (Kim et al. 2011, Hamada et al. 2019, Franck et al. 2019), anti-inflammatory and immunomodulatory action (Pu et al. 2017, Oliveira et al. 2018, Pinto-Filho et al. 2018). Concerning xenogenous mesenchymal stem cells, experimental studies reported that transplantation of pig adipose-derived stem cells (ADSCs) into joints of dogs with osteoarthritis was favorable in controlling the disease (Tsai et al. 2014). Human ADSCs were also effective in healing skin wounds in rabbits (Rodriguez et al. 2015, Kim et al. 2017) and mice (Kim et al. 2011), without showing clinical signs of intolerance to xenogen transplantation. According to Li et al. (2012), only six (6.4%) of 88 published experiments showed failure in stem cells' function, constituting a possible therapeutic alternative with potential clinical use. In this sense, the present study aimed to evaluate the effects of mesenchymal stem cells derived from dogs' adipose tissue in the healing process of full-thickness skin autografts in rabbits.

MATERIALS AND METHODS

The experiment was approved by the Ethics Committee on the Use of Animals (ECUA/FCAV), protocol number 173466/15, of "Faculdade de Ciências Agrárias e Veterinárias", "Universidade Estadual Paulista "Júlio de Mesquita Filho" (Unesp), Campus of Jaboticabal/SP, Brazil.

Animals. Sixty New Zealand White female rabbits (*Oryctolagus cuniculus*), fourteen weeks of age and average weight of 3.0kg (standard deviation 0.14), clinically healthy and without physical changes. The animals were divided into three groups, CG (Control Group), GT1 (Group treated with adipose-derived stem cells (ADSCs), intralesional), and GT2 (Group treated with ADSCs, intravenously), containing 20 animals each. Immediately after grafting, each group was randomly divided into four subgroups, according to the time of euthanasia: 3, 7, 14, and 30 days (GC_3, GC_7, GC_14, GC_30; GT1_3, GT1_7, GT1_14, GT1_30 and GT2_3, GT2_7, GT2_14, GT2_30) with five animals each. Before the experimental procedure, all rabbits were kept in individual cages for an adaptation period of four weeks, in a controlled environment to reduce the stress response, receiving water ad libitum and 100 grams per day of commercial feed for fattening (Agromix Animal Nutrition, Jaboticabal/SP, Brazil) and 100 grams of hay every 48 hours. The rabbits were handed at the same hours and by the same staff.

Xenogenous stem cells. The mesenchymal stem cells derived from dogs' adipose tissue were obtained from the stem cell bank of the company Regenera Stem Cells® (Campinas/SP, Brazil), cryopreserved in individual flasks containing doses of 2×10^6 cells, ready for use. According to company information, the origin of the adipose tissue is periovarian (10-15g) collected during the castration of young donors (≤2 years) and healthy (negative for distemper, parvovirus, and leishmania). The collected adipose tissue was washed, fragmented, and incubated for one hour in a saline solution buffered with Dulbecco's phosphate (DPBS) with a 0.1% type I Collagenase, and centrifuged. Subsequently, the cell precipitate was resuspended in culture (modified Eagle medium with high glucose concentration (LGC Biotechnology, São Paulo, Brazil), 15% fetal bovine serum (Hyclone Inc., Logan/UT, USA), 1% penicillin/streptomycin, 1% of non-essential amino acids and 1% L-glutamine (LGC Biotechnology) and incubated at 37°C and 5% CO_2. When it reached approximately 70% confluence, the cells were trypsinized and expanded. Part of the material was separated for characterization, where the potential for differentiation of mesenchymal stem cells (MSC) was assessed through its ability to differentiate into three different cell lines (osteogenic, adipogenic, and chondrogenic) under specific culture conditions. The immunophenotypic profile of MSCs was evaluated through flow cytometry by expression of CD44, CD73, CD90, CD105, and the absence of CD34 and CD45.

Anesthetic procedure. Rabbits were pre-anesthetized with 0.07mg/kg of acepromazine (Acepran®Vetnil, São Paulo, Brazil) and 0.5mg/kg of morphine (Dimorf®, Cristália, São Paulo, Brazil)

intramuscularly. After 15 minutes, the animals were oxygenated for one minute using a face mask attached to a semi-open system connected to the anesthesia machine. Afterward, isofluorane (Isoflorine®, Cristália, São Paulo, Brazil) was supplied 5V% until the animal allowed to instill oral spray Lidocaine (Xylestesin®, Cristália, São Paulo, Brazil). In lateral decubitus and with the head extended, the orotracheal intubation with a Magil probe number 3.0 was performed. Subsequently, the concentration of isofluorane was then adjusted to maintain an adequate anesthetic plan. The atrial artery was catheterized (22G BD Insyte catheter, Minas Gerais, Brazil) to monitor blood pressure throughout the procedure.

Surgical procedure. With the animal in an adequate anesthetic plane, trichotomy of the right lateral wall of the chest (local donor) and the distal region of the right radio (recipient site) was performed, followed by antisepsis and asepsis. A 2cm x 2cm square wound was created in the recipient site, leaving the tendons of the radial carpal extensor muscle, common digital extensor, and lateral digital extensor exposed. Then, at the donor site, a 2cm x 2cm incision was made to obtain the skin fragment and transform it into a full-thickness mesh graft. Subsequently, the graft edges were fixed with 4-0 nylon suture in a particular simple suture pattern. The donor site wound was also closed with 4-0 nylon thread, in a simple interrupted suture pattern, in a centripetal way. Finally, to compress the graft to the recipient site, non-adherent compressive bandages were performed, the first contact layer consisting of a non-sterile adherent gauze moistened with lubricating gel (K-Y® Johnson & Johnson, Brazil), the second layer of sterile dry gauze, and cotton bandage, and the third layer of protection with waterproof adhesive. All animals received a prophylactic dose of 5mg/kg of enrofloxacin (injectable Chemitril 2.5%, Chemitec, Brazil) subcutaneously, 20 minutes before the start of surgery.

