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ORIGINAL ARTICLE

Radiofrequency treatment induces fibroblast growth factor 2 expression and subsequently promotes neocollagenesis and neoangiogenesis in the skin tissue

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Abstract Radiofrequency (RF) treatment appears to be involved in production of new collagen fibrils and the improvement of existing collagen structures; however, the molecular bases of the effect of non-invasive RF on the skin tissue have not been fully elucidated. This study reports the effects of RF associated or not with hydrolyzed collagen (HC) in the skin tissue. Wistar rats were randomly divided into four groups, according to the treatment received: control group (G1, $n = 5$), no treatment; subjects in group G2 ($n = 5$) were treated with HC; and capacitive RF was applied to the back of each subject in G3 ($n = 5$) and RF associated with HC in G4 ($n = 5$). Biopsies were taken 30 days after treatment and then were histologically processed and studied for inflammatory cell counting, collagen content, and morphometry. In addition, FGF2, CD105, and COX-2 expression was assessed by immunohistochemical staining. The most relevant changes were the increase in cellularity and accumulation of intercellular substance in RF-treated animals (G3 and G4). The greatest dermis thickness rate was observed in G4, followed by G3 and G2 ($p < 0.05$). RF-treated skins (G3 and G4) exhibited a significant overexpression of FGF2 ($p < 0.0001$) and increased microvessel density ($p < 0.0001$) in comparison with G1 and G2. Moreover, the amount of COX-2 was significantly higher ($p < 0.0001$) in dermis of RF-treated areas compared to G1 and G2, and demonstrated differences in G3 (RF) compared to G4 (RF + HC) ($p < 0.0001$). Our results suggests that RF treatment associated or not with HC induces FGF2 overexpression, promotes neoangiogenesis and modulates the COX-2 expression, subsequently promotes neocollagenesis, and increased thickness rate of dermis.

Keywords Radiofrequency · Hydrolyzed collagen · Neocollagenesis · Angiogenesis · Fibroblastic growth factor 2 · Cyclooxygenase 2 · Endoglin

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Introduction

Multiple external and internal factors are associated with the physiological process of aging skin. Ultraviolet radiation and high temperatures induce dermal cells to produce cytokines and enzymes that cause degradation to the extracellular matrix. Furthermore, there are minor levels of growth factors causing cell and tissue aging [1, 2]. Collagen levels have proven to decline dramatically after 25 years of age. Current therapies have been studied to minimize the deleterious effects of skin aging process [3, 4] including chemical peeling and dermabrasion. Others therapies such as CO_2 lasers have

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demonstrated benefited effects in neocollagenesis by thermal modification of collagen [5, 6].

Laser and light-based systems are known methods to promote increase in cellular activity in various tissues resulting in an ability to promote neocollagenesis, angiogenesis, and fibroblast proliferation [7]. On the other hand, non-ablative radiofrequency (RF) is a known method that promotes increased dermal collagen levels. Several studies have demonstrated that RF stimulates production of collagen in the dermal matrix and is effective in the skin rejuvenation treatment as well as esthetic procedures [8, 9].

Previous studies have demonstrated that cultured fibroblasts treated with low-level laser therapy showed a significant increase in basic fibroblast growth factor 2 (FGF2) expression [10]. In this context, the FGF2 has been implicated in the angiogenic process, and its expression during wound healing can be associated with the improvement in this process. Furthermore, FGF2 also is associated with numerous biologic processes, including cell proliferation, differentiation, and migration [11]. Despite previous studies demonstrating that RF treatment induces fibroblast proliferation [8, 12], its effect on FGF2 expression is not related. During the skin rejuvenation procedures, angiogenesis has been recognized as a critical event to supply nutrients required for improvement of neocollagenesis, thickening of the dermis, and enhancement of the metabolism of fibroblasts. Thus, pro-angiogenic molecule expression such as endoglin—CD105—can provide important data on skin treated with RF and induce events that induce skin rejuvenation and collagen remodeling.

