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EVALUATION OF PRILOCAINE-INDUCED TOXICITY IN MICE TISSUES AND CHICK EMBRYO CHORIOALLANTOIC MEMBRANES (CAM)

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Abstract

Prilocaine is a local anesthetic and its compounds might be toxic and can cause cardiovascular damage, central nervous system disorders, and hepatic/renal toxicity. High prilocaine doses lead to methemoglobin formation, a potent pro-inflammatory endothelial cell response activator, and form the carcinogenic metabolite arylamine o-toluidine. We studied prilocaine's effects on murine detoxifying organs and chick embryo chorioallantoic membranes (CAMs). Control Swiss mice were given saline, and experimental mice were given prilocaine over either 20 or 30 days. Experimental liver sections revealed inflammatory infiltration indicating lymphocytic periportal hepatitis and lobular necrosis. Kidney sections revealed multifocal interstitial nephritis, characterized by interstitial lymphocytic inflammation. CAMs of fertile eggs were used for experiments with blood vessels. After a six-day incubation (38°C, 55% relative humidity) prilocaine implants were placed on CAMs. Seven days later, the eggs were reopened and observed stereoscopically. The membranes were analyzed, and the blood vessel number was quantified. The blood vessels/mm² decreased, and the CAM sections showed collagen fibers, indicating possible inflammatory disorders. Furthermore, the embryos were removed, and morphological changes in various embryonic body structures were observed. These data indicate that prilocaine may trigger murine detoxifying organ injuries. Additionally, the drug exhibited anti-angiogenic and inflammatory action on CAMs and caused embryo body changes.

Keywords: Prilocaine, detoxifying organs, chick embryo chorioallantoic membrane (CAM), prilocaine side effects.

Introduction

Intra-operative pain control with local anesthesia is an intrinsic part of clinical practice in dentistry. In the last few years ago, more than 300 million local anesthetic cartridges were administered each year by dentists in the United States [1]. Although administered locally, these anesthetics are transferred through blood to other areas, such as the kidneys and liver, and may exert systemic effects that are concentration-dependent. A typical local anesthetic is composed of a lipophilic and a hydrophilic group linked by an intermediate chain, the molecular nature of which is used to classify the anesthetics into two groups of compounds: amino-esters and amino-amides [2]. Despite low toxicity when applied in small doses or in isolated treatments, the continuous administration of these anesthetics might induce cirrhosis and renal insufficiency [3-9]. It has been observed that the side effects of dental treatment may be aggravated by local anesthetics in some patients, such as those compromised by hepatic disorders. Frequently, liver disease results in a depressed level of plasma coagulation factors, a condition that requires careful evaluation of hemostasis prior to treatment. The oral cavity may also show evidence of liver dysfunction with the presence of hemorrhagic changes, petechiae, hematoma, jaundiced mucosal tissues, gingival bleeding, and/or icteric mucosal changes [8, 9]. Because the compounds used as local anesthetics can be toxic, causing hepatic/renal problems and cardiovascular and central-nervous disorders, the therapeutic serum concentrations should not exceed certain limits. In the blood, local anesthetics usually cause sedation, nausea, vertigo, and anxiety [1, 10-18]. Prilocaine ($C_{13}H_{20}N_2O$, MW 220.3), N-(2-methyl-phenyl)-2-(propylamino)-propanamide), is one of the local anesthetics most commonly used in dental procedures. It has a comparatively weak vasodilatation power, which leads to a slower absorption and more rapid hepatic and lung biotransformation, followed by kidney elimination [3, 15-22]. However, researchers have studied the metabolites of anesthetics, and it has been reported that prilocaine is metabolized to o-toluidine, which is classified as a human carcinogen [12, 21-24]. Hagiwara et al. [12] evaluated the ability of 14 chemical agents used in dental practice to induce chromosome aberrations using Syrian hamster embryo (SHE) cells. The percentages of cells with polyploidy or endoreduplication were enhanced by prilocaine hydrochloride, formocresol, sodium arsenite, p-chlorophenol, p-phenolsulfonic acid, sodium hypochlorite, and erythrosine B in both the absence and presence of exogenous metabolic activation. The results indicated that these substances had a positive response and were potentially genotoxic to mammalian cells. Note that in some instances, such as oral cancer treatment, previous specialized dental care using long-term anesthesia might be required [23, 24, 25]. Furthermore, local anesthetics can cross the placental barrier by passive diffusion, and it is known that high doses of prilocaine hinder placental circulation and may cause methemoglobinemia [2, 3, 14, 26]. In order to analyze a blood vessels system, the CAM (Chorioallantoic Membrane of Chick Embryo) is a well adapted model [27, 28, 32, 33]. The chorioallantoic membrane is formed by fusion of chorionic and allantoic membranes between the fourth and fifth days of chick embryo development [29-33]. This respiratory organ has vascularization and is composed of three layers: the ectodermal, mesodermal, and endodermal layers. The ectodermal layer comprises a microvasculature for gas exchange and a chorionic epithelial layer. The mesodermal stroma is composed of a complex vasculature sustained by fibroblasts and thin collagen fibers [31-34]. The CAM has multiple functions, including mediating gas exchange and nutrient uptake. This membrane surrounds the embryo and differentiates itself entirely on the twelfth day of development. However, its vascularization is only finalized on the eighteenth day, three days before the eggs hatch [29, 32, 33]. Xenobiotics or materials applied on the CAM can induce injuries and associated tissue responses, such as inflammation and fibrosis [27, 30, 31, 33, 37]. In the present study, we report an investigation of the effects of prilocaine in various tissues at different doses.

