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Histological changes in connective tissue of rat tails after bipolar radiofrequency treatment

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Summary. Radiofrequency (RF) has been included in the techniques used in aesthetic surgery/medicine. To date, no studies have performed a histological assessment of changes in the tissue after application of bipolar radiofrequency (BRF) with low energy and frequency. The aim of this study was to examine changes that are produced in connective tissue, principally in the fibroblasts, following BRF treatment. Four groups of rats received a different number of RF sessions (1, 2, 3 and 5). The following parameters were determined: the number of fibroblasts/unit area (FA), the proliferation index (PI), the Heat shock Protein 47 index (HSPI) and the percentage of connective tissue (PC). For statistical analysis, two subgroups (A and B) were made for the variables FA, PI and PC, and another two subgroups (C and D) for the variable HSPI. Significant differences for FA, PI and PC were observed between subgroups A and B, FA and PI having higher values in A, while PC had higher values in B. The HSPI in subgroup C showed significantly higher values than in D. Low energy and frequency BRF led to an increase in the number, proliferation and biosynthetic activity of fibroblasts. The resulting stress suffered by fibroblasts as a result of heat may be associated with the phenomenon of hormesis.

Key words: Fibroblast, Radiofrequency, Hormesis, HSP47, Proliferation

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Introduction

Recent years have seen the development of non-ablative techniques in aesthetic medicine, among them facial rejuvenation using non-ablative laser resurfacing or non-ablative radiofrequency (Christine and Anderson, 2005; Atiyeh and Dibo, 2009). However, the cellular and molecular mechanisms through which these techniques act on the connective tissue of the dermis are still not well understood (Atiyeh and Dibo, 2009). The increased heat affects the tissue and induces a response that involves a first phase of collagen denaturalisation and hyalinisation with a subsequent phase of tissue scarring and remodelling of the treated connective tissue (Hecht et al., 1998, 1999). In the field of aesthetic medicine using monopolar RF and applying high energy, a similar effect to the above has been seen, with an increasing percentage of collagen fibres being modified as the applied energy increases (Zelickson et al., 2004). BRF using lower energy has recently been introduced for the same treatment, which makes it more straightforward since application is topical and anaesthesia is unnecessary. In theory, the effect on the connective tissue of the dermis is similar to that of the above treatments. Two histological studies have recently been published on the application of this type of RF, the first one using an invasive system of fractional BRF that led to vigorous wound healing after an inflammation with subsequent neocollagenesis (Hantash et al., 2009), and the second, which used less aggressive conditions of 80 J/cm^2, which only produced oedema and vascular congestion, followed by collagenesis (Alvarez et al., 2008). According to these findings, it seems that the way in which low energy BRF acts on the dermis is less

aggressive and involves a lower rate of collagen fibre destruction. The use of low energy BRF, then, may be considered a non-aggressive technique as far as the dermis is concerned. Given that very few studies exist on the changes that take place in the connective tissue of the dermis as a result of heat produced by the action of this type of BRF, and none of a quantitative nature, we used a BRF system of 4 MHz and 90 W, with a energy of 95 J/cm^2 in order to analyse its effect on the connective tissue, especially on fibroblasts. The intention was: a) to describe histological changes in the connective tissue of the dermis after exposure to low energy BRF; b) to determine any increase in the same after treatment; and c) to ascertain alterations in the fibroblasts as regards number, proliferation index and collagen synthesis activity. As an experimental model of dermal connective tissue we used the tail of Sprague-Dawley rats.

Materials and methods

Animals and RF application

All sixteen female Sprague-Dawley rats used in this experiment were housed in the same conditions and provided with food and water "*ad libitum*". They were divided into four groups of equal size: T_1, T_2, T_3, and T_5. Two animals per group were treated with RF (1, 2, 3 or 5 sessions, respectively, with a week between sessions) and the other two animals in each group acted as control. The tail was chosen for treatment because this area is rich in connective tissue and has short hair, with a high content of collagen fibres and fibroblasts. The RF treatment consisted of continuous sweeps with a device of 0.95 cm^2 (4 MHz, 90 W and an energy of 95 J/cm^2). Each session lasted 30 minutes. The device has a small area of application and in human skin rejuvenation it is used for small wrinkles. The skin temperature was measured several times during treatment with a laser thermometer, which showed that the skin temperature increased by between 3 to 4 degrees over normal skin temperature.

