RESEARCH ARTICLE

Regulation of matrix metalloproteinase-2 and -9 during healing of dermal wounds after incision using radiofrequency energy in neonatal and adult rats

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Abstract

Background: Radiofrequency energy (RFE) has many medical applications in the treatment of adults and children. The impact of RFE on healing-regulation systems in the developing tissues is not fully known. Matrix metalloproteinases (MMPs) are involved in the remodeling of the extracellular matrix and the inflammatory processes. MMPs are regulated differently among the different age groups. We evaluated possible changes in MMP activity after an incisional wound using a radiofrequency scalpel in neonatal and adult rats.

Methods: In 30 Wistar rats [15 4-day-old (neonates) and 15 4-month-old (adults) rats], a ventral wound was created using a radiofrequency scalpel. Wounded areas and non-wounded tissues were harvested one, three and seven days after the intervention. Enzymatic activities of MMP-2 and MMP-9 were evaluated using gelatin zymography.

Results: Adults expressed higher activity than neonates for MMP-2 on day 7 (Mann –Whitney U-test, p =0.009) and for MMP-9 on days one (p =0.005) and three (p =0.005). MMP-9 was expressed in higher amounts in the wounded tissue in comparison with non-wounded tissue during days one and three (Wilcoxon signed rank test, p =0.028 and p =0.043, respectively). MMP-2 was produced in equal amounts in the wounded and non-wounded tissue at all time-points. Only in the adult wounds at day seven, higher activity was noted compared with non-wounded skin (Wilcoxon signed rank test, p=0.043).

Conclusions: RFE, despite its local burning effect, does not interfere with known patterns of MMP regulation. Neonates have lower activity of MMPs than adults. Energy conduction through adjacent non-wounded tissues does not have an impact upon MMP regulation. HIPPOKRATIA 2017, 21(1): 85-92.

Keywords: Matrix metalloproteinases, radiofrequency, wound healing, neonate, adult, rat

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Introduction

Healing has been studied extensively, but the entire spectrum of its mechanisms is not understood fully[1,2]. Immunologic status may influence the formation of a dermal scar in that immunologic immaturity can promote "scarless" healing (as shown in fetuses of early gestational age)[3]. Between scarless healing of early-stage fetuses and the impaired healing of advanced adulthood, there is a broad spectrum of gradual change in the healing outcome[4,5]. Numerous factors can influence wound healing[6].

In the last few decades, evidence has supported the hypothesis that a family of enzymes named matrix metalloproteinases (MMPs) has a central role in the regulation of wound healing[7-9]. MMP-2 and MMP-9 are involved in the initial inflammatory response and remodeling of the extracellular matrix[10,11]. Furthermore, studies assessing dermal wounds produced by cold knife have shown that the regulation of MMP-2 and MMP-9 differs among different age groups[12-15].

Radiofrequency energy (RFE) has many applications in surgery. It produces a burn-like effect in tissues and induces an inflammatory response[16-19]. RFE has been used in the pediatric population for resection of liver tumors and excision of sacrococcygeal teratomas, with encouraging results[20,21]. Nevertheless, application of RFE in developing heart tissue has been shown to produce an expanding scar that could be prevented by use of corticosteroids[22]. Despite the acceptable macroscopic results of RFE application in the pediatric population, documented reports are rare[23]. Furthermore, studies focusing on the assessment of the biochemical implication of RFE in neonates or children are lacking.

Neonates have a unique response to injury and healing because a minimal inflammatory response is elicited.

The excessive inflammation produced by a radiofrequency scalpel in comparison with the cold knife could influence the immature inflammatory response of the neonate. Also, energy conduction through the adjacent tissues could damage the normal physiology and homeostasis of neonates. Therefore, the pattern of MMP expression could be altered.

The present study aimed to provide initial data on the effect of RFE in the developing tissues and support the use of this method in other applications within the pediatric population. Therefore, we evaluated the influence of the inflammatory response produced by RFE in the regulation of local (within the scared tissue) and regional (in neighboring non-wounded tissues) MMP activity during dermal wound healing in neonatal and adult rats. MMP-2 and MMP-9 were chosen as the best representatives from the MMP family.

