

ORIGINAL RESEARCH REPORT

Wound-healing effects of low-level laser therapy in diabetic rats involve the modulation of MMP-2 and MMP-9 and the redistribution of collagen types I and III

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Abstract

The present study aimed to determine if LLLT restores the balance between mRNA expression of matrix metalloproteinases (MMP-2 and MMP-9) and also the balance between collagen types I and III during the healing process of diabetic wounds. One hundred and twenty male Wistar rats were distributed in Control (untreated non-diabetic rats: UND); Laser (laser treated in non-diabetic rats: LTND); Diabetic (diabetic rats non-laser treated rats: UD); and Diabetic+ Laser (diabetic rats laser treated: DLT) groups. The diabetes model using streptozotocin efficiently induced diabetes, as demonstrated through increased levels of blood glucose. Diode laser (50 mW, 660 nm, 4 J/cm², 80 s) was applied a single time after scare induction. Twenty-four hours after LLLT application, rats were euthanized, the scarred areas were collected for MMP-2 and MMP-9 mRNA analysis and also for histological analysis (inflammation and types I and III collagen). The results demonstrated that scare in untreated diabetic rats significantly increased the MMP-2 and MMP-9 expression compared with that in non-diabetic rats ($p < 0.05$), while LLLT significantly reduced MMP-2 and MMP-9 expression compared with that in untreated diabetic rats ($p < 0.05$). To conclude, the results also showed that LLLT was able to alter the expression of MMP-9 as well as accelerate the production of collagen and increase the total percentage of collagen type III in diabetic animals.

Key Words: *low-level laser therapy, matrix metalloproteinases, wounds repair*

Introduction

Wound healing, irrespective of the way injury is caused, proceeds via overlapping events broadly classified as inflammation, formation of granulation tissue, angiogenesis, and tissue remodeling. Different cell types interaction, extracellular matrix proteins, and their receptors are involved in these biological significant processes, which are mediated by cytokines and growth factors. Release and presence of growth factors affect recruitment, activation, mitogenesis, migration, and differentiation of various cell types in the wound bed (1).

Factors such as age, obesity, malnutrition, and macrovascular and microvascular diseases may

contribute to wound infection and delayed wound healing, especially in the type II diabetic patient. In addition, hyperglycemia caused by decreased insulin availability and increased resistance to insulin can affect the cellular response to tissue injury. Studies of the immune cells necessary for wound healing, such as PMN leukocytes and fibroblasts, as well as studies of injured tissue suggest that there is a delayed response to injury and impaired functioning of immune cells in diabetes mellitus (2). Such alterations in these events give rise to low collagen synthesis and also contribute toward increased risk of infections among diabetic patients (3).

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Epidermal wound healing is a complex and highly coordinated process where several different cell types and molecules, such as growth factors and extracellular matrix (ECM) components, play an important role. Among the many proteins that are essential for the restoration of tissue integrity is the metalloproteinase (MMP) family. MMPs can act on ECM and non-ECM components affecting degradation and modulation of the ECM, growth-factor activation and cell-cell and cell-matrix signaling. MMPs are secreted by different cell types such as keratinocytes, fibroblasts, and inflammatory cells at different stages and locations during wound healing, thereby regulating this process in a very coordinated and controlled way (4).

According to Okada et al. (5) skin wound healing depends on cell migration and extracellular matrix remodeling. Both processes, which are necessary for reepithelization and restoration of the underlying connective tissue, are believed to involve the action of extracellular proteinases. These processes are achieved by extracellular proteases, particularly those belonging to the serine protease and matrix metalloproteinase (MMP) families.

Expression of collagen and other components of the extracellular matrix is essential for maintaining tissue integrity and organ architecture, and has an important role in physiological wound healing and tissue repair processes. However, chronic and uncontrolled inflammation disrupts this homeostasis and results in excessive collagen deposition, producing fibrosis (6).

Normal wounds can heal by secondary intention (epidermal migration to cover a denuded surface) or by approximation of the wound edges (e.g., suturing). Epidermis-derived MMPs are important in healing by secondary intention. Keratinocyte migration begins within 3–6 h post injury, as basal cells detach from underlying basal lamina and encounter a dermal substratum rich in type I collagen (7).