Analgesia with tramadol hydrochloride (Tramal® ampoule 50mg/ml), dosage of 3mg/kg/subcutaneously, every 8 hours for three days postoperatively. The evaluation of acute pain was performed based on changes in behavior and facial expression in rabbits recommended by Leach et al. (2009) and Keating et al. (2012). No anti-inflammatory drugs were injected. The bandage swap was repeated every 72 hours, followed by the macroscopic assessment of the grafts recommended by Reis Filho et al. (2017) by a single evaluator blindly.

Xenogenous stem cell transplantation. In all GT1 animals, the first dose of 2 x 10^6 adipose-derived stem cell (ADSC) cells was intralesional before positioning the graft at the recipient site. Immediately after thawing the ADSC and later dilution of 2 x 10^6 ADSCs in 0.5 mL of lactated ringer's solution, 0.1 ml was injected in five different locations (confluent lateral edges of the wound and in the middle of the lesion) using a 21 G needle. GT1_14 animals received the second dose on the seventh postoperative day. The GT1_30 animals received the second and third doses on the seventh and fourteenth postoperative day, respectively, through the grafts' lateral slits. All GT2 animals received the first dose of 2 x 10^6 ADSC cells, diluted in 1.0mL of lactated ringer's solution, slowly through the ear's marginal vein using a 22 G catheter and a 21G needle, immediately after graft fixation at the recipient site. GT2_14 animals received the second dose on the seventh postoperative day. GT2_30 animals received the second and third doses on the seventh and fourteenth postoperative day, respectively. However, in the CG animals, only skin autograft was performed. The thawing and washing of the ADSCs were performed using the same company's defrosting kit that guarantees the viability of ADSC above 70%; for this reason, after thawing the ADSC, a viability test was not performed at the time of intralesional transplantation or intravenous infusion.

Histopathological evaluation. After euthanasia on the previous days, the grafts were excised with an extra 0.5cm lateral margin of intact skin and a deep facial plane. The samples were immersed for 36 hours in buffered formaldehyde 10%. After this period, they were transferred to a 70% alcohol solution (70% ethyl alcohol) until the moment of paraffin inclusion processing. The 4µm cross-sections were stained with hematoxylin and eosin (HE). The evaluation of inflammation, vascularization, and fibroblasts was the quantitative method of counting mononuclear and polymorphonuclear cells, blood vessels, and fibroblasts both in the superficial and deep dermis, selecting five histological fields at 40x magnification, and was expressed as the average number of vessels per sample. The evaluation of re-epithelialization and necrosis was performed as recommended by Reis Filho et al. (2017). For the evaluation of collagen, picrosirius was used (Easy Path - Picrosirius Red Staining). The sections were analyzed under an optical microscope under polarized light (Olympus BX-53®, Japan). From each slide, 5 histological fields were selected and photomicrographed at a 20x magnification. The images were analyzed with the *software* Image J®, with the plug-in Threshold Colour. The observation of type I collagen fibers (yellow-orange to orange and red birefringence) and type III collagen (green or yellow-birefringence) was identified as recommended by Bedoya et al. (2016).

Immunohistochemistry. The cuts were extended on glass sheets previously cleaned and degreased, prepared with an organosilane-based adhesive (3-aminopropyltriethoxysilane, Sigma Chemical, C.O., USA). Monoclonal antibody CD31 evaluated Immunomarking of vascular endothelium for angiogenesis (Abcam), 1:50 dilution, antigenic recovery with porcine gastric mucous pepsin (Sigma life the science-United Kingdom) 37°C greenhouse, incubation 120 min, a detection system for polymer kit method (Novolinktm Polymer Detection Systems, Leica Biosystems New Castle Ltda-United Kingdom). The immunomarking evaluation of the vascular endothelium (angiogenesis) was performed by the quantitative method of microvascular counting, according to Pazzini et al. (2017), analyzing the areas with the largest number of vessels in the superficial and deep dermis, selecting five fields for analysis in the 40x magnification and was expressed as the average number of vessels in each sample.

Macroscopic evaluation and statistical analysis. The eight continuous variables (macrophages, neutrophils, monocytes, fibroblasts, vascularization, type I collagen, type III collagen, CD31) were categorized into three levels considering the position of the values of each variable: lowest (0<25% first quartile), medium (>25-75% second quartile) and highest (>75% third quartile). This division was performed for each group (GC, GT1, GT2). For categorical variables such as necrosis, re-epithelialization, secretion, and crust, they were cataloged in four classes: absent, discreet, moderate, and accentuated. The graft color variable was classified as usual pink-red, blue, and ischemic. Burt's tables were created, and multivariate multiple correspondence analysis (MCA) for times 3, 7, 14, to determine the association and total variation between the categorical variables and 30 for GC, GT1, and GT2 (general correspondence). After the general correspondence analysis, the variables were grouped into three classes: inflammatory cells (macrophages, neutrophils, monocytes), proliferation (fibroblasts, vascularization, type III collagen, type I collagen, CD31), and external appearance (graft color, secretion, crust, necrosis, re-epithelialization) performing a new ACM analysis for each class by groups including the four times in the same analysis. The Statistical program (STATSOFT, Version 7) was used with a significance level of 0.05.

RESULTS

During the experiments, the animals of GT1 and GT2 did not show local or systemic clinical signs suggestive of rejection or hypersensitivity after transplantation of xenogenic stem cells. On the third day of the macroscopic evaluation, serosanguinolent secretion was moderate in all groups but gradually decreased over the first week of evaluation (Fig.1). However, the animals of GT1_14 continued to present a discreet serous secretion, possibly because they received the second dose of intralesional stem cells on the seventh postoperative day. In most cases, the re-epithelialization of the graft slits was prominent after the seventh day (Fig.1). The grafts' color was similar between all groups; however, grafts in GT2 had a better macroscopic appearance (no crusts) after the second week of evaluation (Fig.1).