Materials and Methods

The study protocol was approved by the Veterinary Organization of Thessaloniki (reference number: 13/12011, 22/11/2010, Thessaloniki, Greece). Following approval, the experiment was conducted during the following calendar year. Animals were provided by Simeonideio research Center of Theageneion Cancer Hospital (Thessaloniki, Greece). All animals were kept in the facilities of the Department of Histology and Embryology of Aristotle University of Thessaloniki (Thessaloniki, Greece). Housing and handling of the animals followed the relevant regulations (Directive 2010/63/EU).

In previous comparable studies, the difference in the MMP-9 activity between scaring and non-scaring tissues has been shown to be 50 %[13]. For this level of difference, a power of 80 % and p =0.05, the estimated sample size was five animals. Therefore, 30 animals (five for each group) were used. Male Wistar rats were used to exclude possible confounders (e.g., estrogen levels) that could influence healing[24]. Fifteen of the 30 rats were 4-month-old (adults), and 15 were 4-day-old (neonates) rats. Adult animals were kept in individual cages. Five mother rats with their litters were kept in separate cages. Each mother provided three male litters for the experiment.

All animals were anesthetized by inhalation of diethyl ether. A ventral surgical paramedian incision using a radiofrequency scalpel (Surgitron Radiolace II; Ellman, Hicksville, NY, USA) was created in the left side of the abdomen. The incision extended from the upper to the lower abdomen covering approximately 90 % of the total abdominal length (approximately five cm in adults and one cm in neonates). We used the minimum settings (level 1 on the device; 50 Watt maximum output) on the device and the same minimum energy level was used in both neonates and adults. During the experiments conducted there was no need to increase the settings at any time to achieve tissue excision or hemostasis. All layers of the abdominal wall were cut, and the peritoneal cavity was opened to reproduce the conditions and surgical stress of a patient undergoing abdominal surgery (Figure 1). Wounds were closed in two layers (muscle tissue and skin). The skin was closed using 6/0 absorbable sutures in the neonatal group and 5/0 absorbable sutures in the adult group (Figure 2). After the intervention, the neonates were returned to their mother following a 30-minute recovery, and adult rats were returned to their cages. Ten animals (five neonates and five adults) were anesthetized and euthanized one day after surgery, another ten three days after surgery and the reminder seven days after the intervention. Neonatal rats of the same experimental days were litters of five different mothers in an attempt to randomize possible confounding factors. The incisional wound along with a non-wounded area of the same animal (on the contralateral side of the abdomen) were harvested. The specimens with dimensions 0.5 x 0.5 cm were collected from the middle of the incision.

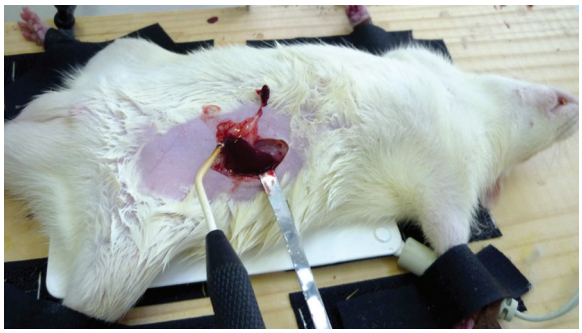

Figure 1: Adult animal on the operating table. The wound on the left abdomen is open and the first organ to be exposed is spleen. Note that the operated field is shaved so that the hair of the animal would not interfere with the proteins measured at later analysis.

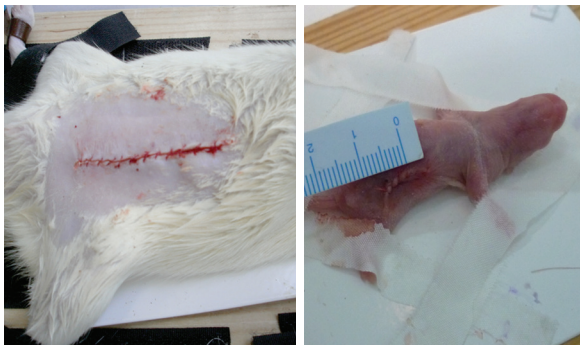

Figure 2: A) Immediate postoperative picture of adult animal with the wound sutured on the left abdominal wall. B) Immediate postoperative picture of neonate rat with the wound sutured on the left abdominal wall. Both animals were sutured with continuous sutures.