Two hypotheses have been formulated aiming to understand the RF mechanism of action. Some authors claim that improvement in the dermal ground substance arises from the light energy absorbed by water, and perhaps by collagen, to cause a direct thermal effect on cells and collagen fibers [13–15]. Other hypotheses affirm these effects are indirect. Inflammatory and dermal cells (fibroblasts, endothelial cells) produce cellular mediators and growth factors that stimulate a tissue response, resulting in fibroblast proliferation and neocollagenesis [16]. The expression of cyclooxygenase 2 (COX-2) could induce these events, besides that COX-2 is a pro-inflammatory enzyme that is associated with cell proliferation and differentiation. Nonetheless, it is still not clear to what RF treatment induces cell proliferation and differentiation, and these events are strictly associated during neocollagenesis.

Other therapies include the use of hydrolyzed collagen (HC), a derivative of collagen which contains eight to nine essential amino acids, including glycine and arginine [17]. Recent studies indicate that absorption of HC is greater if compared to conventional collagen, in addition to being a safer and non-toxic product. Therefore, several studies have been conducted to determine its bioavailability and neocollagenesis activity [18, 19]. Benefits in stimulating the fibroblasts' availability of basic amino acids for collagen resynthesis were observed in pre-clinical trials that assessed the hydrolyzed collagen activity [20–22].

Several studies have demonstrated the effect of RF on fibroblast stimulation and subsequent collagen replacement and augmentation; consequently, this treatment promotes tissue rejuvenation and restoration. However, the effects of RF on fibroblastic proliferation-related proteins (such as FGF2), angiogenic signaling molecules (such as endoglin—CD105—expression), and pro-inflammatory enzymes (such as COX-2) have not been fully elucidated. In this study, we aimed to analyze the effect of hydrolyzed collagen associated with RF in the skin tissue of rats through qualitative and morphometric histological analysis. Furthermore, we evaluated through immunohistochemical method the FGF2, endoglin, and COX-2 expression in animals treated or not with RF.

Materials and methods

Ethical considerations

This experimental protocol followed the guidelines of the Animal Experimentation Code of Ethics and the Brazilian College of Animal Experimentation and was duly approved by the Research Ethics Committee (CEP/UNP; protocol number 013/2013).

Subjects

Twenty Wistar rats (*Rattus norvegicus*) with an average age of 12 weeks (body weight, 300 ± 50 g) supplied by Potiguar University animal facility (Natal, Brazil) were kept in an environment with controlled light (cycles of 12 h light/dark), ventilation, and temperature (24 °C). Subjects were fed on a balanced diet (Labina®; Purina) and water ad libitum.

Experimental groups

Twenty female Wistar rats with an average weight of 300 g were evaluated and sorted into four groups. Control group (G1, $n = 5$) subjects received distilled water during the experiment. Subjects in group G2 ($n = 5$) were treated with hydrolyzed collagen (HC). Capacitive RF was applied to subjects in G3 ($n = 5$) (RF) and G4 ($n = 5$) (RF + HC).

Hydrolyzed collagen procedures

In order to calculate the amount of collagen received by each subject, we relied on suggested literature which verses that the recommended consumption for an adult subject is 10 g/day. Other studies report 0.1 and 0.2 g/kg; it is recommended, however, that lower doses are administered, so 0.1 g/kg was

chosen as the reference dose [23]. Gavage technique was followed for the administration of the substance, with stainless steel needle BD-12, 1.2 mm diameter tube with 2.3-mm ball, 40 mm radius, and 54 mm length.

Radiofrequency procedures

The capacitive RF was performed using the Poya Innovation 2.4L (Poya®; HABALAN Med & Beauty Co., Seoul, South Korea) device with the power setting of 150 W and output frequency of 0.3/0.5 MHz. Carbopol gel was used to improve the contact between the handpiece and the skin, and the temperature was monitored with a highly sensitive digital infrared thermometer.