Through multiple correspondence analysis (MCA) of the variables grouped in the external appearance class, it was possible to identify a significant correspondence ($p<0.05$) of the re-epithelialization with GT1_3, GT1_30, and GT2_3 (Fig.2)

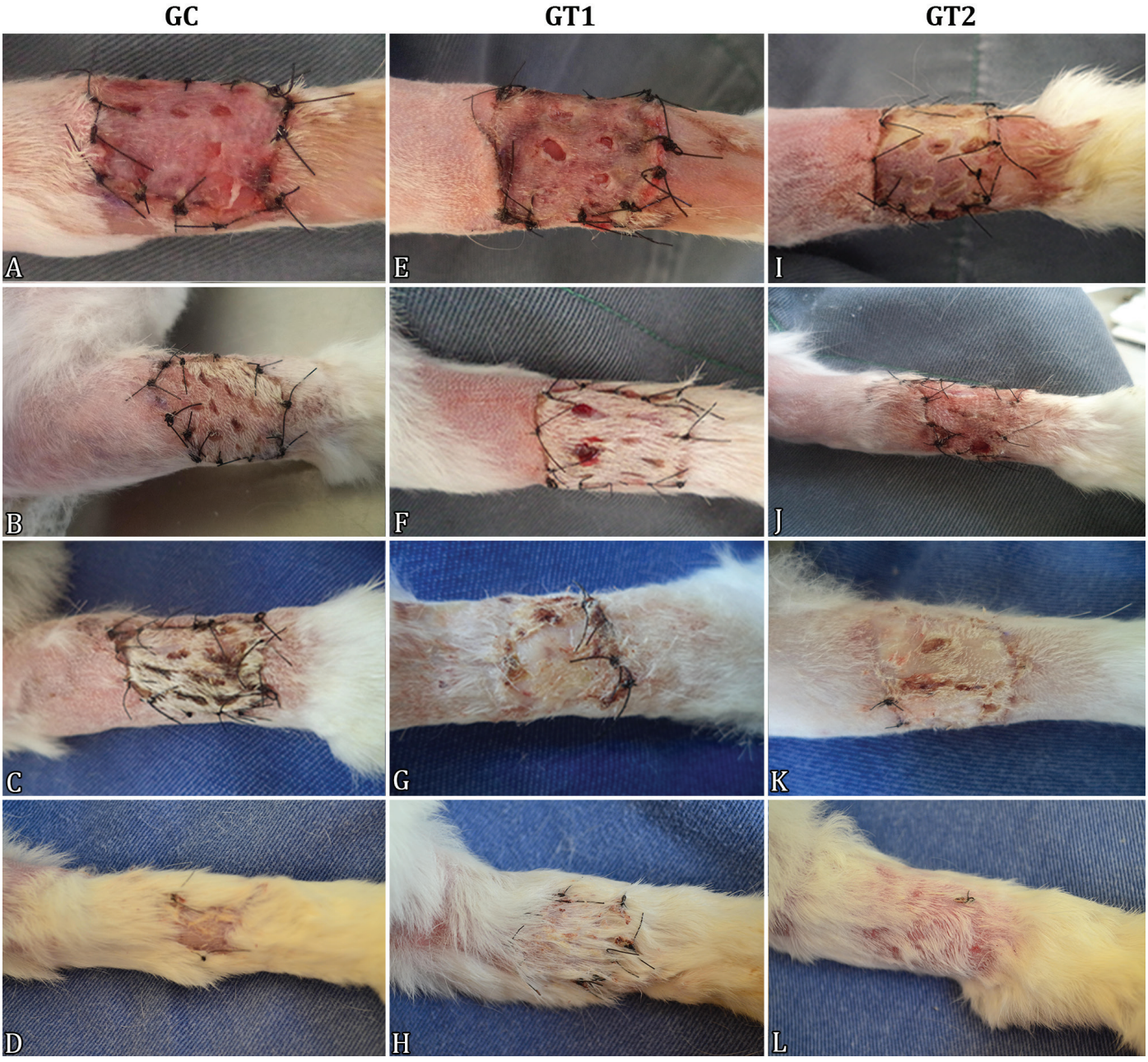

Fig.1. Macroscopic evaluation and clinical evolution of mesh skin grafts in the distal region of the right thoracic limb in rabbits (*Oryctolagus cuniculus*). The presence of secretion through the open slits and the reddish-pink color mixed with the blue or ischemic color was notorious on the third day of evaluation (**A**) GC_3, (**E**) GT1_3, (**I**) GT2_3. On the seventh day, the secretion decreased, and the cracks started to heal (**B**) GC_7, (**F**) GT1_7, (**J**) GT2_7. On the fourteenth day, the grafts' color was similar to normal tissue, with the presence of crusts and healing of the slits in the grafts. (**C**) GC_14, (**G**) GT1_14, (**K**) GT2_14. In general, on the 30th, there was hair growth, showing the treated groups better appearance (**H**) GT1_30, (**L**) GT2_30 than the control group (**D**) GC_30.

and re-epithelialization with GT1_7 and GT2_7 (p<0.10). Although there are other correspondences described in table 1, they were not significant. Regarding the infiltration of inflammatory cells, formed by neutrophils, macrophages, and monocytes, were present in most samples of GC, GT1, and GT2. The cellular infiltrate in the superficial and deep dermis was more evident in samples from the seventh day, gradually decreasing until the 30th. There were some inflammatory foci in the superficial dermis. Through the MCA of the variable inflammatory cells, summarized in table 1, a significant correspondence (p<0.05) of macrophages and GT1_3 and GT1_14 was observed (Fig.3). Other significant associations between the variable high neutrophils with high monocytes and low neutrophils with low monocytes were observed in GT2_7 and GT1_14, respectively. Regarding the control group, there were no significant matches.