All specimens were placed immediately after excision in RNAlater™ solution (Sigma-Aldrich, Saint Louis, MO, USA) and kept at -21°C for later analyses according to the manufacturer's guidelines. Specimens comprised only the dermal layer of skin and not the underlying muscles. The specimen of the non-wounded skin of the contralateral side of the abdomen was harvested and handled in an identical way. For the second part of the experiment we removed the specimen from the storage and handle them immersed

in water at 4°C. All specimens were subsequently mechanically homogenized and, after centrifugation (3,000 rpm/5minutes), the supernatant was collected. Protein concentration was measured in the supernatant using the Bradford assay[25]. The same amount of protein (4 μg) was used to assess the enzymatic activity of MMP-2 and MMP-9 in all samples by gelatin zymography[26].

A gelatin gel with ten slots was prepared manually, and protein (4 μg) accompanied with dye was placed in each slot. The gel was embedded in a buffer solution (15.1 gr TRIS, 72 glycine and 5 gr SDS in water to a total volume of 1L; pH =8), and the applied electric current separated proteins according to their size. Electrophoresis was stopped when the first dye ran out of the gel. The gel was then transferred to an enzymatic buffer 2.5 % (50 mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$, and 2.5 % Triton in 1L) for 10 minutes. Subsequently the gel was immersed in enzymatic buffer 0.1 % (50 mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$, and 0.1 % Triton in 1L) for further 10 minutes. Finally, the gel was transferred in a second enzymatic buffer 0.1 % and transferred to the incubator (Temperature 36°C). After 24 h of incubation, the gelatin gels were ready for staining (0.5 % Coomassie blue, 2.5 % Acetic acid, 25 % Isopropanolol). All activity zones were visible as white zones on a blue background. Colored zymography gels were scanned and enzymatic activity quantified using ImageJ software (accessed at www.imagej.net). White activity zones were converted to pixels of light intensity. Subsequently, an arbitrary number for each activity was produced.

Activity zones in the same gels were compared. Neonatal samples of days one, three, and seven (three samples from each day randomly selected between the five available samples) were compared in the same gel to prove compatibility with previous published data. A similar gel was created for adult samples. Three further gels (one for each study day; first, third, seventh) were created using five samples from neonates and five samples from adults. In these gels material from the wounded/operated region was used. Finally, we examined the differences in MMP expression between the operated and non-operated regions within the same animal. For this reason pairs of samples from wounded and non-wounded areas (pairs from the same rat) were allocated randomly in six gels. Each of these gels comprised paired samples from rats of different ages and stages of healing to adjust for the possible variability of the method.

Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics for Windows (IBM SPSS, IBM Corp., Armonk, NY, USA) version 19.0. Normality was assessed with the Shapiro-Wilk test. Our variables consisted of the detected activity levels of each individual MMP fragment for each day after the intervention. None of our parameters followed a normal distribution ($p < 0.001$ for all variables tested). Non-parametric tests were therefore used because of the non-normal distribution of our data. Mann-Whitney U test was used for comparison between groups (adult-neonatal rats of the same day) and Wilcoxon signed rank test was used for the comparison of the paired samples from wounded and non-wounded areas. Statistical significance was considered at the level of $p < 0.05$.

Results

The calculated power of this study based on the MMP-9 results was 83.1 %. This figure was slightly higher than our initial estimation of 80 % at the calculation of the sample size.

During the experimental study, no wound infection or animal death was encountered. Neonates developed almost scarless wounds (Figure 3). Clot formation was