Materials and Methods

Materials

Prilocaine hydrochloride (AstraZeneca Laboratory™, Barueri, São Paulo, Brazil) was used in the study. In this study an Olympus SZ40 stereoscopic microscope, a Sony 7.2 DSC-W7 digital camera, a laminar flow hood, an automatic incubator (Premium Ecológica Ltda IP70™) and Image Pro-Plus software (version 4.5, Media Cybernetics program™) were used as well. All buffers and solutions were prepared by analytical grade.

Animals

All animal procedures were approved by the Pontifical Catholic University of Parana Ethical Committee,(N^0:003-04/2004;N^0:850/2013).

Murine

Thirty-six Swiss male mice (*Mus musculus brevirostris*) aged from 13 to 15 weeks and weighing 70 ± 20 g were provided by the Central Vivarium of the Pontifical Catholic University of Parana (under the supervision of the Animal Committee). The mice were kept under controlled temperature (25° ± 2°C) and relative humidity (50% ± 15%) conditions, normal photoperiod (12 hr dark: 12 hr light), with drinkable water and food (Purina™) available ad libitum. The animals were housed throughout the experiment in polypropylene cages containing sterile paddy husk (locally supplied) as bedding, with four animals per cage.

CAMs

Fertilized chicken eggs (*Gallus gallus* L. 1758) were kindly donated by the DaGranja Ltda. (Mariental-PR, Brazil) company and kept in an automatic incubator at 38°C and 55% relative humidity for up to 24 hr after laying.

Experimental Design

Murine

The animals were randomly divided into three groups (n = 12): a control group (A) that received a daily supply of 0.9% NaCl solution intraperitoneal (ip), and two experimental groups that received 1.2 μg/g of prilocaine hydrochloride ip for 20 days (group B) or 30 days (group C). The LD50 of prilocaine has been reported as 210 μg/g [36]. After 20 or 30 days, the animals were anesthetized with ketamine:xylazine (10 mg:0.1 mg) intramuscular (IM) and sacrificed using ketamine:xylazine (20 mg:0.2 mg). Their organs were extracted and fixed in buffered 10% v/v formalin for histological processing.