Capacitive RF was applied to the dorsal area of each subject (area = 5 × 10 cm) equivalent to twice the size of the RF handle pointer. After RF reached 40 °C, subjects received a 2-min application on the selected dorsal area. One application of RF was run per week during 4 weeks [24]. After the application period of 4 weeks, subjects were anesthetized and a sample of 2 cm^2 from the controlled dorsal area was removed from each for analysis.

Histological procedures

The skin samples were dehydrated, cleared, embedded in paraffin, and cut with a rotary microtome into sections of 4–5 μm of thickness. Hematoxylin and eosin (HE) and Masson's trichrome staining techniques were applied to verify differences in morphology of the tissue and cells, and collagen tissue in comparison to control group. Picrosirius red technique was realized for differentiation between the type I and type III collagen fibers using a commercial test kit (#ab150681; Abcam plc, Cambridge, UK) in strict accordance to the manufacturer's instructions. After staining, the slides were analyzed under polarized light, and type I collagen appeared yellow or red because of its strong refractive index, while type III collagen was young and thin which displayed green owing to its weak refractive index. The amount of type I and type III collagen fibers under high power fields per animal was also quantified using ImageJ® software (NIH, Bethesda, USA).

Histological qualitative and quantitative analysis

The slides were examined through binocular light microscope (Olympus CX31, Hamburg, Germany) with attached camera. Photomicrographs of various microscopic fields were taken at different magnification levels (×40, ×100, or ×400). The qualitative parameters included for analysis were inflammatory response, fibroblast distribution, organization, and morphological changes in collagen tissue, as described in Table 1, based on Medeiros et al. [7].

Histomorphometry was performed on samples (20 sections were analyzed at a magnification of ×100 and ×400) via ImageJ® software (NIH, Bethesda, USA) with the purpose of analyzing the dermis area and the number of fibroblasts and hair follicles. Arithmetic mean between 20 sections determined the final dermis area and quantification of fibroblasts and hair follicles.

Immunohistochemical study

For immunohistochemical study, 3-μm-thick tissue sections from samples were submitted to antigen retrieval with sodium citrate buffer solution (10 mM, pH 6.0) in microwave during 24 min and were then immersed in 3% hydrogen peroxide for 30 min to quench endogenous peroxidase. Slides were incubated for 60 min at 37 °C with the primary antibodies. Primary antibodies included anti-COX-2 (clone CX-294; Dako, Carpinteria, CA, #M361701-2) diluted at 1:200; anti-CD105, endoglin (clone SN6h; Dako, #M352701-2), diluted at 1:50; and anti-FGF2 (rabbit polyclonal; Santa Cruz Biotechnology, USA, #SC-79) diluted at 1:200. Subsequently, the slides were incubated with horseradish peroxidase conjugated with the secondary antibodies (Envision; Dako) for 30 min at room temperature, and developed by diaminobenzidine (Dako), counterstained with hematoxylin, followed by dehydration and mounting.

Sections stained with anti-COX-2 and anti-FGF2 were photographed, and the positive staining was quantified in ten high power fields for two sections/animal. The microvessel density was reached by counting the CD105-positive vessels divided by the analyzed area, and presented as number of vessels per square micrometer.

Statistical analysis

Briefly, all data were submitted for the Shapiro-Wilk test to verify data normality. The statistical significance of the qualitative parameters (inflammatory response, fibroblast and epithelial thickness) was evaluated by Kruskal-Wallis test with Dunn's post test. Two-way analysis of variance (ANOVA) with Tukey post test was applied to verify differences on the expression of the proteins studied and on microvessel density. Significance level of 5% for the differences was observed when $P < 0.05$ for all tests.