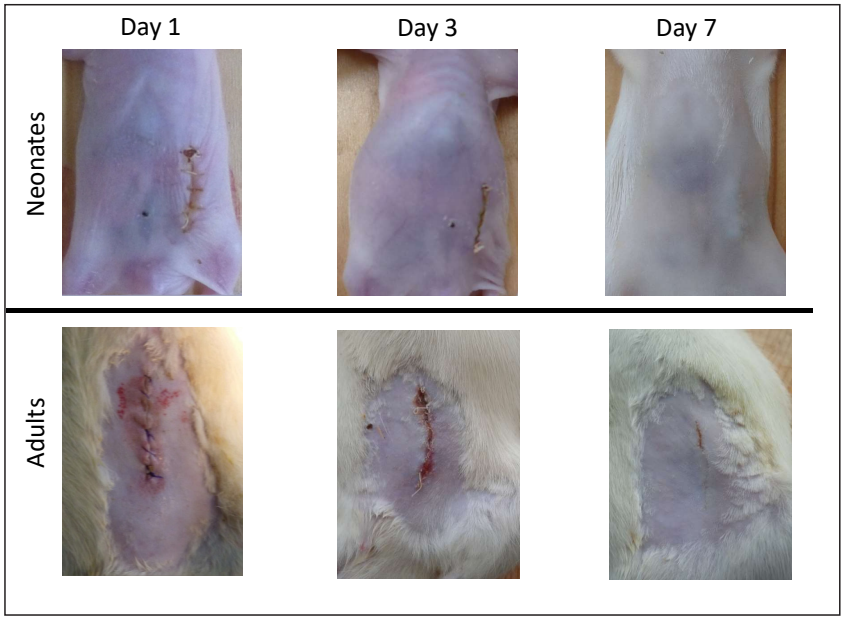

Figure 3: Wounded regions of the neonatal and adult animals in the three distinct time points (Day1, 3 and 7) after surgery.

evident in rats of both groups at day one. At day three, inflammation (observed as reddish zone surrounding the wound) was more evident in adults: such zone was not observed in neonates. At day seven, neonates and adults appeared both to have excellent healing with minimal scarring. The overall macroscopic appearance of the wounds was most favorable for neonates compared with adults for all time points examined.

Analyses of gelatin zymography revealed six distinct zones of enzymatic activity: MMP-9 heterodimer at 220 kDa; MMP-9 homodimer at 135 kDa; proMMP-9 at 92 kDa; MMP-9 at 82 kDa; proMMP-2 at 72 kDa; MMP-2 at 62 kDa (Figure 4). These zones showed different intensities corresponding to variable activity in different animals and at different time points.

In neonates, MMP-9 with predomination of the proMMP-9 fragment was identifiable at days one and three. MMP-9 fragments were not identifiable at day seven in neonates. MMP-2 had baseline activity at all time points (days one, three, and seven). In adults, MMP-9 activity increased immediately after the induced injury and then showed diminished (but traceable) activity at day seven. MMP-2 activity increased gradually from day one until day seven. MMP regulation through days one to seven was similar for neonates and adults (Figure 5 and Figure 6). For this initial analysis due to the limitation of ten available sample slots per gel we used only three randomly selected samples from each representative day. This allowed us to grossly assess the timely regulation of MMPs separately for neonatal and adult rats.

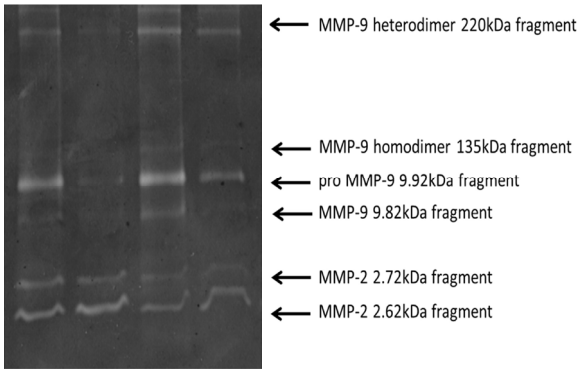

Figure 4: Representative gelatin zymography gel showing all the activity zones that were identified. In this representative gel samples from adult animals were used. The first and third column from the left are samples from the operated areas. The second and fourth columns have the non-operated areas from the same animals.

Neonate and adult samples from wounded areas on the same day (five samples for each) were analyzed in the same gel. Quantitation of the gelatin-lysis bands is presented in Table 1.

At day one, MMP-2 had baseline activity that did not differ between neonates and adults (Figure 7A). MMP-9 (which is activated early) showed higher activity in adults ($p = 0.005$). ProMMP-9 was the predominant fragment in this reaction and was monitored both in neonates and adults ($p = 0.047$). In adults, the other MMP-9 fragments were also monitored (MMP-9 heterodimer and activated MMP-9) that could not be detected in neonates. At day three, overall regulation of enzymes remained almost identical (Figure 7B). MMP-9 activity continued to be high, and adults showed higher activity than neonates with the same pattern of fragmentation ($p = 0.016$ for pro-MMP-9, $p = 0.005$ for MMP-9). MMP-2 activity continued to show baseline activity with no difference between neonates and adults. This pattern changed at day seven (Figure 7C): MMP-9 was no longer detectable and MMP-2 showed higher activity. Also at day seven, MMP-2 activity was higher for adults than for neonates ($p = 0.009$).

