Effects of prostaglandin E_1 (PGE_1) in the genesis of blood capillaries in rat ischemic skeletal muscle: histological study

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RESUMO

Contexto: A angiogênese terapêutica é uma modalidade de tratamento para pacientes com insuficiência arterial crônica que não têm indicação para revascularização direta ou angioplastia e que não tiveram uma resposta satisfatória ao tratamento clínico. Entre as drogas utilizadas para essa finalidade está a prostaglandina E1 (PGE_1).

Objetivo: Estudar os aspectos morfológicos na gênese de capilares sanguíneos em músculo esquelético do membro caudal de ratos submetidos à isquemia sob a ação da PGE_1, administrada por via intramuscular (IM) ou endovenosa (EV).

Métodos: Foram utilizados 48 ratos, linhagem Wistar-UEM, distribuídos aleatoriamente em três grupos de 16, redistribuídos igualmente em dois subgrupos, observados no 7º e 14º dias, sendo um grupo controle onde apenas foi provocada a isquemia no membro, outro com a isquemia e a injeção da PGE_1 via IM e outro com a isquemia e a injeção da PGE_1 EV. Para análise dos resultados, foram realizadas a coloração com hematoxilina e eosina (HE) e coloração imuno-histoquímica.

Resultados: Constatou-se um aumento estatisticamente significativo no número de capilares nos subgrupos com o uso da PGE_1 IM e EV, através da contagem nos cortes corados com HE. A imunomarcação não foi eficiente para a quantificação dos capilares.

Conclusões: A PGE_1, administrada por via IM ou EV, promoveu, após 14 dias de observação, um aumento no número de capilares no músculo esquelético de ratos submetido à isquemia, identificáveis histologicamente com a coloração em HE. A imunocoloração não permitiu estabelecer uma correlação com o aumento de vasos encontrados na coloração com HE.

Palavras-chave: Prostaglandina E1, angiogênese, isquemia.

ABSTRACT

Background: Therapeutic angiogenesis is a treatment modality for patients with chronic arterial insufficiency who do not have indication for direct reconstruction or angioplasty and who were not successfully submitted to clinical treatment. Prostaglandin E1 (PGE_1) is one of the drugs used for this purpose.

Objective: To study morphologic aspects in the genesis of blood capillaries in the lower limb skeletal muscle of rats submitted to ischemia under the action of intramuscular (IM) or endovenous (EV) PGE_1.

Methods: Forty-eight Wistar-UEM rats were randomly distributed into three groups of 16, equally redistributed into two subgroups, observed at the 7th and 14th days as follows: one control group, which had only limb ischemia; one group with ischemia and IM injection of PGE_1; and one group with ischemia and EV injection of PGE_1. To analyze the results, hematoxylin-eosin (HE) and immunohistochemical staining were used.

Results: There was a statistically significant increase in the number of capillaries in the subgroups using IM or EV PGE_1, through counting in the samples containing HE staining. Immunostaining was not efficient for the quantification of capillaries.

Conclusions: IM or EV PGE_1 resulted in an increase in number of capillaries in the skeletal muscle of rats submitted to ischemia after 14 days of observation, which was histologically identifiable through HE staining. Immunostaining was not successful in establishing a correlation with the increase in vessels found in HE staining.

Keywords: Prostaglandin E1, angiogenesis, ischemia.

Introduction

Many patients with chronic peripheral arterial disease with impairment of the primitive arterial bed do not have a satisfactory response to pharmacological clinical treatment and usually do not have clinical and surgical conditions for the operative alternative of direct revascularization or angioplasty.[1,2] Treatment under these conditions is usually palliative and with limited outcomes, and may also result in limb gangrene.[3-5] In those cases, a therapeutic option would be artificial stimulation of vascular neoformation by the organism itself, i.e. angiogenesis, to compensate impaired vascular territory.[3,6-8]

Angiogenesis is a term that designates the stages through which new blood vessels are developed based on a preexisting endothelial structure, such as the case of vessel formation in adults, in response to tissue demands.[3,6,9,10] It is a complex process, with many stages and with the participation of several growth factors.[1,7,10] It distinguishes itself from vasculogenesis, which is the formation of vessels based on progenitor cells, and from arteriogenesis, which is the development of collateral circulation based on arterioles.