The LLLT operating in different wavelengths and energy densities has also been used in several studies to accelerate the healing process changed in diabetes. Reddy (8) who examined the effect of Ga-AS laser radiation (904 nm; 7 mW; 1J/cm; 5 days/week for 3 weeks) in wounds on the back of diabetic rats promoted an increase of 14% in the deposition of collagen in diabetic rats compared to that in non-irradiated rats.

Maiya et al. (9) Conducted a study on skin wounds in diabetic and non-diabetic animals using a HeNe laser (632.8 nm; 4.8 J/cm, 5 days / week). The researchers were able to observe that wounds on the back of diabetic rats and found complete healing after 18 days, while the diabetic animals had not spent an average of healing the wound in the 59th day. Animals irradiated in this study showed an Increased amount of collagen in scar tissue, an power

increase in the proliferation of fibroblasts and in the number of capillaries formed.

Byrnes et al. (10) analyzed the healing of wounds on the back of rats with type II diabetes and found that healing of diabetic animals was irradiated faster (HeNe 632 nm; 16mW; 4J/cm), and that there is an increase in the production of FGF wounds of irradiated animals.

As can be seen in the earlier studies LLLT can accelerate healing and increase collagen deposition in diabetic wounds. However some issues in this regard are not clear, so we conducted this study to determine AIMS at what stage of the repair process LLLT acts on what type of collagen and collagen can be found during this process. In light of the foregoing process, this study analyzes through the birefringence collagen when subjected to polarized light the collagen type and the gene expression of metalloproteinases MMP-2 and MMP-9 in cutaneous wounds of diabetic and non-diabetic rats submitted to low-power laser.

Methods

Animals

One hundred and twenty male Wistar rats weighing between 280 and 320 g remained under controlled environmental conditions (light/dark cycle of 12/12 h, sanitized environment, $24 \pm 2^\circ\text{C}$), receiving pellet laboratory chow (Purina, Labina, São Paulo, Brazil) and water *ad libitum*. This study was approved by the Ethical Committee for Animal Experimentation of the Nove de Julho University (UNINOVE), São Paulo, SP, Brazil (Protocol 023/2006).

Experimental groups

Twenty-four of the 120 animals were chosen at random to make up the group of non-diabetic animals and the 60 remaining animals had diabetes induced using Streptozotocin (STZ). This division resulted in two experimental groups: diabetic and non-diabetic rats. These groups were divided into four subgroups and received the following names: subgroup 1, treated LLLT diabetic rats (TLD); subgroup 2, untreated diabetic rats (UD); subgroup 3, treated LLLT non-diabetic rats (TLND); and subgroup 4, untreated non-diabetic rats (UND).

Experimental induction of diabetes

Animals were made to fast overnight and diabetes was induced by a single intraperitoneal injection of a freshly prepared solution of STZ (60 mg/kg) in 0.1 mol/L citrate buffer (pH 4.5). The dosing volume was 1 mL/kg. To prevent fatal hypoglycemia, rats were kept on 5% glucose solution for 24 h after

STZ injection. Successful induction of diabetes was confirmed by measuring the fasting blood glucose concentration in rats 6 h after injection of STZ. Rats with a fasting blood glucose level > 250 mg/dL were considered diabetic and included in the present study (10,11).

After weighing, each animal received preanesthetic administration of Butorphanol (Turbogestic, 2 mg/kg) associated with Acepromazine (Acepran, 1 mg/kg), both in a single dose intramuscularly. After 15 min the animals were administered Zolazepam and Tiletamine (Zoletil 50, 40 mg/kg). To carry out the injury, a "PUNCH" of 8 mm diameter was used to remove a circular area of skin, with localization to the middle portion of the median sagittal plane (12).

Laser irradiation indium gallium aluminum phosphide (InGaAlP)

Laser irradiation was done using indium gallium aluminum phosphide (InGaAlP) DMC Photon Laser III model, with power of 50 mW (power density of 1.43 W/cm²), the beam area of 0.028 cm², and length λ wavelength of 660 nm. The application was in the form of a single point by the transcutaneous method in the wounds, with fluency (energy density) of 4 Joules/cm², of energy and time of 80 s. The application was initiated immediately after surgery and extended according to the experimental group.