Sacrifice and processing of samples

The animals were sacrificed (in a bell jar using carbon dioxide pellets) seven days after the last treatment, except in the case of the group receiving 5 sessions, when the rats were sacrificed two months after the last session. This study was performed according to Spanish ethical and legal standards regarding animal protection: RD 1201/2005, of 10 October, on the protection of animals used for experimental and other scientific purposes (BOE 21 October 2005), and the European convention on protection of animals (BOE 25 October 1990, pp 31348-31362).

The treated portion of the tails were excised and placed in 10% formol for 8 hours. Fragments were sectioned and representative samples of the treated zones, along with control samples, were selected. These were dehydrated, passed through toluene and immersed in paraplast® overnight in an automatic processor. Sections (5 μm) were obtained in a microtome. After stretching in warm water, the sections were mounted on polylysinated slides. For the histological study, the samples were conventionally stained with haematoxylin-eosin.

PCNA and HSP-47 labelling

Proliferative activity was studied by immunohistochemical labelling of proliferating cell nuclear antigen (PCNA). PCNA is a protein of 35KDa that forms part of polymerase δ, and which participates in the regulation of the cell cycle (Casasco et al., 1993). All the sections used were deparaffinized, rehydrated, washed in phosphate buffered saline (PBS) (3x5 min) and the peroxidase activity was quenched using 0.3% H_2O_2 for 30 min. Subsequently, the samples were washed in PBS and incubated overnight at 4°C with the monoclonal mouse antibody anti-human PCNA (Biomeda, FuterCity, CA; USA) diluted 1:200 in PBS buffer. The PBS buffer contained BSA (bovine serum albumin) diluted 1/100. After washing in PBS, the samples were incubated for 45 min at 18-24°C with biotinylated rabbit anti-mouse (Dako, Golstrup, Denmark). This was diluted in PBS buffer at 1:200. The samples were washed in PBS (3x5 min) and incubated with streptavidin horseradish peroxidase (Dako, Golstrup, Denmark) for 30 min at 18-24°C. The streptavidin peroxidase conjugate was diluted in PBS buffer at 1:300. Finally, the samples were washed in PBS. The antibody-peroxidase complex was developed using TBS containing 0.05 % diaminobenzidine (DAB) (Sigma) and 0.3% H_20_2, for 3 min at 18-24°C. Finally, the sections were washed in water, counterstained with haematoxylin for 1 minute, dehydrated through ascending grades of alcohol and mounted in dibutyl polystyrene xylene (DPX) (Merck). The specificity of the immunohistochemical procedures was confirmed by incubation of sections with non-immune serum instead of the primary antibody. The immunohistochemical protocol for detecting the HSP-47 protein was the same as for PCNA, except for the first antibody stage of incubation, which used a rabbit polyclonal against protein HSP-47 (Santa Cruz, USA) diluted in PBS buffer 1:150. The second antibody was a biotinylated Goat anti- Rabbit (Chemicon International) diluted 1:500 in PBS buffer.

Semi-quantitative analysis

To ascertain the possible effect of BRF on the number of fibroblasts, ten random sections stained with H&E from the treated zones (and corresponding controls) were used, counting 25 areas per section with a magnification of x400. The number of fibroblasts/area (FA) were counted, each area measuring 0.0273 mm^2. To

determine any increase in fibroblast proliferative activity, the number of fibroblasts positive to PCNA was compared with the total number of fibroblasts (proliferation index: PI). To evaluate any effect of BRF on fibroblast biosynthetic activity, the increase in HSP-47 protein was determined by calculating the number of fibroblasts positive to this protein compared with the total number (HSP-47 expression index: HSPI). In both cases, ten random sections of treated area and their corresponding controls were used. The indices described above were calculated in 25 areas of 0.0273 mm^2 with a magnification of x400. A morphometric study determined whether BRF used involved a long term increase in connective tissue. Five randomly chosen sections stained with H&E were used, along with their controls. For the study and for taking images of the sections, a Zeiss microscope capable of digitalising the results was used. Using the image analysis program the following was determined in all cases: a) the total area of tissue in the section and b) the area within that corresponded only to connective tissue. Later, the area of connective tissue with respect to the whole section area was calculated and expressed as a percentage of connective tissue (PC) with respect to the total tissue area.

