

The role of prolactin in the healing process: A preliminary morphological study

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Abstract

Introduction

The authors hypothesized a possible use of prolactin as a therapeutic treatment of skin lesions. We investigated the relationship between topic prolactin-treatment of skin lesions and wound healing process in rats.

Materials and methods

Skin lesions were produced in both control and experimental rats. PRL-treatment (100 µl of PRL 2 ng/ml in PBS) was performed by dripping on lesions of experimental rats. Control rats were treated with only PBS. After 1 week, fragments of tissue from the bottom of lesions were obtained from three rats of both control and experimental groups. The authors continued the PRL-treatment to other rats until complete healing. In skin specimens, analysis of the ultrastructural features of cells, cellular aggregates and extra-cellular matrix components were made by transmission electron microscopy. In skin specimens, computerized morphometry measurements of different cell types were also made.

Results

Results showed that progression of the healing process was more advanced in treated rats as evidenced by the different percentages of distinct cell types after 1 week of PRL-treatment. Moreover, in treated rats,

the healing process was slightly faster than that in the control ones (11–12 days vs. 14–15 days, respectively).

Conclusion

Results of our study supported the idea of a role of PRL on wound healing, thanks to its effects on both angiogenesis and keratinocyte proliferation in re-epithelialization and then on restoration of injured skin.

Introduction

Wound healing of skin lesions is a process well known in relation to histological alterations, even if the molecular mechanisms underlying its regulation are still unclear. Several growth factors and cytokines of the immune response are involved in it¹, as well as macrophages producing cytokines stimulating growth of new capillaries (angiogenesis), collagen synthesis and production of fibres². Wound healing proceeds through a dynamic process that includes the inflammatory phase, the proliferative phase with generation of granulation tissue, the phase of re-epithelialization and of remodelling. If the healing process does not occur regularly, this may be responsible for some pathological conditions such as ulcers or chronic injuries^{3,4}. In the inflammatory phase, neutrophils and macrophages implement a cleaning action in the injury, removing cellular debris, foreign bodies and any contaminating bacteria. Macrophages also stimulate migration of endothelial cells and fibroblasts, thus the beginning of two fundamental processes of wound healing: angiogenesis and fibroplasia. The proliferative phase of wound healing is characterized by fibroplasia, angiogenesis, epidermal regeneration and wound contraction.

Angiogenesis is a fundamental step in the proliferative phase. New blood vessel generation and thus blood flow allow restoring blood supply in the area of the lesion. The last event of the proliferative phase is the re-epithelialization. It results in the regeneration of an intact epidermal layer above the new tissues. This process is supported by keratinocytes that move through the granulation tissue. When wound surface is covered by a monolayer of keratinocytes, migration stops and a new stratified epidermis is restored. The remodelling phase is characterized by collagen degradation and synthesis of new ECM components. The remodelling phase depends mainly on the breakup/building of ECM; macrophages play a crucial role in both processes^{5,6}.

Each stage of wound healing is due to paracrine effectors released by specific cell-types of immune systems and also to some endocrine effectors known from the literature⁷⁻⁹. Prolactin (PRL) belongs to the latter group and is a peptide hormone secreted by the anterior hypophysis. PRL is a hormone secreted by acidophilic cells of the pituitary gland, and it is known for its lactogen action on the mammary gland. It has specific roles in regulating both humoral and cell-mediated immune response. In recent years, a lot of evidence¹⁰ indicates that several other tissues are able to produce PRL. The discovery of a PRL extrapituitary production^{11,12} has proposed a reevaluation of this versatile bio-regulator, which acts systemically as a hormone, and locally as a cytokine¹³. The different isoforms of PRL exhibit distinct biological