Histologic analysis

The wound tissues were fixed with 10% formalin, embedded in paraffin, and sectioned. Sections of 5- μ m thickness were stained using hematoxylin-eosin, Masson's trichrome, and Picrosirius Red.

Morphometric analysis

Morphometric analysis was performed on the slides, by means of image digitization and computational analysis using a specific image processing and analysis program (Image Pro plus 4.5). To quantify the areas representing collagen, five fields observed using a microscope Nikon Eclipse E200 (40 \times lens) were digitized. The microscope was coupled to an image-capturing Sanyo digital active BLC camera, and this was connected to a microcomputer equipped with a video board. All the images were digitized before the quantification process, thereby standardizing the microscope light intensity and condenser height. The collagen areas were separated in the image, using the color distribution as the discriminating parameter. Picrosirius Red, an anionic composite that distinguishes the thickness and density of collagen fibers through coloration emitted under polarized light, was used to estimate the percentage of collagen

fibers. While the thin dissociated fibers typical of type III collagen are greenish, the thickest and strong associated fibers of type I collagen emit colors with bigger wavelength as red and yellow (13,14).

RNA isolation and real-time polymerase chain reaction analysis

First, injured skins were thawed, and Trizol was immediately added (Gibco BRL, Life Technologies, Rockville, MD, USA[®], 1 ml/100 mg tissue). Then, injured skins were homogenized for the recovery of total RNA, according to the manufacturer's instruction. DNase I was employed to digest DNA to obtain RNA purification and the integrity of RNA was verified by agarose gel electrophoresis. Total RNA (2 μ g) was used for first-strand cDNA synthesis (reverse transcriptase (RT)) using SuperScript II. In addition, RNaseOUT was also added to protect the RNA during this process. Three pooled RNA aliquots were routinely sham reverse transcribed (i.e., reverse transcriptase omitted) to ensure the absence of DNA contaminants. Diluted RT samples (1:10) were submitted to real-time polymerase chain reaction (RT-PCR) amplification using Platinum Sybr QPCR Supermix-UDG and specific oligonucleotides for MMP-2 and MMP-9.

The conditions for PCR were as follows: 50°C, 2 min; 95°C, 2 min; followed by 30 cycles of 95°C, 15 s; 60°C, 1 min; and 72°C, 15 s. Ct values were recorded for each gene, and the results of genes of interest were normalized to results obtained with the internal control gene. ddCT were calculated and the results are expressed as fold increase. All oligonucleotides and reagents utilized in this protocol were purchased from Invitrogen Co. (USA).

Statistical analysis

The original data were tested with GraphPad software. All the results are presented with mean \pm standard deviation. One way analysis of variance (ANOVA) and post hoc Tukey's test was used for statistical analysis, and all tests were considered to be statistically significant at $p < 0.05$.

Results

Analysis of total collagen

The density of total collagen of subgroup treated LLLT diabetic rats (TLD) was significantly higher ($p < 0.05$) than that of subgroup untreated diabetic rats (UD), using multiple comparisons by the Tukey test (Figure 1). Figure 1 (a and b) displays high density of collagen in histological preparation of subgroup treated LLLT diabetic rats (TLD) that can be compared with the low density in respective

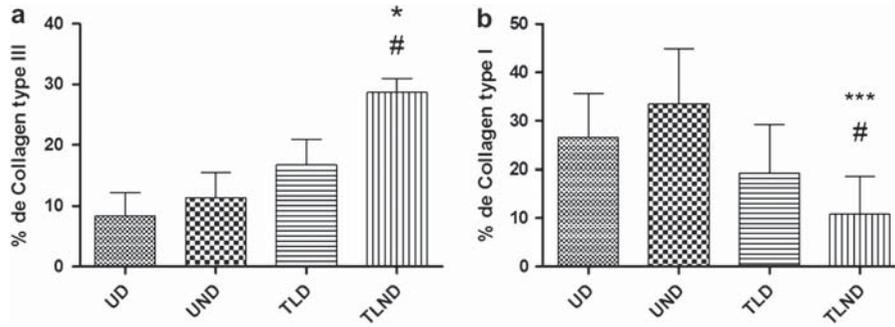


Figure 1. LLLT on Collagen I and Collagen type III in diabetic and non-diabetic wounds, density analysis of collagen fibers emitted through coloration under polarized light, was used to estimate the percent of collagen fibers. a - Collagen type III *p value < 0.05 compared to group UND - not diabetic does not undergo treatment with LLLT, #p value < 0.05 compared to group TLD - diabetic animals treated with LLLT. b - Collagen type I ***p value < 0.001 compared to 05 UND-diabetic group not subjected to treatment with LLLT #p value < 0.05 compared to group TLD - diabetic animals treated with LLLT.