Statistical analysis

Two subgroups were formed: subgroup A, comprising the groups exposed to 1, 2 and 3 sessions (treated subgroup: T_1, T_2, T_3) and subgroup B (post-treated subgroup: T_5), which was formed of the group exposed to 5 sessions. This enabled us to compare the effects in animals soon after treatment and the effects observed in animals sacrificed after two months. It was thought that in this way it would be possible to observe the theoretical tissue response consisting of: a) increase in the number of fibroblasts and maintenance in A, and b) a long term increase in connective tissue in subgroup B. The statistical analyses carried out (ANOVA, DMS and Bonferroni) identified significant differences between both groups. For the statistical study of HSPI the initial groups were restructured by including the animals receiving 1 and 5 sessions in subgroup C (groups: T_1 and T_5) and the animals receiving 2 and 3 sessions in subgroup D (groups: T_2, T_3). This was done in order to observe whether significant differences existed between the animals which theoretically would synthesise less collagen (groups T_1 and T_5), and those in which greater collagen synthesis might be expected (groups T_2 and T_3).

Results

Light microscope

No histopathological lesions or changes were observed in any of the treated animals. The sections of the treated animals of subgroup A showed a higher density of fibroblasts than their corresponding controls (Fig. 1A-D), while this change was not observed in subgroup B (Fig. 1E,F). In subgroup A, more fibroblasts were observed in the course of proliferation compared with their controls (Fig. 2A,B). The dermis of tails of animals from subgroup B showed an apparently greater density of connective tissue than their corresponding controls (Fig. 1E,F). As regards the expression of HSP-47, the tail sections of subgroup C showed few positive cells, while the equivalent sections from subgroup D showed many positive cells (Fig. 2C,D).

Semi-quantitative analysis

Table 1 shows the mean values obtained for the different groups studied. FA was significantly higher in subgroup A (20.9±0.739 and 14.54±0.73 in treated and control animals, respectively) than in subgroup B (12.16±0.10 and 11.51±0.09, treated with respect to control animals), ($p<0.005$). The PI was also significantly higher in subgroup A (19.90±1.11 and to 8.50±0.90) than in subgroup B (7.16±0.025 and 4.93±1.99) ($p<0.05$). In contrast, PC was significantly higher in subgroup B (56.60±2.06 and 50.92±0.17) than in A (51.94±1.30 and 53.08±0.91) ($p<0.05$). As regards the HSPI, subgroup C showed significantly more positive cells (2.82±0.79 and 0.51±0.067) than

Table 1. Percentage values of the variables studied in animals receiving bipolar RF treatment.

	FA	FAC	PI	PIC	HSPI	HSPIC	PC	PCC
1T	23.22±0.40	15.98±0.55	19.04±2.26	10.4±1.60	0.82±0.18	0.87±0.42	53.67±3.21	54.18
2T	19.93±0.54	15.08±1.23	21.4±2.00	9±0.50	2.94±1.88	0.42±0.08	52.62±2.43	51.54±1.7
3T	19.72±0.16	12.58±0.29	19.28±2.46	6.1±0.06	2.7±0.46	0.61±0.04	49.53±0.45	53.33±1.5
5T	12.16±0.10	11.51±0.09	7.16±0.02	4.93±1.99	1.3±0.89	0.3±0.16	56.6±2.17	50.92±0.1

FA: the number of fibroblasts/area; PI: proliferation index; HSPI: HSP-47 expression index; PC: percentage of connective tissue. Controls: FAc, PIc, HSPIc, PCc. T: number of treatments.

subgroup D (1.06±0.39 and 0.58±0.24 in controls) ($p<0.05$).