Therapeutic angiogenesis primarily aims at favoring induction of formation of new vessels in ischemic tissues.[8,11] Substances such as the vascular endothelial growth factor (VEGF), fibroblast growth factor and hepatocyte growth factor have been used in induction and/or maintenance of angiogenesis.[8,12-15]

Limitation to use of such drugs is related to the response to artificial angiogenic stimulus, since, unless there is a remodeling for maturation and stability of new vessels, there is a natural tendency to process regression.[7,16]

Prostaglandin E_1 (PGE_1) is one of the substances used to provide therapeutic angiogenesis. It is a substance with high biological activity, already in use for the treatment of chronic occlusive arterial disease as a vasodilator and inhibitor of platelet aggregation.[7,17-19] It participates in activation of fibrinolysis, modulation of cell proliferation, fibrinogenesis, hemorheologic activity on the erythrocyte, inhibition and activation of neutrophils and it also improves use of oxygen and glucose by tissues.[19,20-22]

In addition to those effects, there is evidence that PGE_1 stimulates angiogenesis under situations of myocardial ischemia.[19,23,24] In situations of peripheral ischemia, whether acute or chronic, there are no reports of experimental or clinical studies using PGE_1 as a stimulant of angiogenesis.

This study aimed at investigating the activity of PGE_1, using intramuscular (IM) or endovenous (EV) administration, on the genesis of blood capillaries in rat ischemic skeletal muscle.

Methods

Animals

All procedures involving use of animals were submitted and approved by the Ethics Committee in Animal Experimenting at Universidade Estadual de Maringá (UEM).

Forty-eight Wistar-UEM, male and adult rats (*Rattus norvegicus albinus*), weighing between 280-300 g were used. They were taken from the experimental laboratory at UEM.

The animals were kept in the experimental laboratory of the Department of Pharmacy and Pharmacology at UEM under a 12-hour light/dark cycle, temperature of 20 ºC and NUVITAL® and water ad libitum.

The animals were randomly divided into three groups of 16 rats and distributed again into two subgroups:

Group I - Rats submitted to ischemia (I):

-I-7 – Observed until the seventh postoperative (PO) day;

-I-14 – Observed until the 14th PO day;

Group II - Rats submitted to ischemia and IM injection of PGE_1:

-IM-7 – Observed until the seventh PO day;

-IM-14 – Observed until the 14th PO day.

Group III - Rats submitted to ischemia and EV injection of PGE_1:

-EV-7 – Observed until the seventh PO day;

-EV-14 – Observed until the 14th PO day.

Experimental procedure

The animals were anesthetized via IM using 2-(2,6-xylidine)-5,6-dihydro-4H-1,3-thiazine chloride (Rompun®) and ketamine chloride (Ketalar®) in a 1:1 ration, using 1 $mL.kg^{-1}$ of body weight for that association, applied in the left lower limb.

Ischemia in the rat lower limb was performed based on a literature model.[20,25-30] A longitudinal incision was performed using a 15 scalpel blade through the skin and subcutaneous screen, extending distally to the inguinal ligament until a site close to the patella. With the aid of a surgical magnifying glass with a 3x magnification, after identification of the vascular-nervous bundle, the femoral artery was dissected through all its extension, from the inguinal region to the popliteal artery. The external iliac artery was dissected and ligated with a 4-0 cotton wire. The femoral artery was completely removed, from its proximal origin in the external iliac artery until its distal portion, where there is a bifurcation into saphenous and popliteal arteries. The anatomic layers were approximated and the skin was sutured using a 5-0 polyamide wire. Blood flow for the ischemic limb became dependent on collateral vessels from the internal iliac artery.

Immediately after the ischemia procedure and before skin suture, PGE_1, at a dose of 5 $\mu g.kg^{-1}$, was carefully injected in the animals using 1-ml syringes and 30G needles;[12] in Group II, via IM, at equidistant sites, directly on the gracile and vastus medialis muscle of the lower limb; in Group III, via EV, through the penis dorsal vein.

During the observation period of 7 and 14 days, the animals were examined daily as to the following aspects: a) in the surgical wound - presence of hematoma, dehiscence, infection or sheath ischemia; b) in the lower limb - presence of skin necrosis, finger(s) necrosis or gangrene; c) in the limb functional aspect - presence of claudication (crawling) or paralysis.

On 7 and 14 days, under anesthesia, a sample was collected containing muscle tissues and vascular-nervous bundle in the area where the femoral artery was removed. After tissue collection, the animals were submitted to inhalation of a lethal dose of ethyl ether.