CAMs

After six days of incubation in an automatic incubator at 38°C with 55% relative humidity, all materials were prepared, including prilocaine, and sterilized in a laminar flow hood under ultraviolet (UV) light for 30 minutes. The eggs were removed from the incubator, taken to flow and cleaned with 70% alcohol. A square piece of plastic tape (2 cm^2) was affixed to the top of each egg (part of the air bag) with a window. Using tweezers, the eggshell membrane was removed to expose the CAM. The control (gel 0.5 ml) and prilocaine (0.2, 0.5, 1, 2, 5, 10, and 15 μg/g) implants were dispensed over 0.5 ml of gel on the CAM with syringes. The window was then closed with plastic tape, and the eggs were incubated again at 38°C. Seven days after the implantation, the eggs were reopened, viewed, and photographed macroscopically with an Olympus SZ40 stereomicroscope coupled to a Sony 7.2 DSC-W7 digital camera to evaluate the number of blood vessels/mm^2. The membranes were then removed and fixed (buffered 10% v/v formalin) for histological processing [30-35]. Finally, the embryos were euthanized with ketamine:xylazine (20 mg:0.2 mg) IM and kept for morphological studies.

Histopathology

Murine

Histological processing was performed on sections of the liver, kidney, cardiac muscle, lung, spleen, small intestine, large intestine, brain, pancreas, stomach, bladder, testicles, and seminal vesicles. The samples were embedded in paraffin, sectioned, and stained with hematoxylin-eosin (H&E) for histopathological evaluation. The stained slides were observed and photographed under light microscopy (Olympus BX50) and analyzed with Image-Pro Plus Software™.

CAMs

The same histological processing method described above was performed on sections of CAM. The sections were stained with H&E to observe the microvessels (arterioles and venules) located around the larger vessels. Masson's trichrome staining technique was used to characterize the collagen fibers and fibrin deposition. Stained slides were analyzed using light microscopy (Olympus BX50) and the Image-Pro Plus Software™.

Histopathological analysis

Murine

The histopathological findings were classified according to the type and anatomic site of the damage (inflammatory, ischemic, or degenerative). To determine the approximate area and perimeter of the injuries, and the number of inflammatory cells, the sections were analyzed using Image-Pro Plus Software™ [37, 38].

CAMs

Gross evaluation and analysis of angiogenesis of CAMs under the effects of prilocaine was performed with the Image-Pro Plus Software™. We calculated the vessel density index (VDI) by counting the number of intersections made by the blood vessels (arterioles and venules) within a square grid (1 mm^2) containing nine smaller squares superimposed on the micrographs [27, 28, 31, 33, 34]. Four of the smaller squares were randomly selected, and the number of

vessels found intercepting the line of the square was counted (at 5X magnification), allowing for an estimation of the average number of vessels in each square grid. At least eight replicates were performed for each implant (n = 8).

Statistical analysis

Murine experimental data were described as the mean ± standard deviation (SD) of 12 independent samples and were analyzed using a one-way analysis of variance (ANOVA) and Tukey's test ($p < 0.05$). The CAM experimental data were described as the mean ± SD of eight independent samples and then analyzed using a one-way ANOVA and Tukey's test ($p < 0.05$).

Results

We observed foci of lobular inflammation infiltration into the portal space in the liver (Figure 1), thereby characterizing lymphocytic periportal hepatitis. The image revealed 274 ± 73 inflammatory cells in an area of 12,799 ± 3,430 μm² and a perimeter of 673 ± 180 μm. Also, an area of hepatic stroke with lymphocytic satellitosis and infiltrated neutrophils (depicted area in Figure 1) was observed. The data from the image of kidney sections showed lymphocytic interstitial nephritis characterized by the presence of lymphocytic inflammation infiltrated into the renal interstice (Figure 2). The analysis revealed 758 ± 229 inflammatory cells in the injured regions, with an area of 96,669 ± 29,210 μm² and a perimeter of 2,000 ± 604 μm. Figure 3 shows the CAM control and prilocaine tests with depicted vasculature maps. Compared to the control samples, the quantification of the response to prilocaine implants demonstrated that there was a significant decrease in the number of vessels/mm² at concentrations of 0.2, 0.5, 1, 2, and 5 μg/g. Corroborating with this data, Figure 4 shows that there is a statistical correlation between prilocaine and the inhibition of vessel growth according to the trend formed from the means number of vessels/mm². The membranes treated with prilocaine (high dose) showed only isolated blood vessels when observed microscopically (Figure 5a), indicating inhibited vessel growth, as observed in the macroscopic analysis. An increase of collagen fibers was observed (stained blue, Figure 5b), suggesting that despite a decrease in the number of vessels, there was potential inflammation [33, 37].