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properties in relation to the ability of activating specific signal-molecules¹⁴. Therefore, the role of PRL on distinct phases of wound healing appears very interesting. In our previous studies^{15,16}, it was shown that PRL receptors were present on macrophages, highlighting a role of PRL in the activation of them. There are evidences¹⁷, which also indicate that human dermal fibroblasts synthesize and release PRL *in vitro*, suggesting a potential local source of PRL in human skin. It is believed that PRL expression in skin can probably be related to an inflammatory response and may play a role in wound healing^{12,18,19}. Our study on effects of PRL in wound healing of skin injuries in rats takes the cue from these considerations. We treated skin lesions, experimentally produced in rats, with PRL and then we made histological and ultrastructural analysis and morphometry measurements in skin biopsies collected after 1 week from the beginning of the experiment. The aim of our study was to evaluate the possible therapeutic role of PRL in the treatment of skin lesions.

Material and methods

The protocol of this study has been approved by the relevant ethical committee related to our institution in which it was performed. Animal care was in accordance with the European Community Council Directive (86/609/EEC) and Italian Animal Protection Law (116/1992).

Breeding and housing of animals

We used 14 adult healthy female Wistar rats (Charles River Laboratories, Wilmington, MA, USA), with an average body weight of 200±40 g. Rats were individually housed in polycarbonate cages during the entire stabling period and were housed at steady temperatures (20-23°C) and humidity, with free access to water and food and photoperiod of 12 hours light/dark.

Experimental design

We proceeded to shave and disinfect the back of each animal; after anaesthesia by ketamine-midazolam-xylozine (KMX), a full-thickness lesion (1.5 cm × 0.5 cm) was produced with a lancet. We proceeded to dressing with a sterile bandage.

Rats were divided into two groups: control group consisting of six rats, and experimental group consisting of eight rats. PRL treatment was made by dripping on lesions, on alternate days from the second day for a total of seven doses, giving the control group 100 µl of PBS, and experimental group 100 µl of PRL 2 ng/ml in PBS as protocol.

Dressing with sterile bandage was done after each treatment.

After 1 week from the beginning of the experiment, fragments of tissue from the bottom of lesions were obtained from three rats of both control and experimental groups. We continued the PRL-treatment on other rats until complete healing. Collection of biopsies was performed under anaesthesia using KMX.

Transmission electron microscopy

Soon after sampling, fragments of skin (thickness ~3 mm) were immersed and fixed in Karnovsky fixative (0.1 M phosphate buffer, paraformaldehyde 2%, glutaraldehyde 2.5%, H₂O) for 3 hours at 4°C. After fixation, specimens were washed overnight in phosphate buffer 0.1M and then they were post-fixed in 1% OsO₄ in phosphate buffer with addition of 10% sucrose. Dehydration took place in increasing concentration series of alcohol or acetone. Embedding was made in Epon 812 (Sigma-Aldrich, Milan, Italy); polymerization was made at 60°C for 12 hours²⁰. Ultrathin sections of 40-50 nm were obtained with ultramicrotome Leica EM UC7 (Leica, Milan, Italy) using diamond knives. Sections were placed on naked, copper grids and stained with uranyl acetate and lead

citrate and then they were observed on transmission electron microscope HITACHI H-600-3 (accelerating voltage 75 kV). Film images of sections were obtained.

Computerized morphometry measurements and image analysis

Other skin specimens were fixed in 10% neutral buffered-formalin (Bio-Optica, Milan, Italy), following overnight washing; specimens were treated as previously described²¹. After tissues were dehydrated, cleared and infiltrated with the embedding material, they were ready for external embedding. During this process the tissue samples were placed into the cassettes in the same orientation along with liquid hot (65°C) embedding paraffin wax. Sections of 4-5 µm thickness were cut from paraffin blocks using a rotary manual microtome (Leica RM2235, Milan, Italy) and mounted on silane-coated slides (Menzel-Glasser, Braunschweig, Germany) and stored at room temperature. Slides were de-waxed in xylene, hydrated using graded ethanol, and stained for routine histological evaluation by haematoxylin and eosin (H&E) staining. Quantitative evaluation of different types of cells was based on observation of sections of all skin samples. Ten sections/animals were analysed in stepwise manner as a series of consecutive fields, and counting was performed at 200× magnification. Data obtained from each observed slide were recorded as count of cells of the different cell types. These evaluations were independently evaluated by four investigators (two anatomical morphologists and two histologists) and scored as a percentage of the final number of 100 cells in five categories: grade 1: <5% (0); grade 2: 5-10% (+); grade 3: 11-20% (++); grade 4: 21-50% (+++), and grade 5: >51% (++++), using an image analyser (Image-Pro Plus 4.5.1, Immagini &