The samples were fixed in 10% buffered paraformaldehyde, processed for inclusion in paraffin. Semi-serial 5-µm sections were performed, which were stained with hematoxylin and eosin (HE) for morphological tissue evaluation and to determine the number of blood capillaries or processed for immunohistochemical technique of avidin-biotin-peroxidase using anti-VEGF antibody, and anti-CD34 to evaluate neovascularization.

Counting of capillaries in HE-stained sections

Counting of capillaries was performed by two observers by the double-blind method. A 10x10-point reticulum was used, attached to the ocular in an optical microscope (Olympus®BX41) with a 40x objective. Vessels were quantified in many histological sections, in a total of 60 microscopic fields of 0.24 mm^2/animal, and results were expressed as a mean of both counts.

Immunohistochemistry

The blades selected for immunohistochemical procedure were deparaffinized and the sections were rehydrated. Identification of endothelial cells was performed by immunohistochemical staining using the avidin-biotin-peroxidase method to detect VEGF and CD34 expression per endothelial cells, using policlonal antibodies, anti-VEGF (Santa Cruz Biotechnology), extracted from rabbit in a concentration of 12:200, and glycoprotein CD34 using a policlonal antibody anti-CD34 (Santa Cruz Biotechnology), extracted from rabbit in a concentration of 1:50. The blades were analyzed in an optical microscope (Olympus® BX41), and reaction positivity was determined.

Statistical analysis

The statistical study was performed by PGS Medical Statistics using the *software* SPSS10, applying nonparametric testes due to the nature of the data. Mann-Whitney test was used to compare subgroups of euthanasia time within each group and Kruskal-Wallis to compare study groups within each euthanasia time.

Significance level was set in 0.05 ($\alpha \leq 5\%$), and descriptive levels (p) lower than that value were considered significant and represented by an asterisk.

Results

Macroscopic observation

There was no limb necrosis in macroscopic observation of lower limbs submitted to surgery. There was no claudication (crawling) or death among the animals under investigation.

Microscopic analysis

Optical microscopy showed most muscle cells with normal morphology, with peripheral core and absence of degenerative changes typical of necrosis, such as edema or inflammatory infiltrate. Only in a few animals there was occurrence of muscle cells with centrally located cores, characteristic of ischemic cells (Figure 1).

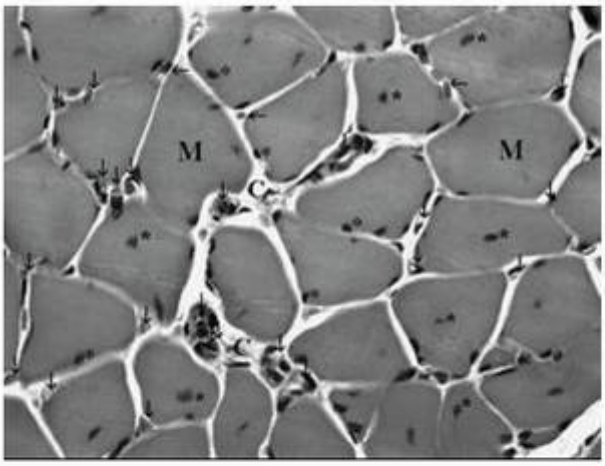

Figure 1 - Photomicrography in light microscopy of rat skeletal muscle showing: presence of central cores (*) and large amount of major capillaries and vessels (arrows) in a rat belonging to the subgroup using endovenous PGE_1 observed at 14 days (HE, 960x). M) Muscle cells; C) collagen fibers

There was increase ($p < 0.05$) in number of capillaries in subgroups IM-14 (43.7±6.1) and EV-14 (43.7±5.6), when compared with subgroups IM-7 (24.9±3.9) and EV-7 (21.0±3.3) respectively (Table 1).

Table 1 - Number of capillaries quantified in HE-stained histological sections in different groups assessed 7 and 14 days after the procedure of ischemia and injection of prostaglandin E_1

	Counting of capillaries in HE					
Group	Mean	MSE	Median	Minimum	Maximum	n
I-7	24.1	1.4	24.5	17	28	8
I-14	26.0	2.2	27.0	18	35	8
IM-7	24.9	3.9	20.5	15	45	8
IM-14	43.7*	6.1	45.0	18	65	8
EV-7	21.0	3.3	16.5	13	38	8
EV-14	43.7*	5.6	37.5	26	68	8

* $p < 0.05$ when subgroups were compared between themselves.
MSE = mean standard error; EV = endovenous injection; HE = hematoxylin and eosin; I = ischemic; IM = intramuscular injection; n = number of animals.