Discussion

It has been shown that local anesthetics, such as prilocaine, can provoke serious injury at the hepatic and/or renal level, possibly leading to cellular death [16, 19, 40, 41]. Its continuous administration may trigger hepatic disturbances, which can lead to a substantial increase in its serum availability [16, 18, 19, 40, 47]. Schmittner et al. [42] have shown that high prilocaine doses form methemoglobin (MHb), a potent activator of the pro-inflammatory endothelial cell response. Pelkonen et al. [43] and Berkman et al. [44] have reported that prilocaine metabolism in the liver inhibits the cytochrome P450 system and may lead to increased levels of intermediates, such as arylamine o-toluidine, and possibly trigger cellular carcinogenic mechanisms [12, 21, 23-25]. In this study, the effects of prilocaine in various tissues of male Swiss mice were analyzed over 20- or 30-day daily treatment periods. Significant histopathological alterations were found only in the liver and kidney tissue. Other organs, such as the cardiac muscle, lung, spleen, small intestine, large intestine, brain, pancreas, stomach, bladder, testicles, and seminal vesicles did not show any significant alterations (data not shown). An examination of the injuries with image analysis (Figure 1) revealed 274 ± 73 inflammatory cells in an area of 12,799 ± 3,430 μm² and a perimeter of 673 ± 180 μm. The foci of infiltrated periportal inflammation are most likely the lightest and most precocious injuries revealed in our investigation because the lymphocytes found might have migrated from the blood vessels of the portal system. As the injury intensified, hepatocyte death occurred in the lobular spaces. During the evolution of this necrosis, an area of hepatic stroke with lymphocytic satellitosis and infiltrated neutrophils was observed. This characterization is associated with a rapid disappearance of destroyed hepatic cells and focal necrosis, and, in some cases, the only identification is the presence of mononuclear aggregates and limited polymorphonuclear aggregates surrounded by cellular debris. It has been postulated that focal necrosis occurs as a result of destruction of isolated or small groups of hepatocytes, affecting variable portions of the hepatic parenchyma [40, 41]. Supporting our findings, Houslay et al. [45] have shown that prilocaine can increase the fluidity of rat liver plasma membranes by selectively disturbing the inner half of the bilayer because of to its preferential interaction with phospholipids. Another study showed significant changes in liver function after an acute dose of local anesthetic was administered. An immediate increase of oxygen consumption, mitochondrial uncoupling and bile flow was observed with the inhibition of potassium channels [45-47]. Few reports have

described the effects of prilocaine on the kidneys(the excretion site). Our analysis revealed 758 ± 229 inflammatory cells in the injured regions, with an area of 96,669 ± 29,210 µm² and a perimeter of 2,000 ± 604 µm. Researchers consider toxic metabolites resulting from biotransformation to be potential causes of renal injury [12, 39, 40, 47, 48]. These metabolites can provoke an interstitial immunological reaction, which is exemplified by acute nephritis arising from induced hypersensitivity [40, 42, 48]. More marked effects of prilocaine on the kidneys (Table 1) likely occurred because the liver was injured and debilitated by the xenobiotic action and may have been compromised by biotransformation processes [40, 47, 48]. Prilocaine could have accumulated in the kidneys because of the slow degradation capacity of the organ [48]. This accumulation may result in the drug being received in a more potentially toxic state, leading to a more vigorous injury process in the kidneys than in the liver. In our experiments, we observed that the period of 20 or 30 days of drug administration was not significantly different, considering that in both cases, the injury effects were similar. However, it has been shown that acute doses of prilocaine over a period of 14 days did not promote significant changes in the plasmatic marker (bilirubin, pyruvic transaminase, and creatinine) levels related to hepatic and renal injuries [36].