Computer, Milan, Italy). Digital pictures were taken using the Zeiss Axioptan light microscope (Carl Zeiss, Oberkochen, Germany) and photographed with a Canon digital camera (Power Shot G5 Canon, Tokyo, Japan). Evaluations were assumed to be correct if values were not significantly different. In the case of disputes concerning the interpretation, the case was revised to reach a unanimous agreement.

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS® release 16.0, IBM, Chicago, IL, USA). Data were tested for normality with the Kolmogorov–Smirnov test. All variables were normally distributed. Comparisons between two means were tested with Student's *t* test, whilst comparison between more than two groups was tested using analysis of variance (ANOVA) and Bonferroni's test. *p*-values of less than 0.001 were considered statistically significant. Data are presented as mean ± SEM.

Results

Transmission electron microscopy

Analysis of the ultrastructural features of cells, cellular aggregates and also of ECM components was made. In electron microscopy, control rat dermis showed an evident epidermal basal layer, an underlying moderate fibrin deposition and some activated macrophages (Figure 1). In contrast, the dermis of treated animal lesions detected, in electron microscopy, the presence of differentiating monocytes into macrophages, activated macrophages rich in lysosomes and the stroma had several collagen fibres (Figures 2 and 3). In the stroma of the dermis, new vessels were detected. Their endothelium had a well-developed vesicular apparatus; collagen fibres were evident near the vessels; cytoplasmic fractions of leucocytes could be detected in the capillary lumen (Figure 4).