During the microscopic evaluation of the samples on the third day, it was possible to observe incomplete epithelialization, predominant in the not re-epithelialized area, especially in grafts from the control group. (Fig.4). The integration of the graft at the recipient site was evident after the seventh day. Necrosis was similar between CG, GT1, and GT2, being on the third day multifocal in most samples and with discrete signs on the seventh day. In all groups. Fibroblast proliferation was marked on the seventh and moderate on the fourteenth day of evaluation. Samples from animals treated with xenogenous adipose-derived stem cells (ADSCs) had a more significant presence of keratinocytes proliferating on day 14. Evaluation of vascularization/angiogenesis by the quantitative counting method microvascular in HE stained samples (Fig.4) and immunostaining with CD31 antibody, respectively, were similar.

Regarding collagen fibers, after histochemical evaluation with picrosirius (Fig.5), it was observed that type III collagen

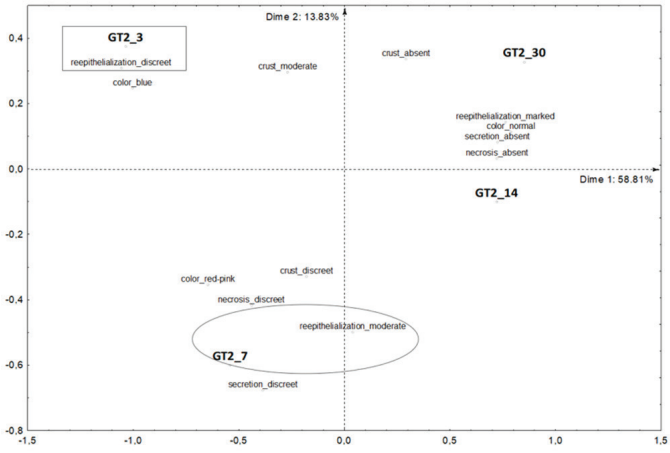

Fig.2. Multiple correspondence analysis of the variables grouped in the external appearance class (graft color, secretion, crust, necrosis, re-epithelialization) for treatment group 2 (GT2), including the four times in the same analysis. There was a significant correspondence (p<0.05) of the variables within the rectangle. However, the variables within the circle were significant for p<0.10.

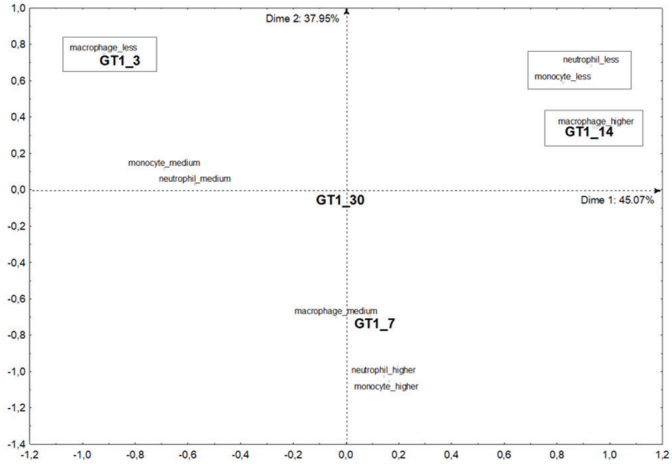

Fig.3. Analysis of multiple correspondences of the variables grouped in the class inflammatory cells (macrophages, neutrophils, monocytes) for treatment group 1 (GT1), including the four times in the same analysis. There was a significant correspondence (p<0.05) of the variables within the rectangle. Although there were other correspondences, they were not significant.

Table 1. Summary of the multiple correspondence analysis of variables grouped into three classes: inflammatory cells, proliferation and external appearance

Grouped variables	Group	Evaluation day			
		3	7	14	30
Macro/Neutral/Mono	GC	L/L/L	H/H/H	M/-/M	M/M/-
	GT1	L/M/M	M/H/H	H/L/L	M/M/-
	GT2	L/M/L	H/H/H	M/L/M	M/-/-
Fibro/Vasc/COL-I/COL-III/CD31	GC	L/L/-/-/L	H/H/-/M/M	M/M/-/-/H	-/-/L/H/-
	GT1	M/-/-/L/-	-/M/H/-/H	L/L/-/-/-	H/H/L/H/-
	GT2	L/L/-/-/-	M/M/-/H/-	H/H/-/-/L	M/M/-/H/-
Necros/reepitel/color/secretion/crust	GC	mo/-/-/mo/-	-/-/r/d/d	-/mo/-/-/mo	-/ch/n/a/a
	GT1	-/d/is/-/-	d/mo/r/-/-	-/-/-/d/mo	a/ch/n/a/a
	GT2	-/d/bl/-/-	d/mo/r/d/d	a/ch/n/a/a	a/ch/n/a/a

Macro = macrophages, Neutral = neutrophils, Mono = monocytes, Fibro = fibroblasts, Vasc = vascularization, COL I = collagen type I, COL-III = collagen type III, CD31 = angiogenesis, L = low, M = medium, H = high, a = absent, d = discrete, mo = moderate, ch = checked, n = normal, r = red-rose, bl = blue, is = ischemic.

fibers predominated at all times of assessment and in all groups. However, edema in the deep dermis was frequently observed in GT1_7 and GT1_14, dissociating the collagen fibers. In this same group, there were also hemorrhage foci on the third, seventh, and fourteenth day of the microscopic evaluation (Fig.4).

Through the multiple correspondence analysis (MCA) of the proliferation variables (fibroblasts, vascularization, collagen type III, collagen type I, CD31), several matches were found (Table1). However, a significant correspondence ($p<0.05$) of the vascularization was observed in the control group (GC_7, GC_14); in the group treated with intralesional stem cells (GT1_30) and, in the group treated with stem cells intravenously (GT2_3, GT2_14) (Fig.6). There was also a significant correspondence of type III collagen in GT1_3, GT1_30, and GT2_30. On the other hand, fibroblast proliferation was significant only in GT2_3 (Fig.6).