control group. When the LLLT was not used in the wounds (subgroups, untreated non-diabetic rats - UND and untreated diabetic rats -UD), the density of the collagen had a significant reduction in the wounds on diabetic rats (UD), compared to that in the control subgroup ($p < 0.05$).

Analysis of type I collagen

The density of type I collagen of subgroup treated LLLT diabetic rats (TLD) was significantly higher ($p < 0.05$) than that of subgroup untreated diabetic

rats (UD), using multiple comparisons by the Tukey test (Figure 1). Figure 2 displays high density of collagen type I in histological preparation of subgroup treated LLLT diabetic rats (TLD) that can be compared with the low density in respective control group. When the LLLT was not used in the wounds (subgroups, untreated non-diabetic rats -UND and untreated diabetic rats -UD), the density of the type I collagen had a significant reduction in the wounds on diabetic rats (UD), compared to that in the control subgroup ($p < 0.05$) (Figures 1 and 2).

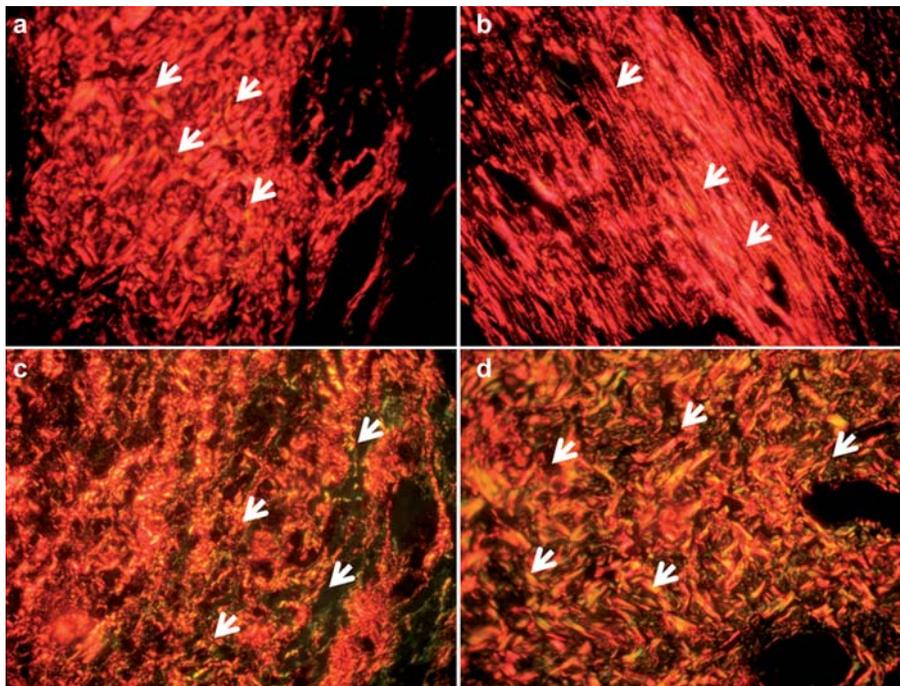


Figure 2. Representative photomicrographs using polarized light illustrating the collagen quantification. a - Non-diabetic rat does not observe the large amount of fibers that are shown in red color typified as type I collagen and the lack of staining greenish (collagen type III, marked by arrows). b - The image obtained from the lamina relating to the animals diabetics not borers, observe the rare areas of collagen marked by green color. c - Diabetic patients treated with LLLT, this group noted that there is an increase of type III collagen fibers (arrows) in the in smaller quantities red fibers. d - Not diabetic treated with LLLT, observe presence of the two types of collagen fibers as well as their interlacing (arrows).