Results

RF treatment increases dermal thickness and hair follicle density

Specimens stained through HE technique revealed normal skin tissue for all groups (Fig.1). However, in groups G3 and G4, increased stratification of epithelium and irregular distribution of collagen fibers in the dermis were observed. In groups treated with RF (G3 and G4), the epithelium evidenced more layers ($P = 0.0096$) in some areas with irregular epidermal hyperplasia,

Table 1 Histomorphological parameters and scores for qualitative analysis

Scores	(1)	(2)	(3)	(4)
Inflammatory response	Absent	Weak inflammatory infiltrate	Moderate inflammatory infiltrate	Intense inflammatory infiltrate
Fibroblasts	Absent	Inactive (pyknotic nuclei)	Active (large nuclei) up to 10 per field	Active (large nuclei) more than 10 per field
Collagen	Organized and non-thickened fibers	Organized and thickened fibers	Disorganized and non-thickened fibers	Disorganized and thickened fibers
Epithelial thickness	Up to 5 layers	More than 5 layers and normal granular layer	More than 5 layers and prominent granular layer	More than 10 layers and prominent granular layer

with granular layer more evident (Fig. 1c, d; in detail—Fig. 1g, h) if compared to G1 and G2 (Fig. 1a, b; in detail—Fig. 1e, f). Inflammatory events occurred primarily in RF-treated groups. Mononuclear inflammatory cells were found in the reticular layer of the dermis (Table 2, Fig. 1g, h). The mean thickness of dermis increased in a statistically significant manner ($P < 0.0001$) in G2, G3, and G4 when compared to G1 (Fig. 1i). No statistically significant variations between G3 and G4 were registered.

The number of hair follicles was also higher in the treated groups and increased in a statistically significant manner ($P < 0.0001$) in G3 and G4 if compared to G1 (Fig. 1j). No statistically significant variations between G2 and G1 were registered. Furthermore, there was a greater number of fibroblasts in the dermis of RF-treated subjects if compared to the group treated with collagen alone as well as to the control group (Table 2; Fig. 1g).

RF treatment induces neocollagenesis

Some specimens stained with Masson's technique showed thickened collagen fibers and some did not (Fig. 2). In G1 and G2, fibers appeared normal, but unevenly distributed thickness was