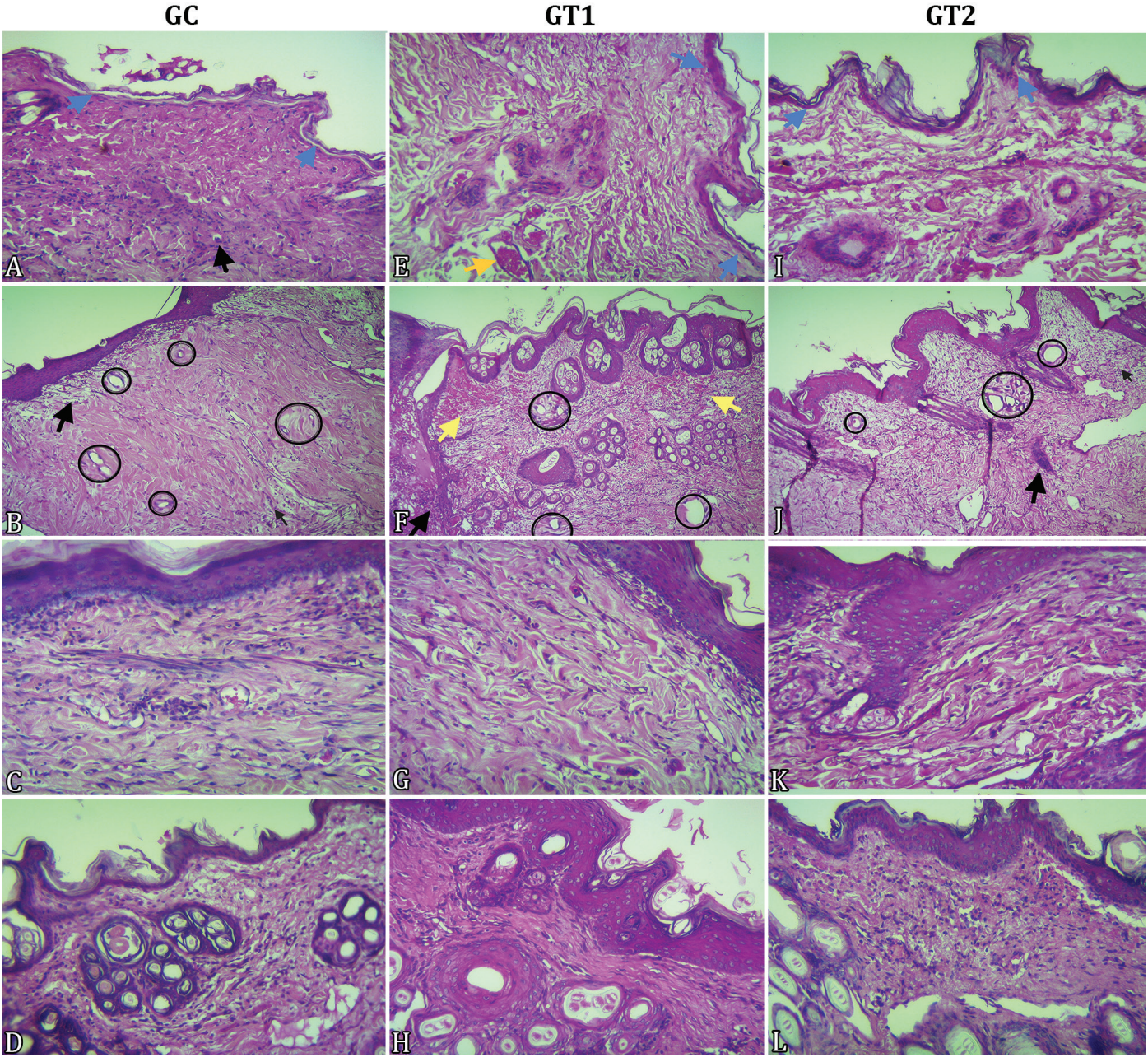

Fig.4. Microscopic evaluation of full-thickness mesh skin grafts in rabbits (*Oryctolagus cuniculus*). On the third day, the formation of discontinuous and thin epithelium is observed - epidermis (blue arrow), hemorrhage foci (yellow arrow) and slight presence of inflammatory cells (black arrow) (**A**) GC_3, (**E**) GT1_3, (**I**) GT2_3. On the seventh day, there was a predominance of the re-epithelialized area, neoangiogenesis (black circle), mild hemorrhage (yellow arrow) (**B**) GC_7, (**F**) GT1_7, (**J**) GT2_7. On the fourteenth day, the re-epithelialization was complete on the connective tissue (**C**) GC_14, (**G**) GT1_14, (**K**) GT2_14. On day 30, a complete formed epithelium is observed, showing follicles developed in the treated groups (**H**) GT1_30, (**L**) GT2_30 than in the control group (**D**) GC_30. (**B**, **F**, **J**) HE, obj.10x. (**A**, **C**, **D**, **E**, **G**, **H**, **I**, **K**, **L**) HE, obj.20x.

DISCUSSION

According to Andreassi et al. (2005) and Liptak (2012), fibrin, leukocytes, and fibroblasts play essential roles in the adherence and plasma imbibition of grafting. However, the adherence phase can be compromised by excess fluids in the recipient site (Yucel et al. 2016) or by the presence of xenogenic biological material (Reis Filho et al. 2017), interfering with graft adhesion to the recipient site, causing chronic inflammation, purulent secretion, ischemic necrosis and consequent failure of graft integration and healing (Bohling & Swaim 2012, MacPhail 2013, Reis Filho et al. 2017). However, grafts treated with intralesional xenogenous stem cells did not demonstrate these harmful clinical signs despite delayed serous secretion in GT1 (GT1_14), similar to another study (Rodriguez et al. 2015). Edema, dissociation of collagen fibers, and foci of hemorrhage in GT1 samples possibly occurred due to the dilution volume (Yucel et al. 2016). Due to repeated local trauma after intralesional injection (in the second and third doses) with a 21G gauge needle, both the dilution volume and repeated injections did not hinder the success of the mesh grafts. Recently, a study demonstrated that a local injection of 200μL of 0.9% saline solution in full-thickness grafts in