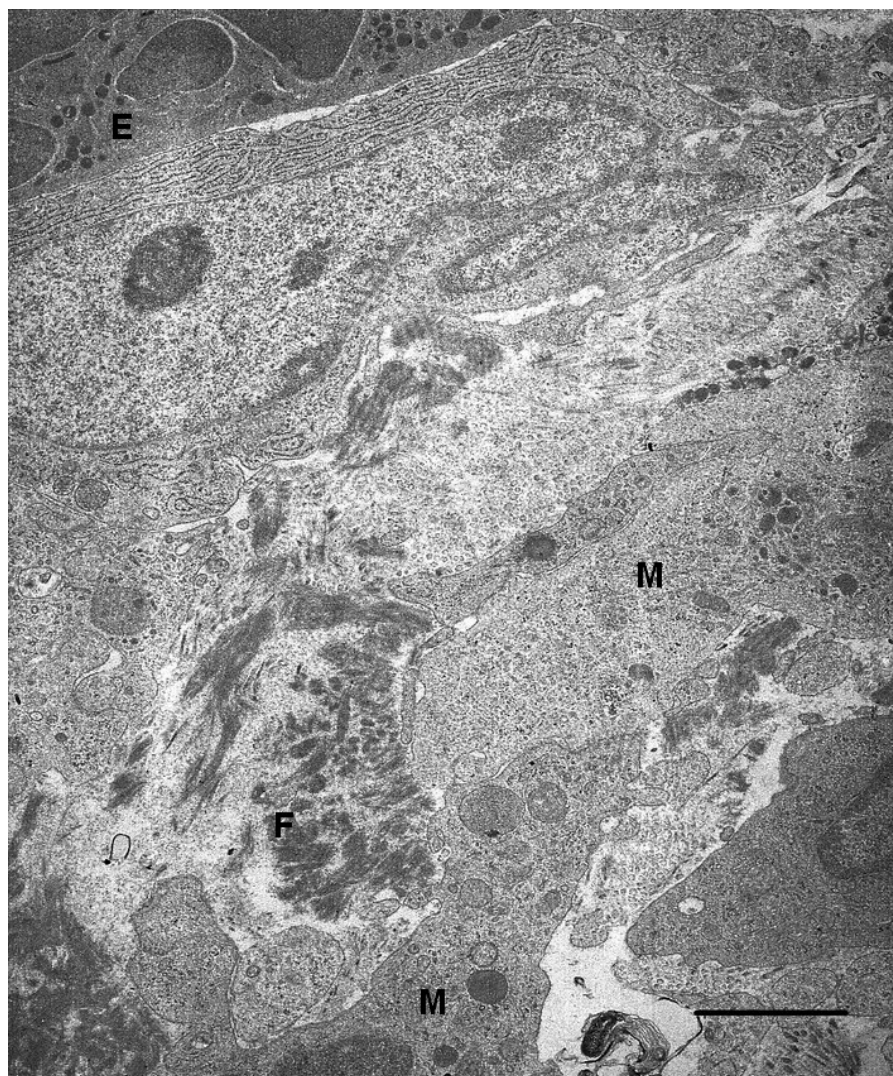


Figure 1: Control rat dermis in transmission electron microscopy. Top left: basal layer of epidermal epithelium (E), below which, it is possible to observe discrete deposits of fibrin (F) in the extracellular matrix and a moderate number of activated macrophages (M). Scale bar = 1.66 μ m.

Computerized morphometry measurements and image analysis

All histological specimens appeared with abundant granulation tissue (data not shown), and the process of wound healing was completed at 14-15 days from the beginning of experiment in control rats, and at 11-12 days in treated rats.

Quantitative evaluation of different types of cells was made. Percentages of epithelial cells were similar in control (3.3 ± 0.75 ; 0) and treated (2.5 ± 0.75 ; 0) rats (*p* not quite significant).

Neutrophils were more present (22.1 ± 0.54 ; +++) in control rats than in treated ones (13.1 ± 0.83 ; +++) (*p* < 0.001; significant). In treated rats, percentages of monocytes (22.6 ± 0.70 ; +++)), macrophages (28.3 ± 1.01 ; +++) and fibroblasts (29.1 ± 1.04 ; +++) were greater when compared with respectively monocytes (20.6 ± 0.89 ; +++)), macrophage (19.8 ± 0.75 ; +++) and fibroblasts (24.6 ± 1.02 ; +++) of control rats. In all these comparisons, *p* < 0.001 is significant. In relation to lymphocytes, their