Paired samples of wounded and non-wounded areas harvested from each rat were analyzed in the same gel using zymography and the MMP activity is summarized in Table 2. Some fragments showed gelatinolytic activity only locally in the wounded areas (Figure 8 and Figure 9). Some fragments exhibited identical activity in both wounded and non-wounded tissue.

During day one, in neonates, proMMP-9 (but not MMP-9) could be detected, and this was only in the wounded areas. In adults, MMP-9 heterodimer,

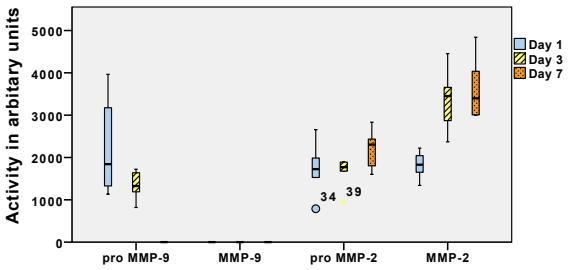

Figure 5: Graphs representing the timely enzymatic activity of different MMPs on 1st (D1), 3rd (D3) and 7th (D7) day postoperatively. Samples derived from operated regions on neonatal animals (three samples for each day were randomly selected to be incorporated in the analysis). Arbitrary units reflect the intensity of the gelatinolytic zone in the gel.

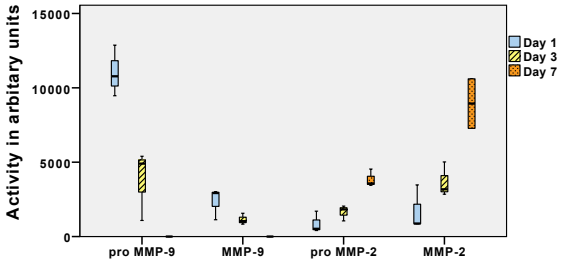

Figure 6: Graphs representing the timely enzymatic activity of different MMPs on 1st (D1), 3rd (D3) and 7th (D7) day postoperatively. Samples derived from operated regions on adult animals (three samples for each day were randomly selected to be incorporated in the analysis). Arbitrary units reflect the intensity of the gelatinolytic zone in the gel.

Table 1: Comparison was made between the neonatal and adult wounded regions of the five animals regarding their enzymatic activity in gelatin zymography (-: no detectable activity, +: detectable activity, ++: higher detectable activity/note that the symbols are indicative for the comparisons between neonates and adults). Statistical analysis was made between the activity levels as they were quantified with the use of ImajeJ program. Each day represents the analysis of one zymography gel.

		neonates	Adults	Mann Whitney U test
Day1	MMP-9 heterodimer	-	+	**p =0.005**
	MMP-9 homodimer	-	-	p =1
	proMMP-9	+	++	**p =0.047**
	MMP-9	-	+	**p =0.005**
	proMMP-2	+	+	p =0.465
	MMP-2	+	+	p =0.117
Day3	MMP-9 heterodimer	-	+	**p =0.005**
	MMP-9 homodimer	-	-	p =1
	proMMP-9	+	++	**p =0.016**
	MMP-9	-	+	**p =0.005**
	proMMP-2	+	+	p =0.602
	MMP-2	+	+	p =0.347
Day7	MMP-9 heterodimer	-	-	p =1
	MMP-9 homodimer	-	-	p =1
	proMMP-9	-	-	p =1
	MMP-9	-	-	p =1
	proMMP-2	+	++	**p =0.009**
	MMP-2	+	++	**p =0.009**

MMP: matrix metalloproteinases.

Table 2: Comparison was made between wounded and not wounded regions of neonate (five animals) and adult rats (five animals) separately regarding their enzymatic activity in gelatin zymography (-: no detectable activity, +: detectable activity, ++: higher detectable activity/note that the symbols are indicative for the comparisons between wounded and non-wounded areas). Statistical analysis was made between the activity levels as they were quantified with the use of ImajeJ program.