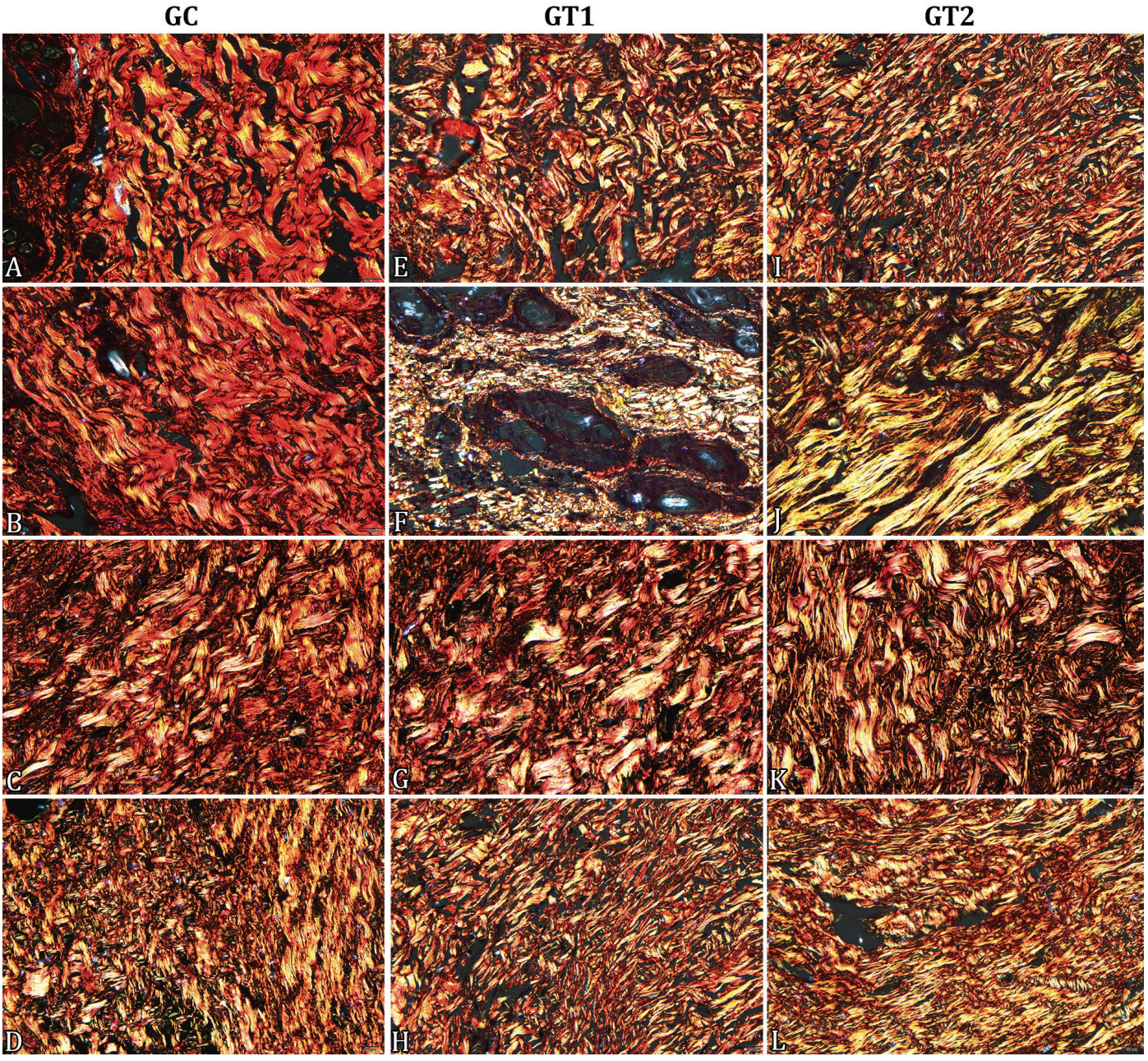

Fig.5. Photomicrography under polarized light of the evaluation of collagen fibers, analysis with Image J® software with the Threshold Color plug-in. The organization of collagen fibers in the central region of the skin graft in rabbits (*Oryctolagus cuniculus*) is shown in samples from the third day (**A**) GC_3, (**E**) GT1_3, (**I**) GT2_3, from the seventh day (**B**) GC_7, (**F**) GT1_7, (**J**) GT2_7, on the fourteenth (**C**) GC_14, (**G**) GT1_14, (**K**) GT2_14 and the 30th, (**D**) GC_30, (**H**) GT1_30, (**L**) GT2_30. Picrosirius staining, obj.20x.

rats caused a more significant inflammatory reaction than a negative control group (without injection) (Vidor et al. 2018).

Nevertheless, when allogeneic adipose-derived stem cells (ADSCs) were transplanted simultaneously, this inflammatory process was attenuated at the graft site (Vidor et al. 2018). This fact explains why the GT1 grafts managed to overlap the inflammatory process caused by the intralesional injection. Possibly there was an interaction between the inflammatory cells and the transplanted stem cells, activating a paracrine mechanism with pro-inflammatory and anti-inflammatory cytokines (Kim et al. 2013, Vidor et al. 2018), creating a favorable transition from the inflammatory to the proliferative phase (Kim et al. 2013). Regarding the presence of polymorphonuclear cells at the lesion site, Oliveira et al. (2018) report that mesenchymal stem cells promote chemotaxis of polymorphonuclear cells to the injury site, but for Ma et al. (2014) and Zachar et al. (2016), the actions of mesenchymal stem cells seem to vary according to the inflammatory process and between animal species.