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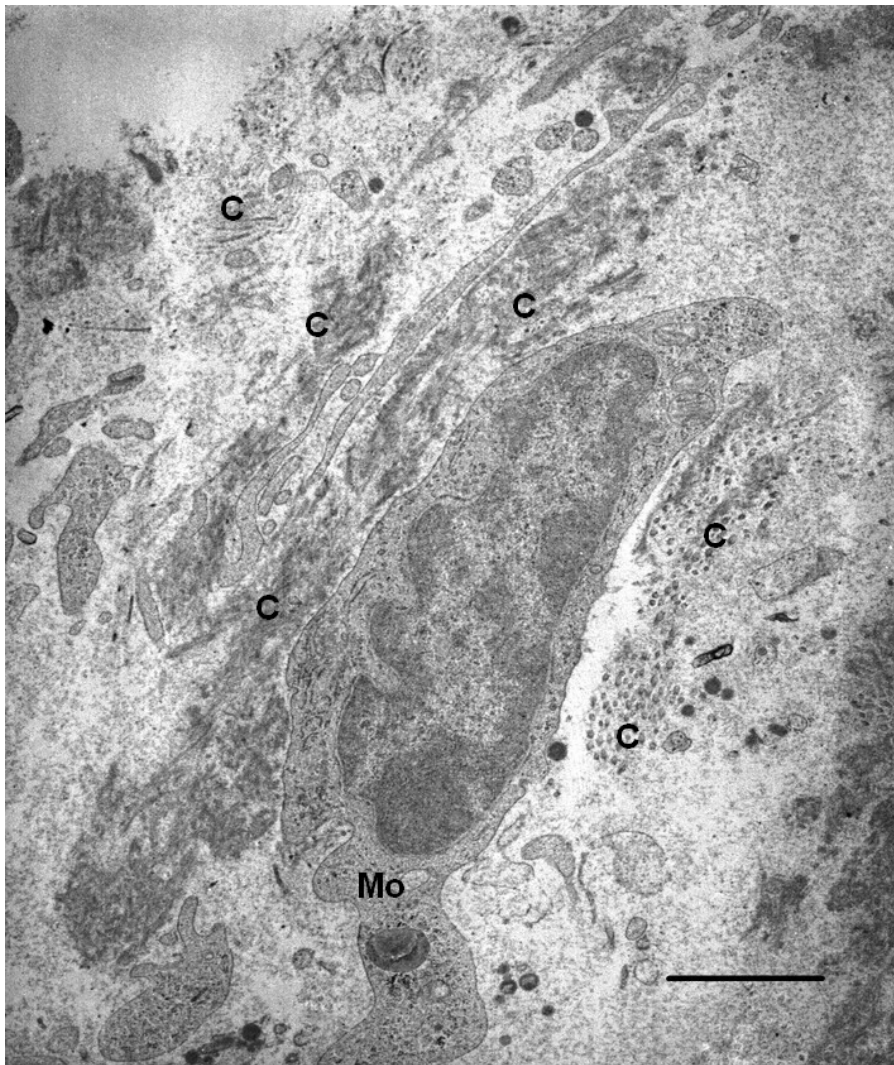


Figure 2: PRL-treated rat dermis in transmission electron microscopy. Stroma shows several systems of collagen fibres (C) both in cross-section (on the right) and oblique section (on the left). A monocyte (Mo) differentiating in macrophage is evident. Scale bar = 1.66 μ m.

percentages were greater in control rats (9.6 ± 0.81 ; ++) than in treated rats (4.4 ± 0.91 ; +) ($p < 0.001$; significant). Quantitative evaluation of percentages of different cell types was illustrated in Figure 5.

Discussion

Wound healing is an extremely complex process involving many different cell types, growth factors, cytokines and ECM components¹. Macrophages influence the healing process in different manners,

depending on the microenvironment in which they act^{5,22}. Initially they are important for removal of dead cells and debris. Subsequently they affect the healing process by production of collagen, angiogenesis and re-epithelialization^{23,24}. Macrophages induce angiogenesis in the lesion, mainly by the release of TGF- α and vascular endothelial growth factor (VEGF).

VEGF is one of the most important proangiogenic mediators. It is secreted by different cell-types