We also observed gross morphological changes induced on the CAMs by prilocaine under stereoscopic examination (Figure 3). Tissue repairing mechanisms (wound healing) involve neovascularization or angiogenesis, and in the presence of inflammation, lead to extensive tissue destruction, usually resulting in fibrosis [30-33]. Fibrosis was also observed in different concentrations, which indicates a likely inflammatory response (Figure 5) [33-35, 37]. Besides, in our experiments, the embryo analysis revealed the different morphological abnormalities caused by prilocaine (data not shown). We observed malformation [27, 29] of the ventral wall, abnormal growth of the brain, malformation of the beak, cysts in the eyes, malformation of the wings, and developmental delay. In addition to these changes, at concentrations of 10 and 15 µg/g, the drug caused embryo deaths.

Conclusions

Prilocaine is a local anesthetic widely used in dentistry and is characterized by relatively low vasodilation potential, which makes it particularly strong, with a low degree of toxicity. However, hepatic and renal tissue alterations that occur with its use have been described in the literature. Oral mucosa tissue changes, hemorrhagic changes, petechiae, hematoma, jaundiced mucosal tissues, and gingival bleeding have all been reported to be derived from liver aggression. Our results in the liver showed lobular inflammatory infiltration characterized by lymphocytic periportal hepatitis and lobular necrosis. In the kidneys, we observed lymphocytic interstitial nephritis characterized by the presence of lymphocytic inflammatory infiltration into the renal interstice. On the CAM, there was a significant decrease in the number of vessels/mm² with fibrosis, which indicates a likely inflammatory response. The drug also affected the morphological development of the embryos and was lethal at high doses. The results of the injury process reported in this study (potentially caused by the anesthetic agent) provide additional perspectives to better understand the lesion-causing mechanisms triggered by prilocaine.

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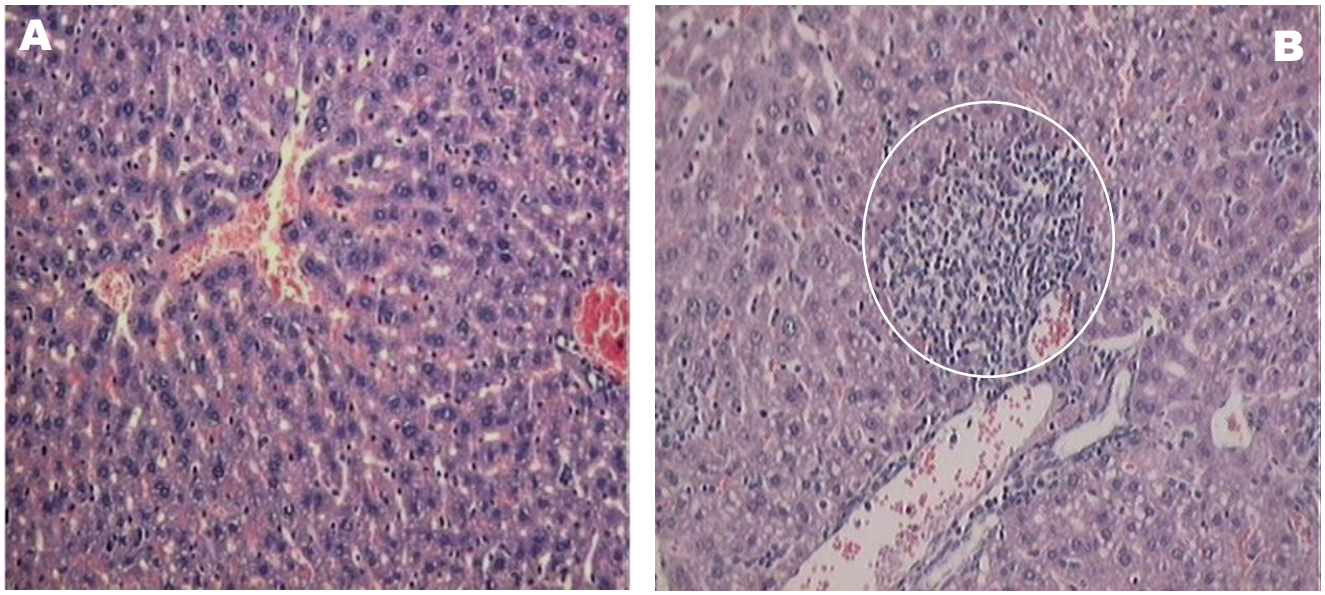

Figure 1. A – Hepatic tissue, Control. **B** - Hepatic tissue representing periportal lymphocytic hepatitis (marked area), H&E staining, 10x.