Immunohistochemistry

Staining by the immunohistochemical method did not show uniformity between animals in the same group, making quantification impossible. In subgroups IM14 and EV14 capillaries were marked by CD34 (Figure 2A) and with VEGF in large-diameter vessels (Figure 2B).

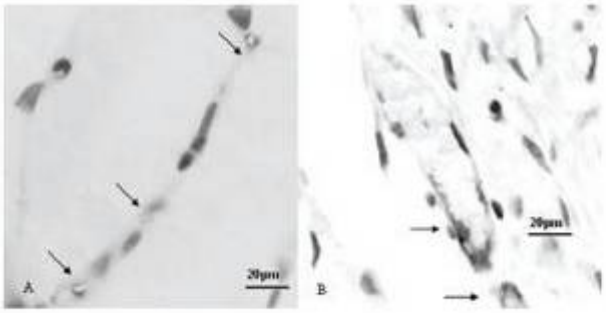

Figure 2 - Photomicrographies in light microscopy of the skeletal muscle of an animal belonging to the subgroup using intramuscular PGE_1 observed at 14 days, showing marked capillaries through immunohistochemistry (arrows), using the antibody CD34

Discussion

Therapeutic angiogenesis is a new approach for the treatment of chronic ischemic vascular diseases, which can be used for a growing number of patients who have no indication for revascularization.

Ischemia alone is an event that stimulates vascular neoformation in skeletal[7,15,31-33] and cardiac[19,23,24] muscle. This process is regulated by growth factors that are located in endothelial cells and in the extracellular matrix.[8,34,35] Among the several factors involved VEGF stands out, starting the angiogenesis process, as well as nitric oxide (NO), which regulates and modulates VEGF.[20,33]

The ideal agent for application in therapeutic angiogenesis should have the following characteristics: stimulate and enhance the potential of angiogenesis; maintain a lasting activity and have ischemic tissues as specific target; do not cause collateral effects; do not cause pathological angiogenesis; achieve high local concentration; have proper exposure time; be easily reapplied; be orally or

parenterally administered; have low cost.[14,36,37]

PGE_1 is a vasodilating drug used in the treatment of ischemic arterial diseases of the lower limbs,[37,38] and experimentally tested to induce angiogenesis in ischemic cardiac diseases.[19,23,24] It also stimulates angiogenesis in rabbit cornea[39] and in the chorioallantoic membrane of chicken embryos.[40] Its activity on angiogenesis occurs indirectly by induction of VEGF expression.[19,23]

Based on that theoretical foundation, there was the idea of using the drug by injecting it directly on the ischemic area or via EV, within the protein therapy modality, which is more conventional and offers less risk than gene therapy.[41] It also seemed like a more feasible strategy for eventual use in daily medical practice.[42,43]

The model used proved to be reliable and reproducible. Previous studies confirmed immediate reduction in arterial flow using arteriography.[20,26,32]

Systemic routes, IM, EV or intraarterial, although being restricted in offering the drug at the ischemic site – because this area has no proper blood circulation – are more commonly used, both experimentally and in clinical trials. Both have advantages and adverse effects.[29,41]

In the proposed model, IM administration at a single dose was directly performed in the ischemic muscle. That access theoretically provided higher concentration of the drug in the ischemic area, reaching sites where the systemic access cannot reach.[13,14,41,44] As to intraarterial access, it has the advantage of being used when the arteries have a severe impairment that prevents catheter placement close to the atherosclerotic lesion.[8]

Use of EV access did not cause systemic changes that could put the animal life at risk, which could be confirmed by observing the vital data that had discrete variation during the experiment. EV application has the advantage of being less invasive, and can be repeated if necessary. However, one advantage of this access is that a large part of PGE_1 (70-95%) is disabled in its first passage through the lungs, with consequent reduction in its bioavailability in the ischemic area.[21]

The expected effect by using PGE_1 in the ischemic area is exactly on the quantity or quality of neoformed vessels. Therefore, histological and immunohistochemical parameters were established as an attempt to find the relation between the drug and its administration route in angiogenesis.

After ischemia, the process of angiogenesis starts in a period of 1-3 days, reaching proliferation peak around 7 days; from then on, there is a fast and progressive regression in number of neoformed vessels until 28 days.[26,32,35,44,45]

In morphological evaluation of HE-stained blades, there were muscle cells with detached cores from the periphery to the central region, a typical characteristic of the muscle cell in process of ischemia. Such change was observed in all observation times, showing that this regularity of samples is related to adequacy of the proposed model in producing muscle ischemia.[46]

At 7 days of observation there were no significant differences in number of capillaries. However, at 14 days there was an increase of approximately 40% of capillaries in the ischemic area, regardless of type of administration, in relation to animals that were not given PGE_1, and in relation to animals observed at 7 days (IM-7 and EV-7). It can be inferred that the stimulating effect of PGE_1 on angiogenesis was only detected in this experiment 14 days after its administration.