in response to several growth factors^{25,26}; from our recent studies, it seems that VEGF is also produced by macrophages stimulated by PRL^{15,16}. PRL-receptor (PRL-R) is widely expressed in several and distinct tissues, and it was identified in human skin too^{27,28}. Several recent studies suggest that PRL contributes to a wide variety of cutaneous processes, both physiological and pathological¹². In wound healing that requires appropriate responses by fibroblasts and endothelial cells, it was shown that these cells express PRL-R^{12,29}, so the cutaneous vascular system could provide new important details in relation to PRL-induction on angiogenesis. The presence of PRL-R on endothelial cells also suggests a direct action of PRL on angiogenesis, in addition to that mediated by growth factors such as VEGF. Furthermore, dermis fibroblasts, necessary for the maintenance and remodelling of ECM of skin during wound healing, produce PRL *in vitro*¹⁸. Human skin is, therefore, both the source and target of PRL. Recent studies suggest that intracutaneous PRL-expression seems to follow the central neuroendocrine regulation^{30,31}, and that PRL regulates keratin expression in human skin and that it has an influence on human epithelial stem cells¹⁹. It could be possible that keratinocytes, expressing PRL-R and proliferating in response to PRL *in vitro*²⁷, are the potential target cells of PRL produced by fibroblasts, and this PRL-production may represent a time signal for keratinocyte proliferation necessary for restoration of epidermis during re-epithelialization, in which intact skin regenerates over the newly formed tissues. Once again macrophages and then PRL are involved; in fact, stimulation of dermal fibroblasts and the resulting keratinocyte proliferation is supported by cytokines⁹, produced also by macrophages activated by PRL^{15,16}. In our previous studies we investigated on PRL role in activating macrophages

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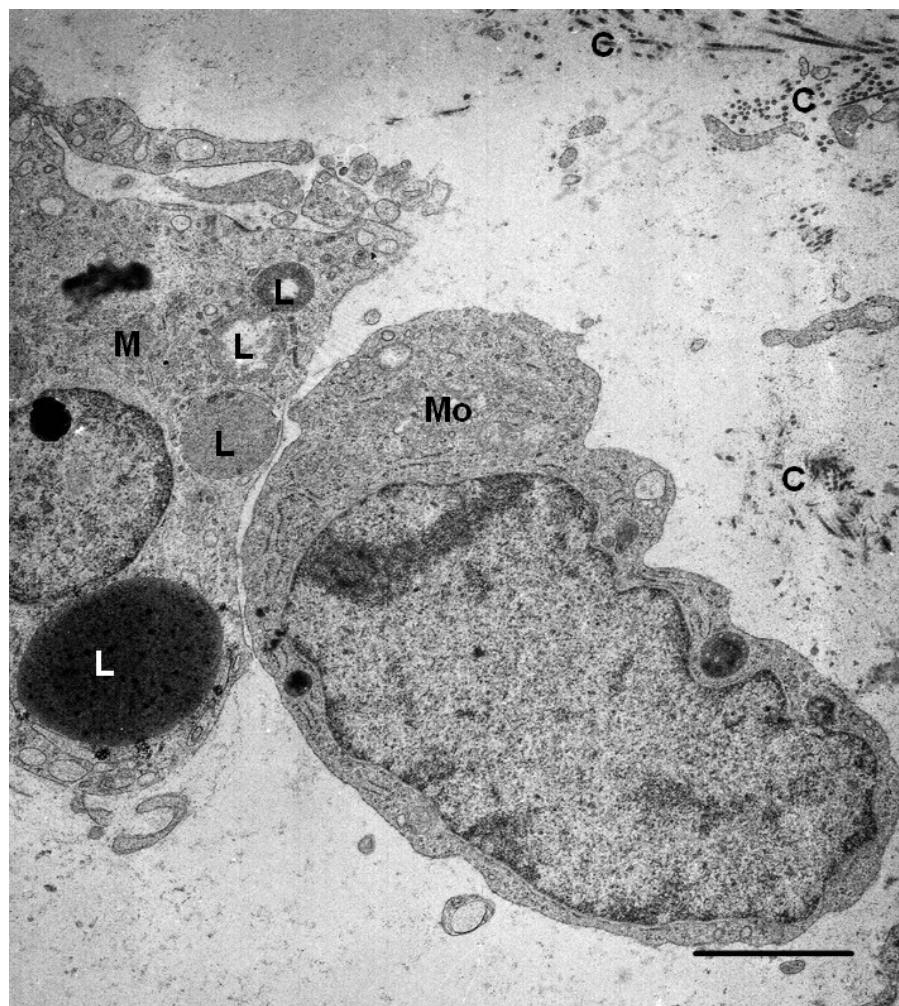