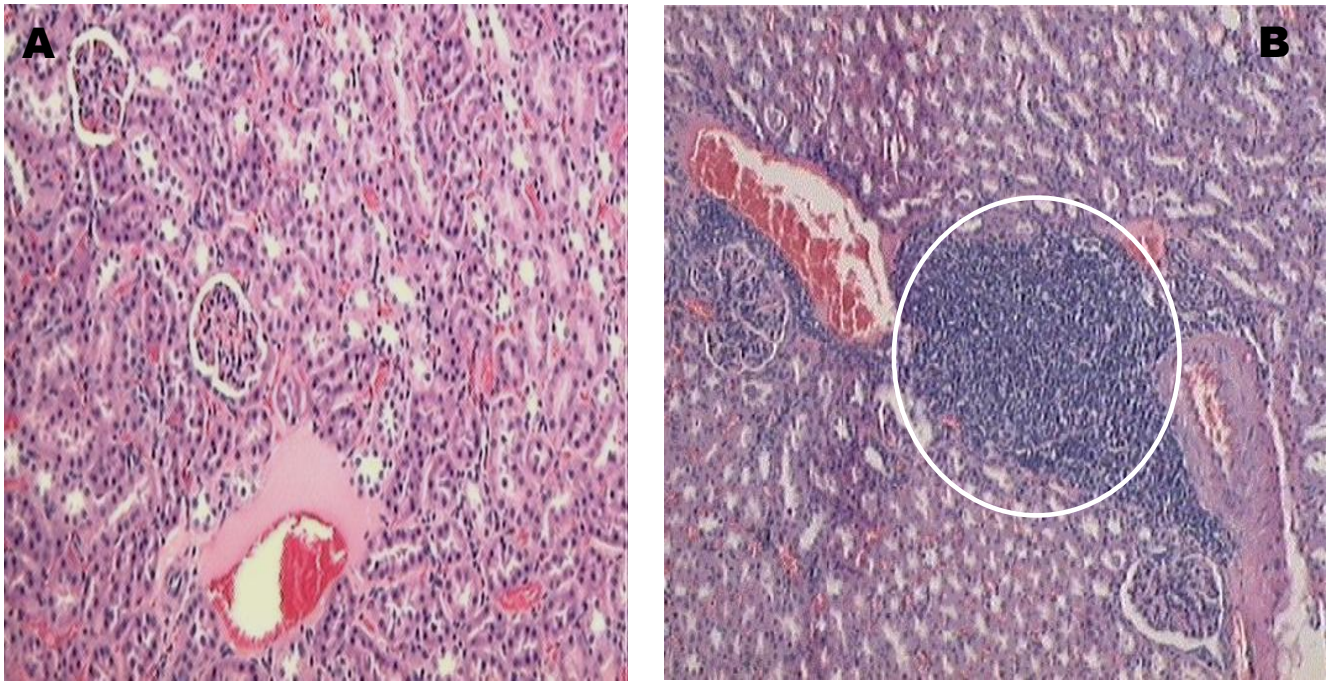

Figure 2. A – Renal tissue, Control. **B** - Renal tissue representing lymphocytic interstitial nephritis (marked area), H&E staining, 10x.