The method we used was not able to distinguish occasional advantages in this increase in number of capillaries that could be attributed to type of administration, although there are reports in the

biomedical literature of a more favorable result when using local PGE_1[26,47].

Angiogenesis is modulated by growth factors that are located in endothelial cells and in the extracellular matrix.[34,35] VEGF is associated with start of vascular neoformation process and is considered a reliable monitor of the process. Is ischemic cardiac tissues, exogenous administration of PGE_1 can stimulate angiogenesis measured by VEGF expression.[19,23] PGE_1 does not directly stimulate endothelial growth. Its angiogenic effect seems to be mediated by the paracrine activity of angiogenic factors released by other cells,[48] such as by stimulating macrophages to secrete growth factors, such as the growth factor originated from platelets (PDGF) and adenosine, among others.[27,49]

Immunochemistry showed that there was immunostaining of a few vessels, most of them being arterioles and venules located in the connective tissue surrounding muscle fiber bundles. According to the results of optical microcopy, the subgroups IM-14 and EV-14 showed a higher number of capillaries when compared to subgroups IM-7 and EV-7; therefore, a staining with a higher number of blood capillaries could be expected in those animals. However, there was no such correspondence and most vessels stained with VEGF had large diameter, with few stained capillaries. Reactions with CD34 stained a higher number of blood capillaries, but there was no reproducibility in all histological sections, making counting impossible. Lack of homogeneity in results and incoherence with other microscopic findings, as well as variations presented in groups used as witness put occasional conclusions under suspicion, although immunostaining of larger vessels may suggest a mechanism of new vessel formation by arteriogenesis.

Therapeutic angiogenesis in peripheral arterial diseases has been studied for approximately 1 decade and it offers great perspectives for a near future. Experimental studies have demonstrated improvement in blood flow; however, the first clinical trials in human beings, despite some effectiveness, are still far from a definitive conclusion.[41,11,50,51] Limiting factors are growth factor dose, its activity duration, type of administration, varied sites of agent activity, selection of patients, patient heterogeneity, endogenous inhibitors of angiogenesis, and a powerful placebo effect.[14,36,37] Continuous use of certain drugs (aspirin, isosorbide, nitrates, spirolactone, furosemide, captopril, bumetamide, lovastatin, cycloxygenase inhibitors and clarithromycin), as well as old age, dyslipidemia, smoking and diabetes, may also affect the result of clinical trials, since they would act as angiogenesis inhibitors.[13]

There is still no consensus on the ideal angiogenic factor or whether it would be needed to use a combination of growth factors to foster and maintain angiogenesis.[52] Knowledge of cellular and molecular bases of blood vessel maturation can be the most important point in the life cycle of these vessels, and may be the key to the development of therapies to stimulate or inhibit angiogenesis.[16,53] Fast advances in these areas and in systems of drug releases can make therapeutic angiogenesis the standard choice of treatment in ischemic diseases.[13,54]

Conventional and classical histological evaluation using HE staining showed that in animals given PGE_1, there was higher presence of vessels in the systemic skeletal muscle tissue, associated for longer observation times, more precisely at 14 days, even if the technique did not allow knowing whether these vessels were formed by angiogenesis or vasculogenesis. However, it did not allow detecting advantages about IM or EV administration.

In addition, material staining by immunohistochemical technique provided staining of large-caliber capillaries and vessels; however, in assessed times it did not allow determining whether that number of vessels, found in HE staining at 14 days, occurred due to angiogenesis or vasculogenesis, probably because during that observation time blood capillaries were not expressing the markers used.

Another possible failure in staining by that technique concerns reaction complexity, since the primary antibody should bind to the epitopes that were closed during the stage of material fixation and processing and need to open during the stage of antigenic recovery, which can be performed in many

forms. Several tests were performed using technical variations, such as many types of antigenic recovery, varied incubation times, changes in dosages of primary and secondary antibody samples, variations in batches with exchange of the primary anti-VEGF antibody by a different batch and then by another primary antibody, the anti-CD34, in which staining was not sufficient to quantify angiogenesis.

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