Figure 3: PRL-treated rat dermis in transmission electron microscopy. A monocyte (Mo) differentiating in macrophage, an activated macrophage (M) with several lysosomes (L) is evident; collagen fibres (C) are evident in the oedematous stroma. Scale bar = 1.25 μ m.

to evaluate a possible PRL induction in angiogenesis^{15,16}. These studies showed that treatment of cultured monocytes/macrophages with PRL results in an increased expression of HO-1 and a release of VEGF by these cells, that is, macrophage activation. Involvement of PRL in macrophage activation provides evidence of co-operation between components of the immune system and endocrine system in the induction of angiogenesis. In addition, our previous results also indicated that activation of macrophages by PRL did not require the co-presence of other

cytokines, suggesting that PRL is a sufficient signal to induce events related to macrophage activation; our results showed that PRL really modulates and controls the angiogenic functions of macrophages¹⁶. Moreover, the presence of PRL-R on macrophages³² further supports that PRL is involved in the control of monocytes/macrophage functions, with regard to the production of VEGF, which in turn acts on endothelial cells, and then on angiogenesis, confirming the claims in our previous studies^{15,16}. In the present study, we made a comparative analysis of

morphometry measurements and ultrastructure of cutaneous biopsies in control rats versus PRL-treated rats.

Our results showed a progression of the healing process more advanced in treated rats compared with controls, as evidenced by the higher percentages of differentiating monocytes, macrophages and fibroblasts as compared with control rats, and by a more evident presence of collagen fibres and newly formed capillaries, consequences of the healing process. Neutrophils and lymphocytes, on the other hand were present in lower percentages in the samples of treated rats as compared with control rats, probably in relation to the overcoming of the inflammatory phase in which debris and contaminating bacteria were removed.

Conclusion

Our results support the idea of a role of PRL on wound healing, thanks to its effects on both angiogenesis and keratinocyte proliferation in re-epithelialization and then on restoration of injured skin. Moreover, in treated animals, the healing process was slightly faster than that in the control ones.

However, further studies are required to better establish optimal dosages of PRL for a more effective action on the treatment of skin lesions.

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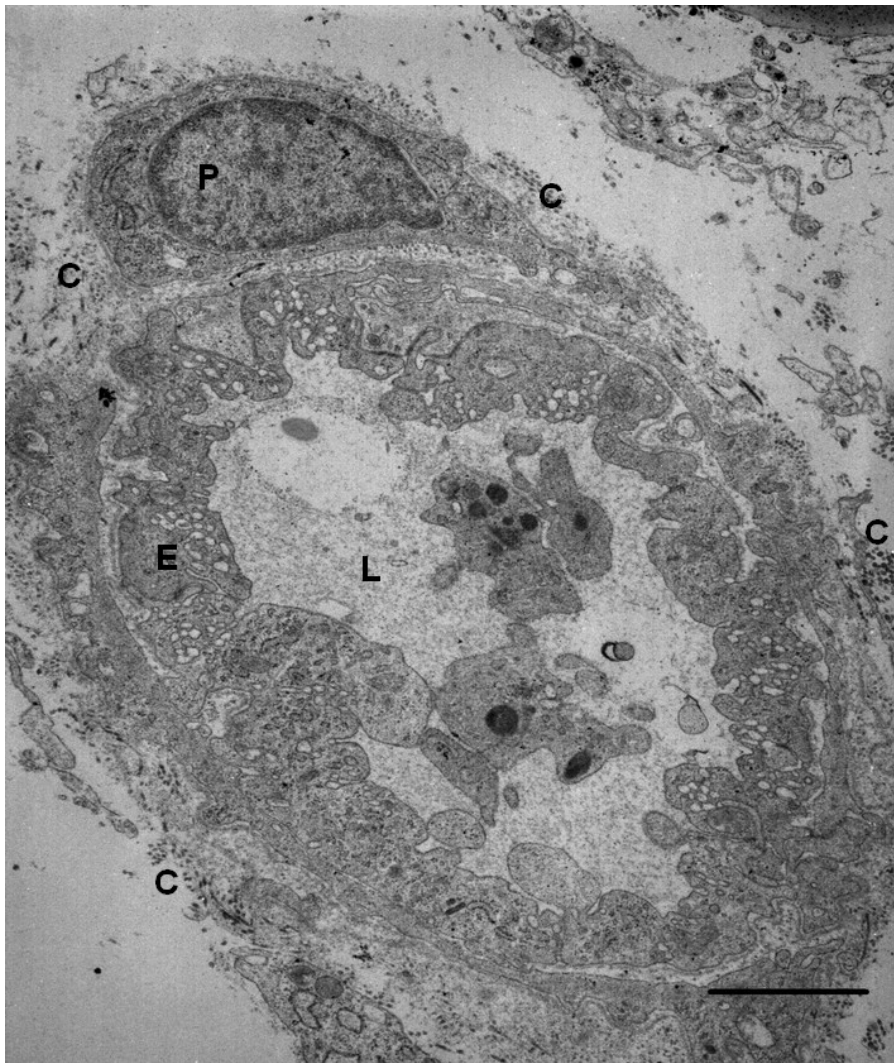