Discussion

Some results obtained using the RF apparatus have pointed to a series of cellular and histological alterations in the connective tissue but have provided no quantitative data. Our study, on the other hand, is based on a histological analysis that is both qualitative and quantitative. After the application of BRF, an increase in the number of fibroblasts per area and in the proliferation index was observed between subgroups A and B. In addition, the number of fibroblasts positive to HSP-47 was greater in subgroup C than in D. Lastly, the percentage of the area of connective tissue was greater in post-treated subgroup B than in treated A.

The use of RF on skin has become more common in

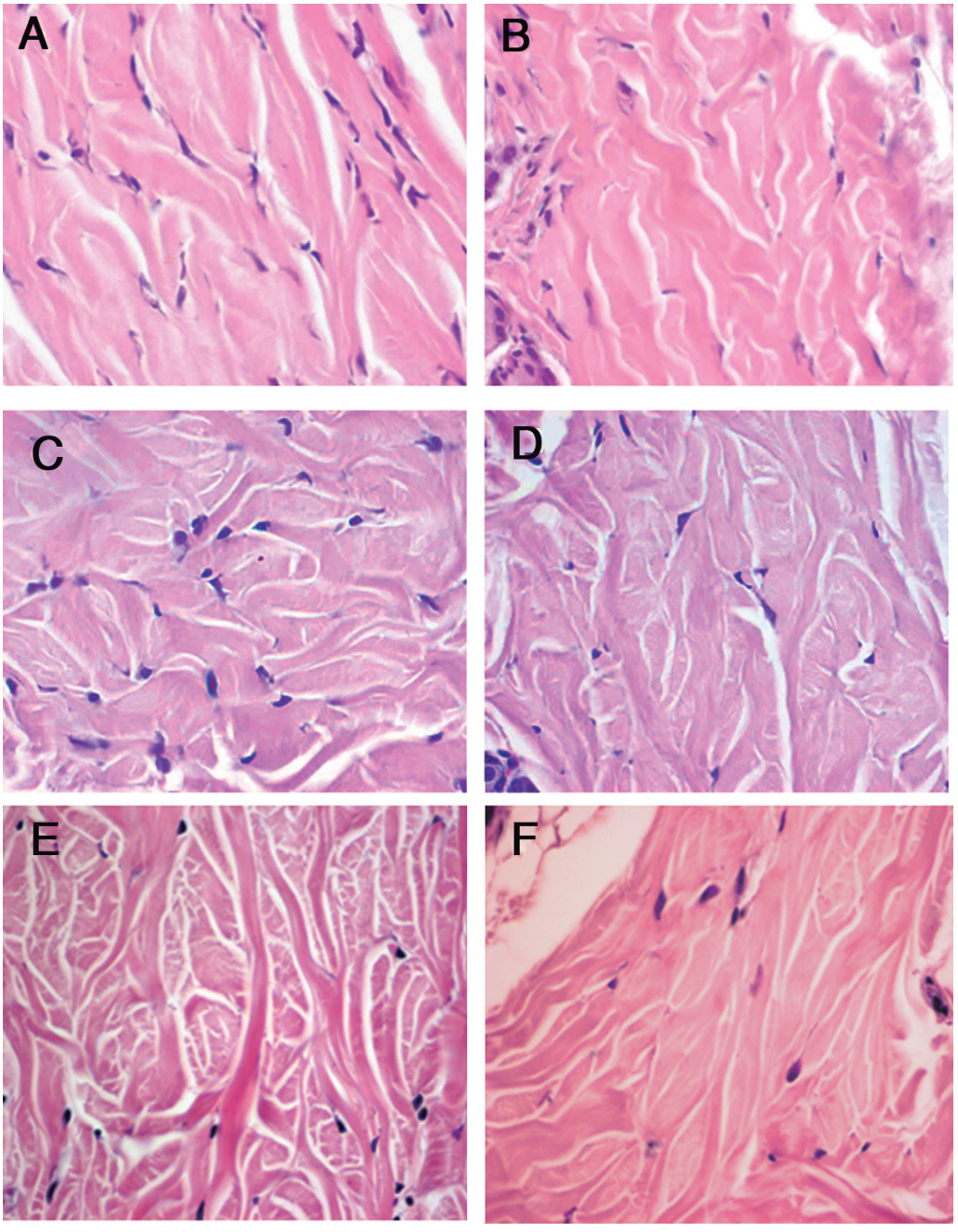

Fig. 1. A. Dermis of the tail of an animal from radiofrequency subgroup A (T_1;one treatment). Note the numerous fibroblasts. **B.** Compared with the previous section, this section of the dermis of a control animal contains fewer fibroblasts per area. **C.** Dermis of the tail of an animal from radiofrequency subgroup A (T_2; two treatments with a week between sessions). Abundant fibroblasts are observed. **D.** Compared with the previous section, this section of the dermis of a control animal contains fewer fibroblasts per area. **E.** Dermis of an animal from subgroup B (T_5; 5 sessions and rats sacrificed two months after the last session). There are fewer fibroblasts than in the treated sections from subgroup A. **F.** Control of subgroup B. The number of fibroblasts per area is similar but the connective tissue seems to have fewer bundles of collagen fibres. H&E. x 400.

recent years, especially for anti-aging treatments. While clinical evidence on whether such treatment is beneficial has been widely discussed (Atiyeh and Dibo, 2009), the histological mechanism by which it acts is little known. The first histological studies were carried out in the connective tissue of tendons and joint capsules, in which a series of cellular events