Analysis of type III collagen

The expression of the type III collagen was analyzed, and a statistical differences between the two subgroup borers were observed and compared with both non-diabetic control groups; however, there was no statistical difference between the control subgroup non-diabetic and diabetic subgroup treated with LLLT ($p > 0.05$). The diabetes significantly reduced the density of type III collagen when those subgroups with the no-treated wounds were compared (UND and UD) (and) treated with LLLT ($p < 0.05$). The expression of the collagen type I was always significantly greater than that observed in type III collagen of all the other subgroups (Figure 1-a). Figure 2(a) demonstrates the low density of type III collagen in diabetic wound of the subgroup UD, where rare green fibers are seen and a high density of the type III collagen is observed in the subgroup treated with LLLT (Figures 1 and 2).

Expression of genes involved in MMP-2 and MMP-9 synthesis

Real-time PCR analysis of genes associated with extracellular matrix turnover demonstrated significant differences between diabetic and non-diabetic treated LLLT groups. MMP-9 gene expression was significantly increased in diabetic rats no treated compared to that in non-diabetic rats (1.61 ± 0.19 versus 0.59 ± 0.5 , respectively; $P < 0.01$); however, we observed a decrease in MMP-9 gene expression in diabetic rats treated with LLLT (0.84 ± 0.2) than in non-diabetic rats treated with LLLT (0.51 ± 0.2 ; $P > 0.05$). However, there was a mild increase in MMP-2 gene expression in the diabetic rats treated with LLLT ($0,84 \pm 0,24$) when compared with that in non-diabetic rats treated with LLLT; no significant difference was found ($0,51 \pm 0,26$; $P > 0.05$). There was in MMP-2 gene

expression between diabetic or non-diabetic rats non-treated ($1,6 \pm 0,19$ versus $0,19 \pm 0,06$, respectively) (Figure 3).

Discussion

The low-power laser has been used in several studies involving tissue repair in normal and diabetic animal model, especially in increasing the revascularization, increased density of collagen and modulation of chemical mediators of inflammation (15).

Studies (16–19) which involve wound healing show that the retardation of the healing process is characterized by increased expression of matrix metalloproteinases (MMPs), a decrease in tissue inhibitors of metalloproteinases (TIMPs), and a reduction in some growth factors, in particular, transforming factor-beta (TGF- β growth). Both MMPs and TIMPs are secreted by cells related to wound healing, and their concentrations vary with the repair phase.

Given the evidence that LLLT acts to enhance collagen synthesis both in normal wound healing as those involving diabetes mellitus, this study tried to identify the influence of LLLT in the differentiation of collagen type I and III, using both histological sections stained with picrosirius red that allows to evaluate the type of collagen and quantify it and that studies show that the amount of attached dye is proportional to the amount of protein present, allowing its use for quantification of collagen as observed by light microscopy polarization due to birefringence of collagen fibers, the present study also aimed to evaluate the gene expression of metalloproteinases MMP-2 and MMP-9 due to its important role in tissue repair.

In our study we found that LLLT was able to increase the expression of total collagen significantly in the groups of diabetic and non-diabetic animals

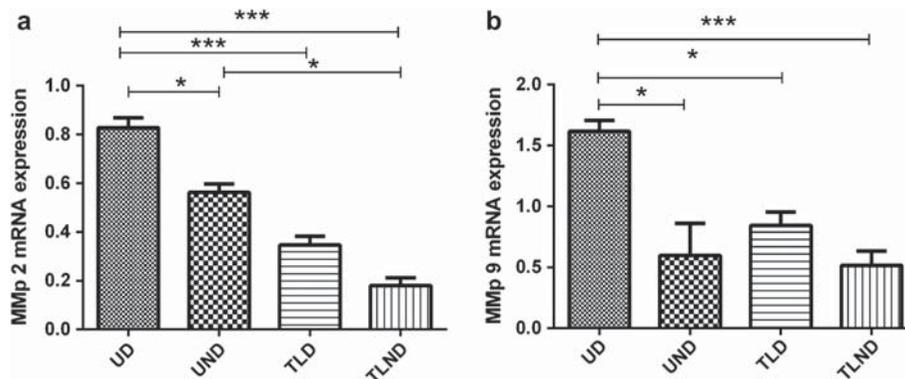


Figure 3. Real-time PCR rats diabetic and non-diabetic skin treated and no treated with LLLT. a - Mean matrix metalloproteinases (MMP) 9 were significantly greater among normal rats no treated com LLLT when compared with diabetic rats (* $p < 0.05$), (** $p < 0.001$). b - MMP2 was greater among diabetic rats no treated com LLLT. The results show that the groups treated with LLLT, the values expressed both MMP2 and MMP9 were similar in diabetic and non-diabetic animals no significant differences were statistically significant. (* $p < 0.05$), (** $p < 0.001$).

compared to their respective control groups. The study also enabled us to verify that after the period of irradiation with LLLT there was no statistical difference between diabetic and non-diabetic groups submitted to LLLT.