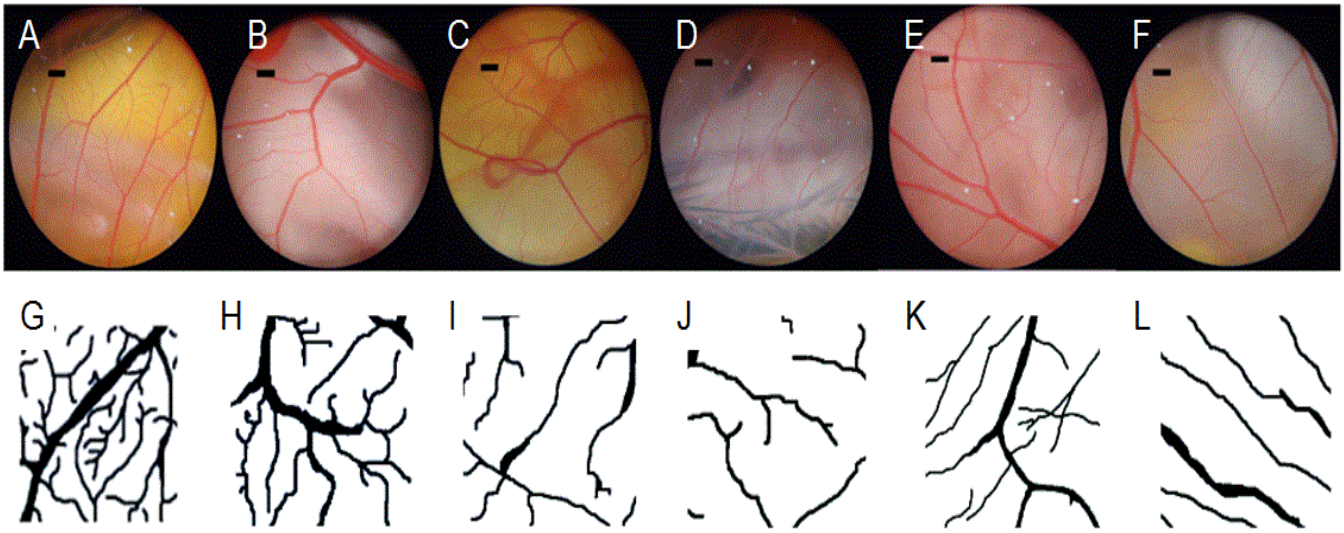

Figure 3. Photomicrography representing the gross aspect of CAMs with its vessel maps. The effects of prilocaine implants under stereoscope (5X) were depicted with vascularity maps (n = 8). A) Control with 0.5 mL gel (0.5%) (7.2 ± 0.8 vessels/mm²); B) Prilocaine 0.2 µg/g (6 ± 0.4 vessels/mm²); C) Prilocaine 0.5 µg/g (5.3 ± 0.6 vessels/mm²); D) Prilocaine 1 µg/g (6.6 ± 1.8 vessels/mm²); E) Prilocaine 2 µg/g (5.9 ± 0.6 vessels/mm²); F) Prilocaine 5 µg/g (3.6 ± 0.5 vessels/mm²); G) Control map; H) Prilocaine 0.2 µg/g map; I) Prilocaine 0.5 µg/g map; J) Prilocaine 1 µg/g map; K) Prilocaine 2 µg/g map; and L) Prilocaine 5 µg/g map. Photomicrographs have a 1-mm bar for determining the studied area.

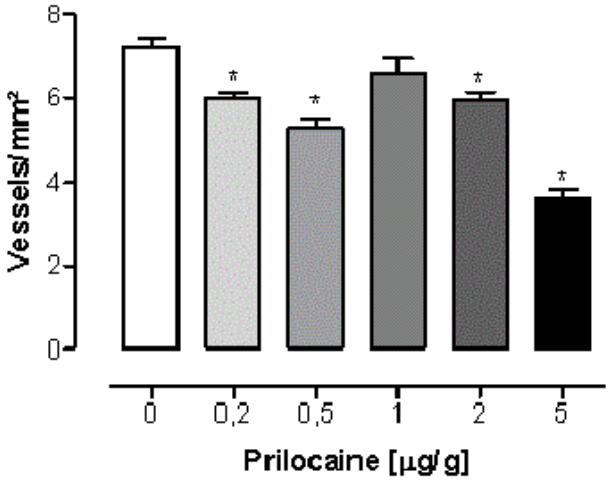

Figure 4. Graphic representation of the effects of prilocaine on the CAMs. Control gel 0.5 mL, (7.2 ± 0.8 vessels/mm²), 0.2 µg/g (6 ± 0.4 vessels/mm²), 0.5 µg/g (5.3 ± 0.6 vessels/mm²), 1 µg/g (6.6 ± 1.8 vessels/mm²), 2 µg/g (5.9 ± 0.6 vessels/mm²), and 5 µg/g (3.6 ± 0.5 vessels/mm²); n = 8 and $p < 0.05$.