		Neonate			adult		
		operated region	not operated region	Wilcoxon Signed Rank test	operated region	not operated region	Wilcoxon Signed Rank test
Day 1	MMP-9 heterodimer	-	-	-	++	+	**p =0.043**
	MMP-9 homodimer	-	-	-	-	-	-
	proMMP-9	+	-	**p =0.028**	++	+	**p =0.043**
	MMP-9	-	-	-	++	+	**p =0.043**
	proMMP-2	+	+	p =0.249	+	+	p =0.686
	MMP-2	+	+	p =0.463	+	+	p =0.500
Day 3	MMP-9 heterodimer	-	-	-	+	-	**p =0.043**
	MMP-9 homodimer	-	-	-	-	-	-
	proMMP-9	+	-	**p =0.043**	+	-	**p =0.043**
	MMP-9	-	-	-	+	-	**p =0.043**
	proMMP-2	+	+	p =0.225	+	+	p =0.345
	MMP-2	+	+	p =0.225	+	+	p =0.686
Day 7	MMP-9 heterodimer	-	-	-	-	-	-
	MMP-9 homodimer	-	-	-	-	-	-
	proMMP-9	-	-	-	-	-	-
	MMP-9	-	-	-	-	-	-
	proMMP-2	+	+	p =0.345	++	+	**p =0.043**
	MMP-2	+	+	p =0.225	++	+	**p =0.043**

MMP: matrix metalloproteinases.

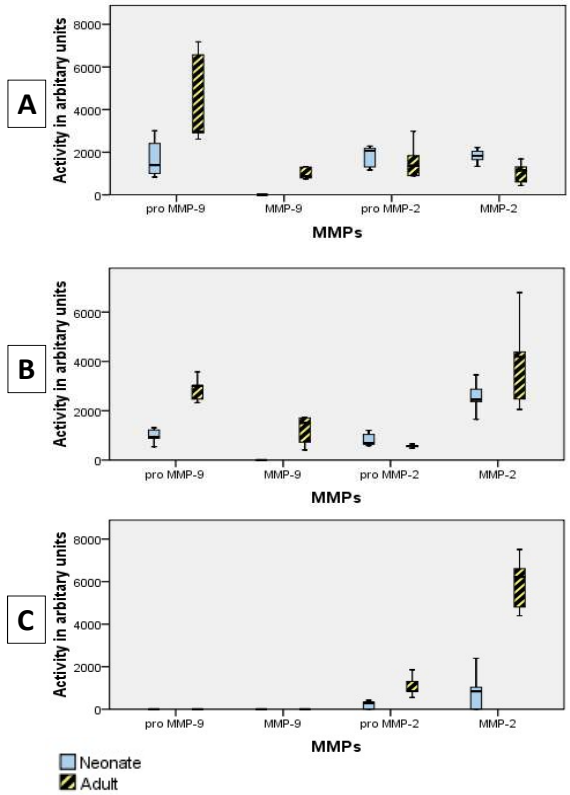

Figure 7: Graphs of comparative MMP enzymatic activity on operated regions from neonatal and adult rat samples animals (five neonatal and five adult samples compared). A: Day 1, B: Day 3, C: Day 7. Arbitrary units reflect the intensity of the gelatinolytic zone in the gel.

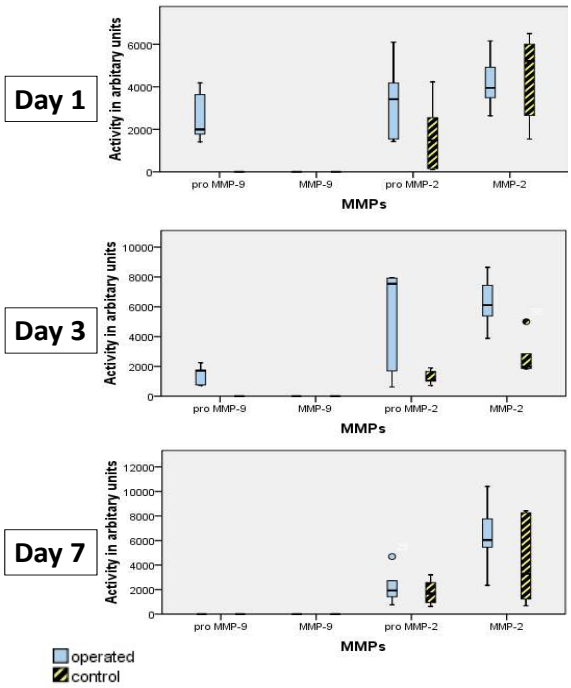

Figure 8: Comparative graphs of MMP enzymatic activity on operated and not operated regions from neonatal animals (five samples from operated areas with matched non-operated areas were compared). Arbitrary units reflect the intensity of the gelatinolytic zone in the gel.