The results of our study indicate that the increase in the total percentage of collagen fibers is corroborated with the findings of Pugliese et al. (20) Studied the effects of LLLT on standardized skin wounds in normal rats. Used laser Ga-Al-As with different energy densities. In morphometric analysis, we observed higher expression of collagen fibers. They found that the best results occurred in the groups irradiated with 4 J/cm². The authors attributed to the laser a significant increase in collagen deposition which could be due to induction of cell proliferation or increased by the process of protein synthesis and secretion, there is also the possibility that both mechanisms occur simultaneously.

We can also compare our results with Those of Maiya et al. (9) in study using animals with diabetes induced by alloxan and Treated with LLLT for 5 days with energy density of 4.8 J/cm², Concluded that the total content of collagen (Hydroxyproline) was Significantly Increased in laser-treated wounds as compared to the control group ($p < 0.0001$). Therefore, the production of collagen in diabetic wounds can be modulated by laser treatment. The authors report That Also the mechanism by laser photostimulation which facilitates collagen production in diabetic wound healing may involve common mechanisms variety of common photostimulating.

With respect to the density of the type of collagen type I tended to decrease in the groups treated with LLLT, with statistical significance compared to the untreated diabetic group, this decrease can be related to the inflammatory process stage and degree of maturation of collagen existing there mind that in untreated groups were higher values.

In this study we also observed an increase in collagen type III in treated groups and there was no statistical difference between diabetic and non-diabetic groups treated with LLLT, however the non-diabetic animals showed a statistically significant difference for both untreated groups.

Concerning the study of matrix metalloproteinases (MMP2), a decrease of gene expression in diabetic and non-diabetic groups treated with LLLT, with statistical difference of these two groups in relation to untreated diabetic groups, since the literature (18) indicates a decrease of MMP2 wounds in diabetic animals when compared with normal animal wounds which leads us to believe that LLLT was outlook capable of normalizing the mechanism of expression of this metalloproteinase.

Study by Dias et al. (21) if the LLLT Examined operating with different doses cause alteration in the expression activity of gelatinases (MMP-2 and MMP-9) in rat masseter muscle. The pesquisadores

observed that the exposure to the LLLT Increased activity of MMP-2 in group treated with 20 J/cm². The zymography in this study showed the expression/activity only of MMP-2 in the untreated control group (group I). However, under exposure to different laser doses a slight but detectable increase in MMP-9 expression/activity after exposure to all doses used can be observed, whereas an MMP-2 increase compared to the untreated control group was detected only when the highest dosage, 20 J/cm², was applied. Furthermore, different studies (22) have demonstrated that during pathological and regenerative processes, or in response to experimentally injured muscle, MMP-2, and, especially, MMP-9, are upregulated.

Our study also investigated the gene expression of MMP9, and a marked decrease was found in the diabetic group submitted to LLLT. Our results demonstrate that this decrease is statistically significant when compared with untreated control groups.

According to Xue et al. (23), it is widely recognized that high levels of MMP9 slow down the healing of diabetic foot ulcers by excessive degradation of extracellular matrix, growth factors, growth factor receptors, integrins, and their receptors, as well as increasing the local inflammatory response in the wound. It is still unknown whether MMP9 can influence the behaviors of biological affect skin fibroblasts and wound healing. Skin fibroblasts play important roles in wound repairing. Anything that can affect their biological properties will ultimately affect wound healing.

Conclusion

We conclude that LLLT was able to alter the expression of matrix metalloproteinase (MMP2 and MMP9) by adjusting the action in these diabetic animals, as well as accelerate the production of collagen and increase the total percentage of collagen type III in wounds of diabetic animals.

Declaration of interest: The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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