was described after RF: early, medium term and late. The first is associated with clear signs of inflammation, infiltration and cell death. After a few days, the treated zone shows hyalinisation, the destruction of collagen fibres and, from a physical point of view, tendon retraction. Later, the zone is repopulated with an increased number of fibroblasts (fibroplasias), accompanied by fresh collagen synthesis. Subsequently, the newly generated zone shows multiple new collagen bundles. The RF action mechanism, according to these studies, would involve the production of a wound followed by a healing (Hecht et al., 1998, 1999).

Few works describe the morphological alterations that take place in skin after RF treatment. Using a monopolar system and one application of 104, 133 or 181 J (Thermacool TC system, Thermage, Inc. Hayward, Calif), few alterations of any significance were described and only slight perivascular or periadnexal inflammation. Three and eight weeks after treatment no changes could be observed by light microscopy. Ultrastructurally, some collagen fibres appeared to be damaged immediately after RF application, but the fibres appeared to recover during the following six weeks (Zelickson et al., 2004). Furthermore, Northern blot analysis demonstrated an increase in collagen type I messenger RNA two and seven days post-treatment. When a bipolar system of lower energy was used on guinea pig skin (80 J per cm^2) (Thermafine Lift device, Grupo Solilaser, S.L: Girona, Spain) for one weekly six-minute session over a period of six consecutive weeks, alterations in the papillary dermis involving vascular congestion and oedema were detected in weeks 1-4. This continued into weeks 5 and 6, when it was accompanied by an increase in intercellular substance and in the population of resident cells. From week 4 onwards, biopsies showed an increased level of weak acid mucopolysaccharide acids and collagen and elastic fibres compared with control levels. Two months after the last