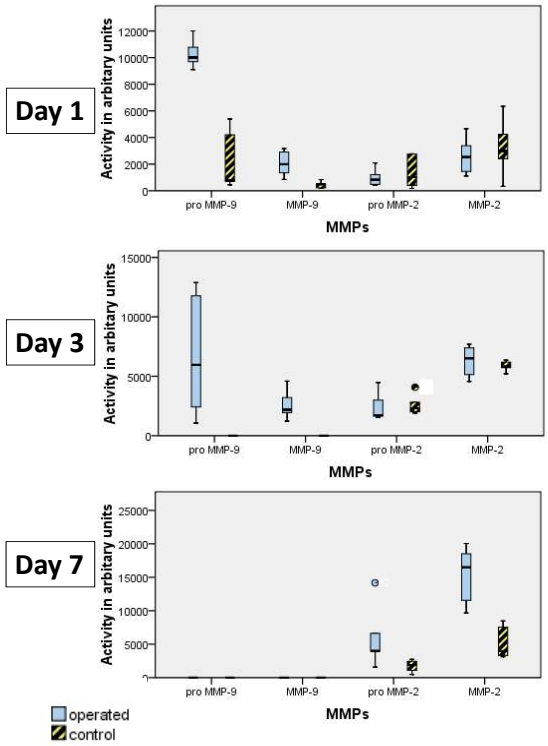

Figure 9: Comparative graphs of MMP enzymatic activity on operated and not operated regions from adult animals (five samples from operated areas with matched non-operated areas were compared). Arbitrary units reflect the intensity of the gelatinolytic zone in the gel.

proMMP-9, and activated MMP-9 were detected in the wounded and non-wounded areas, with the former showing higher activity (p =0.028). MMP-2 had identical baseline activity in the wounded and non-wounded areas, with no difference between neonates and adults. An identical pattern of regulation was observed at day three for all enzymes. At day seven, no MMP-9 fragments were detectable in the wounded and non-wounded areas. MMP-2 was detected in both the wounded and non-wounded areas in neonates and adults. Interestingly, neonates exhibited identical activities of MMP-2 fragments in wounded and intact tissue (p =0.345 for pro-MMP-2 and p =0.225 for MMP-2). Adult rats had higher activity of proMMP2 and MMP2 in the wounded areas than in intact tissue (p =0.043 for pro-MMP-2 and p =0.043 for MMP-2).

Discussion

An excessive inflammatory response impairs wound healing[27]. RFE triggers an inflammatory response in the wound because of its burn-like effect[16-19]. The present study showed that, even though RFE promotes an inflammatory reaction, the effect of this initial damage does not alter macroscopically the expected final outcome in neonates or adults. The esthetic result observed seemed to be comparable to that obtained by cold knife as previously

reported[28]. Furthermore, the previously documented better healing response of the neonates was observed to be preserved despite the induced tissue thermal damage by RFE.

Heating of tissues over a certain limit results in denaturation of the local proteins[16]. The three-dimensional structure of enzymes is of major importance due to the fact that their enzymatic activity is based on it. In the present study, scattered heating by RFE used to induce incisional wound did not alter the overall patterns of the MMP regulation of dermal wound healing both in neonates and adult rats. Gradual upregulation of MMP-2 expression and immediate activation of MMP-9 shown previously[13,14,29] were reproduced in our results both for neonates and adult rats. Therefore, the alteration in protein structure as a consequence of the heating effect from RFE application did not affect the local regulation of proteins such as MMPs in the wounded tissue.