Studies have established that macrophages play a central role in the different phases of physiological wound repair (Zhang et al. 2010, Heo et al. 2011). Depending on the phenotype, macrophages are classified as activated M1 or pro-inflammatory macrophages and, in alternative activated macrophages M2 or anti-inflammatory with regenerative functions that promote tissue homeostasis (Das et al. 2015, Krzyszczyk et al. 2018). Mesenchymal stem cells exert their modulating effect on M1 phenotype macrophages' polarization for the M2 phenotype (Blázquez et al. 2016). A study carried out in rats demonstrated that the macrophages phenotype M2 with the macrophages phenotype M1 were statistically significant on the second day of grafting, with low levels of pro-inflammatory cytokines IL-1β, and FNT-α and high levels of IL-10, thus regulating the local inflammation of cutaneous autografts treated with adipose-derived stem cells (ADSCs) (Wang et al. 2016). Other studies suggest that mesenchymal stem cells can induce monocyte (M0) polarization to acquire phenotype macrophage characteristics M2 (Zhang et al. 2010, Heo et al. 2011), or in the absence of IL-6, mesenchymal stem cells can induce M0 polarization for the phenotype M1 (Wang et al. 2016).

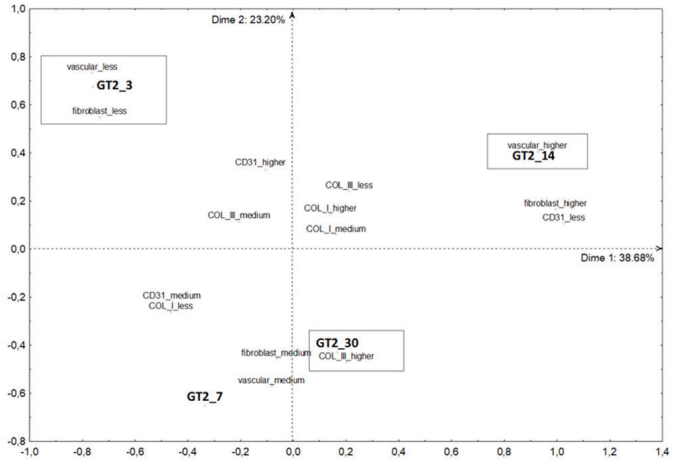

Fig.6. Multiple correspondence analysis of the grouped proliferation variables (fibroblasts, vascularization, type III collagen, type I collagen, CD31) for treatment group 2 (GT2) including the four times in the same analysis. There was a significant correspondence (p<0.05) of the variables within the rectangle.

According to MacPhail (2013), blood flow is reestablished from the recipient site to the skin graft during the inoculation phase, 24 hours after grafting. While revascularization occurs in the next 48 or 72 hours, the dark coloration of the graft's fades, and a reddish-pink tonality appears (Bohling & Swaim 2012), like the macroscopic appearance observed in grafts of the three groups. It is known that some cytokines, particularly vascular endothelial growth factor (VEGF), stimulates vascular proliferation on the fifth or seventh day of grafting (Bohling & Swaim 2012, MacPhail 2013). This fact was corroborated in the control group, where angiogenesis was significant in GC_7 and GC_14. However, it was also significant in GT2_3 and GT2_14, and GT1_30, similar to another xenogen study in rabbits (Kim et al. 2017). However, other studies on skin autografts in rats have reported that intralesional transplantation of autogenous adipose-derived stem cells (ADSCs) have a local paracrine effect on VEGF, stimulating angiogenesis and anti-apoptotic action on the seventh day of grafting (Zografou et al. 2013), thus reducing ischemic necrosis on the fourteenth postoperative day (Wang et al. 2016, Vidor et al. 2018). Regarding the method of assessing vascular proliferation, according to Pazzini et al. (2017), the CD31 antibody did not have an advantage over the H&E method.

The effect of xenogenic mesenchymal stem cells on cutaneous autografts of GT1 and GT2 was similar both in microscopic re-epithelialization (GT1_3, GT1_7, GT2_3, GT2_7) and in collagen deposition type III (GT1_30 and GT2_30), in agreement with another xenogenous study in skin wounds that showed an acceleration of healing after the seventh day of treatment (Rodriguez et al. 2015). This result demonstrated that autologous adipose-derived stem cells (ADSCs) stimulated collagen deposition in skin grafts through the expression of the VEGF factor or by the transforming growth factor β3 (TGF-β3), which also stimulates epithelialization (Zografou et al. 2013, Wang et al. 2016) possibly through a paracrine effect (Kim et al. 2007, Pelizzo et al. 2015). The TGF-β3 expression is also associated with smaller scars and better collagen organization (Hamada et al. 2019). Furthermore, ADSCs seem to significantly influence the tensile strength of wounds after the second week of treatment (Stoff et al. 2009). Usually, during the wound healing and remodeling process, type III collagen gradually decreases as type I collagen increases (Bohling & Swaim 2012, MacPhail 2013). However, in GT1 and GT2, collagen type III predominated in all periods of evaluation. For Franck et al. (2019), the persistent increase in type III collagen in skin wounds treated with ADSCs possibly happens because ADSCs interrupt the regulation of growth factors. Together with a more significant number of type III collagen molecules, this interruption generates extracellular matrix formation and early reduction of scars.

In principle, most of the xenogen type experimental studies were with bone marrow-derived mesenchymal stem cells (BMSC) in models of autoimmune diseases or ischemia and reperfusion (Li et al. 2012). According to Lin et al. (2012), since liposculpture is performed more frequently, more xenotransplantation studies with human adipose-derived stem cells (ADSCs) have been carried out with different animal species with no evidence of inflammation or rejection.

Niemeyer et al. (2008) proved in mice that the subcutaneous injection of undifferentiated human ADSCS and human BMSCs survived for eight weeks; however, when human ADSCS and human BMSC had osteogenic differentiation, they survived only four weeks in a xenogenous environment. Stoff et al. (2009) reported that on the third day and seventh day after intradermal injection of 1.5 x10^6 human mesenchymal stem cells (hMSCs), they observed a diffuse dispersion pattern of hMSCs in the immediate area to the wound; on day 14, they found hMSCs at the dermo-epidermal interface and 21 days after the injection the cells persisted, but to a lesser extent in the dermis and underlying fascia in skin wounds in rabbits.