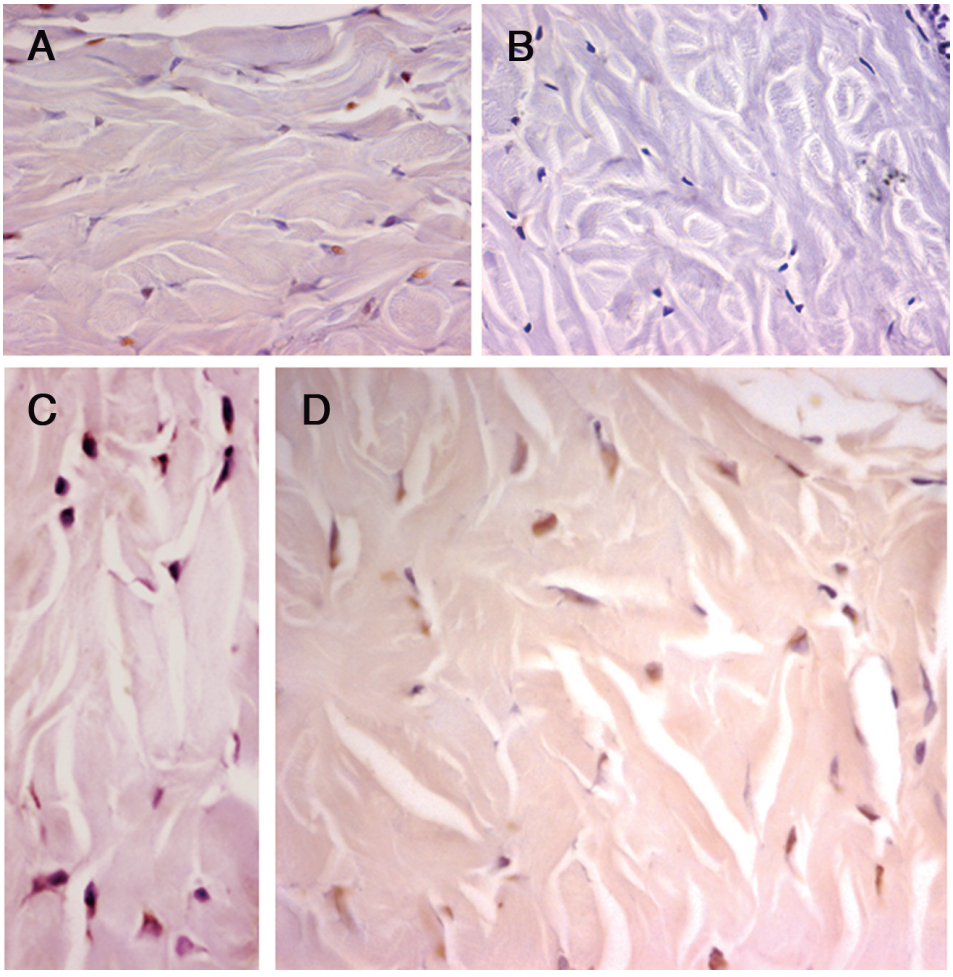

Fig. 2. A. PCNA positive cells in the dermis of an animal from subgroup A. **B.** Dermis of control animal from subgroup A: note the very few PCNA-positive cells. **C and D.** Numerous HSP47 positive cells in dermis of animals treated from subgroup D. x 400

treatment no congestion or oedema was observed, while the increase in cell numbers and both types of fibre persisted. The study concluded that no microscopic alterations of any importance were produced, while the papillary dermis increased due to the increase in connective tissue (Alvarez et al., 2008).

Our study found no histopathological alterations similar to those mentioned after the successive applications of RF. We did, however, observe quantifiable changes in the fibroblasts: an increase in the number of fibroblasts/area and in the number of proliferating fibroblasts in subgroup A compared with subgroup B. This suggests that the fibroblasts are affected in animals that have undergone successive BRF treatments – the number of proliferating fibroblasts increasing, which logically, leads to increased cell density in the connective tissue. The suspension of BRF treatment results in a diminution of fibroblast proliferative activity and, as a consequence, a decrease in cell density in subgroup B. This demonstrates that BRF has a direct effect on the proliferative activity of fibroblasts since, when treatment is suspended, the activity disappears. As mentioned, treatment with BRF, with no prior inflammatory wound being evident, was capable of increasing the number of fibroblasts, which subsequently divided. We are of the opinion that this reflects an adaptive factor on the part of the skin, rather than the healing of a wound. In contrast to observations made in other models in which RF has been applied to connective tissue, the tissue response in our study did not involve a process of inflammation with subsequent fibroplasias and wound healing; rather, we observed a mild gradual stimulatory response of the tissue. Indeed, what occurs is probably an adaptive cell response of hormesis rather than a curative response, which would imply a previous wound and subsequent healing. Such processes of hormesis have been described in several recent studies of cell aging. The effect of stress on organisms seems to be dose-dependent, i.e., mild stress induces stress tolerance and extends the lifespan, whereas excessive stress accentuates the aging process. This paradox is known as hormesis in aging research (Salminena and Kaarniranta, 2010). Hormesis involves the adaptive responses of cells and organisms to mild and moderate stress including heat (thermal hormesis), irradiation, hypoxia, oxidative stress and caloric restriction. Mild-stress increases chaperone proteins, the replicative life span and certain proteins in the endoplasmic reticulum (Rattan, 2008; Salminena and Kaarniranta, 2010). The concept of heat stress, especially in repeated mild heat treatments, increases the lifespan of fibroblasts during culture. These fibroblasts showed a variety of cellular and biochemical hormetic anti-aging effects upon repeated exposure to mild heat stress at 41°C (Rattan et al., 2007). Finally, the same RF used at low frequency (without producing heat) increased fibroblast proliferation and activated the HSP in an effect that was also related with hormesis (Perez et al., 2008).