Figure 4: PRL-treated rat dermis in transmission electron microscopy. Cross-section of a newly formed capillary with a pericyte (P). Endothelium (E) shows a well-developed vesicular apparatus. Cytoplasmic fractions of leucocytes within the lumen of the capillary (L). Collagen fibres (C) are widespread close to the capillary. Scale bar = 1 μ m.

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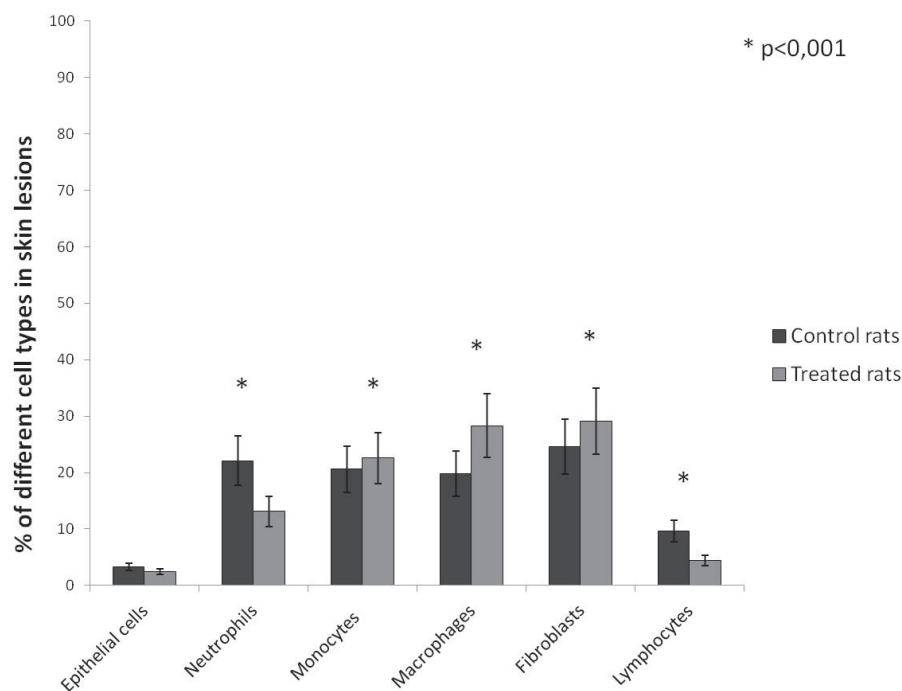


Figure 5: Comparison between the means of the percentages of different cell types from control and PRL-treated rats after 1 week of experiment. * $p < 0.001$.

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