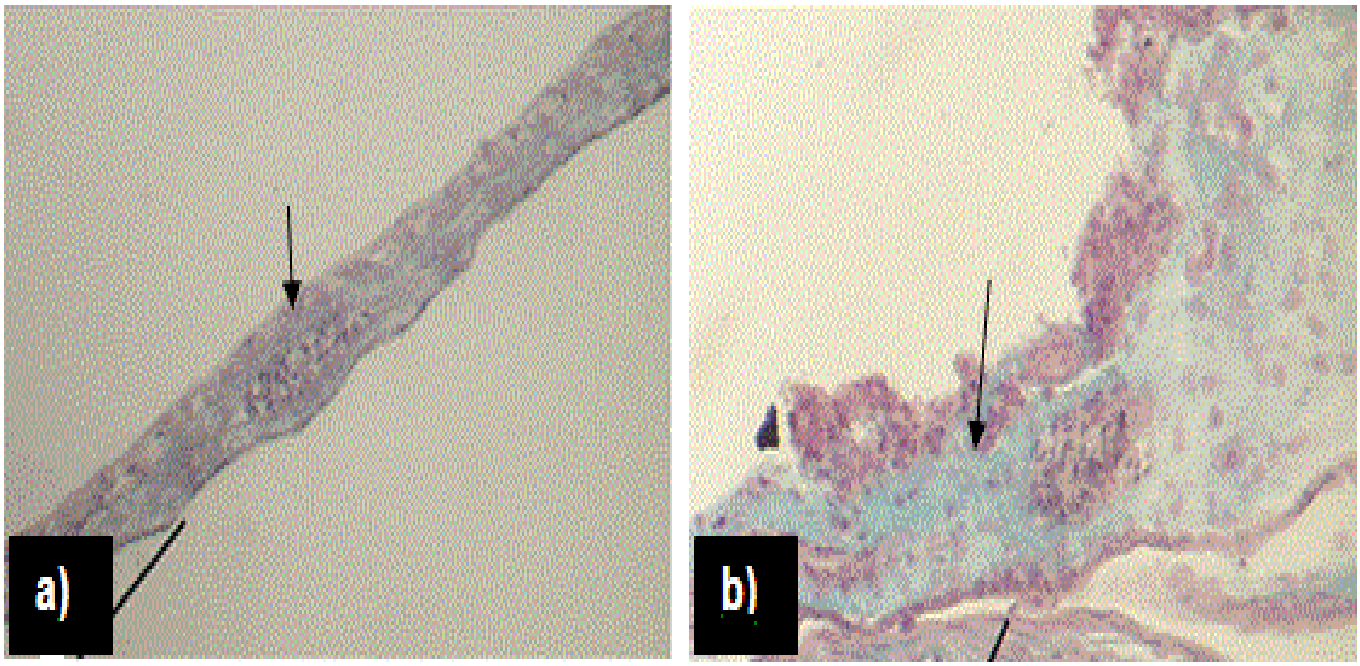

Figure 5. Photomicrography representing an aspect of the CAM microvasculature under prilocaine effects. Masson's trichrome staining. a) Prilocaine 5 µg/g, 10X. The arrow shows a microvessel; b) Prilocaine 5 µg/g, 40X, the arrow shows collagen fibers (light blue).

Table 1. Comparative data for liver and kidney injuries.

(N = 12)	Liver	Kidney
Average area of injuries (μm^2)	12,799 ± 3,430*	96,669 ± 29,210*
Average perimeter of injuries (µm)	673 ± 180*	2,000 ± 604*
Average number of inflammatory cells	274 ± 73*	758 ± 229*

Data represent mean ± SD of 12 individual values. * $P < 0.05$ in the liver versus the kidney.