The increase in connective tissue observed in our study could be related with an increase in neocollagenesis after the proliferation and subsequent differentiation (involving collagen synthesis) of fibroblasts. Such an increase in collagen has been described as one of the effects of slight or severe inflammation following the application of non-ablative laser or different types of RF, and is considered part of dermis healing. That is to say, dermal wound-healing would produce fibroblast activation and new collagen deposition (Atiyeh and Dibo, 2009). According to these authors, the mechanism would have two effects, the first and main effect being the retraction of collagen, which would stretch the skin, and a later secondary effect involving collagen deposition and dermis remodelling, accompanied by an increase in dermis thickness. Some authors have speculated on the possibility that heat might stimulate the fibroblasts and that the mechanism might not be wounding *per se*, but a healing mechanism and repair cascade by heat (Ruiz-Esparza, 2006). In this sense, it has recently been demonstrated that pulsed heat shocks enhance procollagen type I and procollagen type III expression in human dermal fibroblasts *in vitro* (Dams et al., 2010). In short, the application of BRF to skin in our case involved, as in other cases of RF treatment, an increase in neocollagenesis in the dermis, although probably not as a consequence of prior inflammation but as a direct effect of heat on the fibroblasts. The above might help throw light on the results obtained with respect to HSP-47, a good marker of fibroblast activation during collagen synthesis, wound healing and fibroblast aging (Miyaishi et al., 1995; Kuroda and Tajima, 2004). It has also been shown that its expression increases during the ten weeks following the application of bipolar fractional radiofrequency (BFR), during which time a wound healing response is produced (Hantash et al., 2009), as also occurs after pulsed heat shocks in human fibroblasts (Dams et al., 2010). Our results, then, confirm that our non-fractional bipolar radiofrequency also stimulates fibroblasts to express this protein, which is closely linked with the synthesis of collagen, the number of HSP47 cells increasing, as occurs with BFR, but without evident signs of inflammation (Hantash et al., 2009). The expression of HSP47 in our study follows a similar pattern to that observed in the BFR experiment (Hantash et al., 2009). A first phase of low protein expression would be followed by an increase in the same (in our case between the third and fourth week) and finally a reduction after two months, when the formation of new connective tissue has finished. In our opinion, this pattern would be related with the stages of initiation, synthesis and deposition of collagen in the extracellular matrix, the role of HSP47 being particularly important in the second stage.

We conclude therefore that the application of BRF (4 MHz, 90W and an energy of 95 J/cm^2) in our experiment had a similar effect on dermal connective tissue as other treatments involving higher energies. However, no lesion

of the connective tissue was observed, while there was an increase in fibroblast proliferation and differentiation, accompanied by collagen synthesis and the subsequent increase in connective tissue. The application of RF in our conditions, therefore, is less aggressive than other RF treatments but has similar long-term histological effects in the dermal connective tissue. The mechanism through which this is achieved could well be considered to be of a hormetic nature. However, further studies are necessary to determine whether successive treatments foment the stimulatory role of BRF, whether dermis age affects the response to BRF and whether BRF is indeed effective in treating skin aging compared with other treatments, especially non-ablative treatments based on RF.

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