MMP-2 has baseline activity that increases only after day seven[14,15,30]. This finding is consistent for tissue healing in adults and neonates, and was also reproduced in the present study. Furthermore, MMP-2 is related with the remodeling phase and formation of new vessels occurring at the later stages of healing[11,29]. Adult rats had more evident scars than neonates, and adult rats seemed to have higher activity of MMP-2 on day seven in comparison with neonates. MMP-2 is involved in the remodeling phase, so a relationship between the higher activity of MMP-2 in adults and inferior healing is possible.

The other important mediator in healing is MMP-9, and it had higher activity during the first 24 h. MMP-9 is stored in neutrophils and can be released rapidly[31]. Therefore, MMP-9 reflects the initial inflammatory response in tissue damage[7,11]. Higher activity of MMP-9 is related with the reduced proliferation and activity of fibroblasts, which results in the impaired healing seen in diabetic ulcers and keloids[32,33]. In our study, MMP-9 activity on day one was higher for adults than for neonates. The immaturity of the inflammatory response in neonates could be an explanation for this observed lower activity. As a consequence, lower activity of MMP-9 could be beneficial for the healing process in neonates.

Upregulated expression of MMPs in wounded tissue in comparison with unwounded tissue in the same animal suggests a more localized reaction. Conversely, the equal activity of MMPs at certain time points suggests a more integrated response to induced injury. MMP-2 had baseline expression in the normal (not wounded) tissues and its expression was upregulated in the later stages of the healing process. In the adult wounds, baseline expression of MMP-2 in the non-wounded tissue is lower than expression in the wounded tissue[14]. In the present study, only at day seven was MMP-2 activity higher in the wounded area than in the non-wounded skin in adults. Neonates on day seven had similar activity of MMP-2 in the wounded and non-wounded regions. MMP-2 expression in scarless healing has modest upregulation in the first few days and then returns to baseline activity by day seven[34]. Also, MMP-2 is believed to have a maintenance role in the normal skin[14]. Regarding MMP-2 regulation, neonates seem to respond to tissue damage in a holistic manner. In the growing tissues of neonates, remodeling enzymes may already be activated, and the only change is the overall response of the organism. This hypothesis is supported by the fact that MMP-2 was not activated locally in neonates (though MMP-2 activity increased at day seven).

MMP-9 is activated at the initial stages of the wound healing and shows a local response in both neonates and adults. It was more active in the wounded regions at days one and three, with the predominant fragment being proMMP-9. At day seven, MMP-9 was no longer detectable in the wounded or non-wounded areas. Neonates constantly expressed lower levels of MMP-9 than adults, and this phenomenon could be attributed to the immature inflammatory response of neonates. MMP-9 is part of the inflammatory response, so it is expected that neonates will have lower activities of these enzymes[31]. This is also supported by studies showing that wound healing without scar formation is associated with lower activity of MMP-9 in comparison with healing in which a scar is formed[34].

Our study is limited by the fact that MMP regulation was evaluated only by their enzymatic activity according to gelatin zymography. At the cellular level, MMPs are regulated through transcription (measured by RNA production) and inhibition by tissue inhibitors of metalloproteinases (TIMPs). Furthermore, gelatin zymography cannot be utilized to reveal small differences or low levels of activity Hence, in the instances that we were unable to detect activity in one enzyme fragment, it could have been due to the method used that could not detect a low level of activity. The purpose of our study was not to measure the total activity of MMP-2 and MMP-9 in the healing process; other studies have provided evidence in this area[10,29,35]. We aimed solely to detect alterations in already known data that could be induced by the use of RFE and the subsequent thermal tissue damage.

MMPs are a large family of enzymes with many and sophisticated regulation patterns. To better understand MMP regulation under different conditions, studies should focus on their total regulation by observing gene expression and TIMP regulation along with the activity of other MMPs and signaling systems.

Handling of neonatal tissues requires special attention to preserve their unique physiology and growth potential. Application of any type of energy for therapeutic reasons must take into account this peculiarity. Skin is the tissue studied most extensively regarding neonatal healing. Our study is the initial step in the molecular evaluation of RFE effects in the growing tissues. It was shown that RFE did not disturb the physiology of healing within the wounded tissue or in the adjacent normal tissues in neonates. Further studies are needed to assess its properties in the other tissues before consideration of novel applications in young children.

Conflict of interest

The authors have no conflict of interest to declare.

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