According to Li et al. (2012), determining the ideal number of xenogenic mesenchymal stem cells required to guarantee the effectiveness of the treatment is complicated due to the many variations between the origin of the mesenchymal stem cells, doses, routes of administration, recipient species, and objectives of experimental models (Lin et al. 2012). Thus, the idea of using three applications of xenogenic mesenchymal stem cells came from the hypothesis that a dose increase, that is, the presence of more cells in the site after the initial stimulus, could increase and/or accelerate the response obtained. Moreover, two analyses were performed between the first and second doses (3 and 7 days). We can assess whether after the second application (analysis with 14 days) there was an increase or improvement in the response compared to the analysis of 3 and 7 days. The same principle was used for the third dose. However, the results observed in this healing model of cutaneous autografts in acute wounds demonstrated few full advantages between the evaluation periods after the second and third injection of xenogenous stem cells, when compared with other studies that administered a single intralesional dose of autogenous stem cells (Zografou et al. 2013, Wang et al. 2016) or allogenous (Vidor et al. 2018). Recently, a study carried out in mice with skin wounds compared three administration methods (intravenous, intramuscular, and topical) containing 1 x 10^6 allogeneic adipose-derived stem cells (ADSCs). On the first day of the evaluation, no differences were found between the three treated groups and the control group without ADSCs. However, on the third day (intravenous and topical group) and fourth day (intramuscular group), they observed that the healing process was significantly accelerated until the seventh day when compared with the control group (Kim et al. 2019). Only one bone regeneration model in rabbits has carried out a comparative and proven study that the transplantation of autologous mesenchymal stem cells (rabbits) shows better results compared with the xenogenous transplant (human). However, the xenogenous transplant was superior to the control group (Niemeyer et al. 2010), justifying its application.

The literature shows that mesenchymal stem cells are non-immunogenic, able to protect themselves from the immune system without the risk of rejection after extrinsic transplantation because they present low expression of the class I histocompatibility complex (MHC I) and, absence of molecules of the class II histocompatibility complex (MHC II) (Li et al. 2012, Kim et al. 2013, Ma et al. 2014). In general, mesenchymal stem cells (MSC) use several molecular mechanisms to suppress innate immune cells, intelligently orchestrate this entire immune system. This action by MSC is possible, as they secrete several soluble factors such as IL-4, IL-6, IL-1, TGF-β, HGF, IDO, and PGE2 (Spaggiari et al. 2009, Spaggiari & Moretta 2013), the latter being the primary central mediator of the immunomodulatory response on other cells of the immune system (Lee et al. 2016). Dendritic cells (DC) unite the innate and adaptive immune systems, as they function both as producers of cytokines and potent antigen presenters (Spaggiari & Moretta 2013). DCS absorb the antigen and, during maturation and activation, increase the expression of co-stimulatory molecules (CD40, CD80, CD83, and CD86), migrate to secondary lymphoid organs, and present the antigen to T cells (Spaggiari & Moretta 2013). During T cells' initiation, the DCs also produces a mixture of cytokines that affect the T cells' evaluating function. MSCs affect most of these processes: they inhibit endocytosis by the DCs, increase the regulation of major histocompatibility complex (MHC), CD40, CD80, CD83, and CD86 during differentiation, and prevent the further expression of CD40, CD83, and CD86 during maturation. MSCs also interfere with CD's ability to produce IL-12 and activate allogeneic T cells (Nauta et al. 2006, Spaggiari et al. 2009). Also, MSCs block the generation of dermal CD from precursors of CD14 + CD1a derived from CD34 and those derived from immature monocytes (Nauta et al. 2006).

Mesenchymal stem cells (MSCs) also antagonize the macrophage M1 phenotype expression and reduce the expression of CD86 and MHCII of these cells, thus decreasing their stimulatory power (Maggini et al. 2010). MSCs also antagonize the macrophage M1 phenotype expression and reduce the expression of CD86 and MHCII of these cells, thus decreasing their stimulatory power (Di Nicola et al. 2002, Zappia et al. 2005). This inhibition is also independent of the major histocompatibility complex (MHC) since autologous and allogeneic mesenchymal stem cells (MSC) have this same anti-proliferative effect. This T cell suppression mechanism occurs because MSC regulates the synthesis of inducible nitric oxide (iNOS), which produces nitrous oxide (Ren et al. 2008). Another important event is that MSC modulate the production of cytokines released by T cells through the production of immunomodulatory molecules, such as hepatocyte growth factor (HGF), TGF-B, and PGE2 (Di Nicola et al. 2002), being able to suppress IFNy production from TH1 cells, they promote IL-4 secretion from TH2 cells and increase the proportion of Treg (Aggarwal & Pitenger 2005). These MSC actions are well observed in responses to autoimmune diseases where pathology control only occurs when there is severe immunosuppression and immunomodulation (Ben-Ami et al. 2011).

Among the present study's limitations, we have not marked the adipose-derived stem cells (ADSCs) of dogs to track their migration and exact location in the wound and skin graft in rabbits treated by the intravenous intralesional route. Besides, to corroborate, the effects of stem cells on macrophages have not been evaluated for specific markers or evaluation of interleukins involved in the process of acute inflammation and other growth factors using flow cytometry.

CONCLUSIONS

The study demonstrates no rejection even across species, suggesting that xenogenous transplantation is safe after intralesional or intravenous injection of mesenchymal stem cells derived from adipose tissue in dogs in rabbits.

Regarding therapeutic efficacy, treatment with xenogeneic stem cells did not influence fibroblasts' angiogenesis and

proliferation compared to the control group. There was possibly a favorable effect on re-epithelialization in the first week and deposition of type III collagen, both in the group treated by the intravenous route and the group treated by the intralesional route. Further studies are necessary to assess xenogenous adipose-derived stem cells (ADSCs) immunomodulatory, anti-inflammatory, and angiogenic actions in grafting.

Acknowledgements.- To the "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES), for granting the financial support to Jorge Luis Alvarez Gómez, to carry out activities in Brazil and to the "Faculdade de Ciências Agrárias e Veterinárias", Unesp, Campus de Jaboticabal, for the structure and laboratory support.

Conflict of interest statement.- The authors declare no conflict of interest.

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