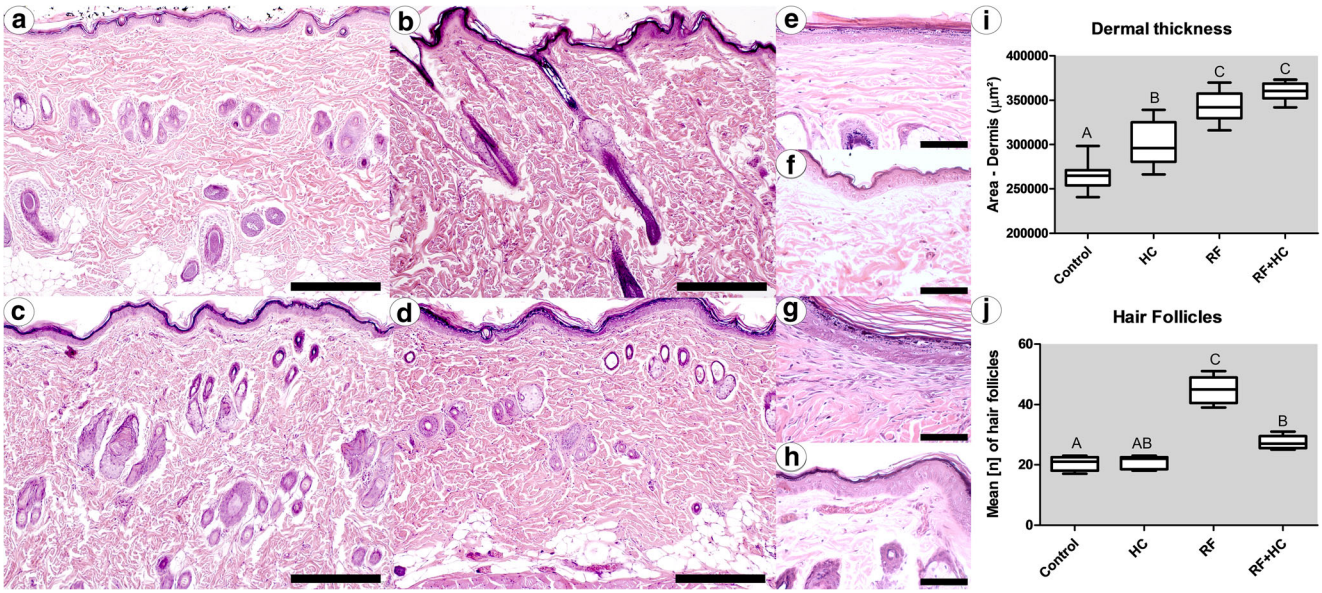

Fig. 1 Microscopic findings, qualitative analysis. **a** Organized collagen fibers and regular number of hair follicles, epidermis revealed some layers in the control group (G1). **b** Disorganized collagen fibers and epidermis with normal number of layers in the HC-treated group (G2). **c** Disorganized collagen fibers and proliferation of hair follicles, epidermis with increased of number layers in the RF-treated group (G3). **d** Disorganized collagen fibers with reduction of adipose tissue, epidermis with increased number of layers in the RF + HC-treated group. **e** Detail of findings related to control group, with normal cellularity and hair follicle distribution. **f** Detail of findings related to the HC-treated group, with collagen fibers showing edema in the subepithelial area and more disorganized collagen fibers. **g** Detail of and findings related to the RF-treated group, with fibroblast proliferation, increased cellularity, and young fibroblasts. **h** Detail of and findings related to the RF + HC-treated group, with edema on the subepithelial area, vascular congestion, and fibroblast proliferation. **i** Graphic representation of the measurement of mean thickness of dermis in histological sections of biopsies taken from treated and control groups. **j** Graphic representation of mean of hair follicles on the dermis in histological sections of biopsies taken from treated and control groups. Photomicrographs—hematoxylin and eosin; the *scale bars* in (**a**), (**b**), (**c**), and (**d**) represent 200 μm, and the *scale bars* in (**e**), (**f**), (**g**), and (**h**) represent 40 μm. $^{**}P < 0.05$, two-way ANOVA. Different *letters* (A, B, C) in *columns* represent statistically significant differences

Table 2 Parameters used for the calculation of the Kruskal-Wallis test for evaluation of median for scores in qualitative analysis

	G1	G2	G3	G4	*p* value
Inflammatory response	1 (1–1)[a]	1 (1–1)[a]	3 (2–3)[b]	3 (1.5–3)[b]	0.0011
Fibroblasts	2 (2–2)[a]	2 (2–2)[a]	3 (3–4)[b]	3 (2–4)[b]	0.0022
Collagen	2 (2–2)[a]	2 (2–3)[a]	4 (4–4)[b]	4 (3–4)[b]	0.0011
Epithelial thickness	2 (2–3)[a]	3 (2–3)[a]	4 (3.5–4)[b]	3 (3–4)[a]	0.0096

Median (Q_{25}–Q_{75}). Q = quartile. Values with different letters represent differences in the groups. Kruskal-Wallis Dunn's multiple comparisons test

noticed in G2 (Table 2, $P = 0.0011$). However, in G4, fibers presented the thickest and most irregular distribution (Table 2, $P = 0.0011$). The irregular distribution was associated to increased thickness of the dermal layer. Median scores and parameters analyzed in qualitative analysis are shown in Table 2.

Type I collagen content in G2, G3, and G4 was significantly higher ($P < 0.0001$) than in the control group (G1). In RF + HC-treated group (G4), the content of type I collagen was significantly higher than in G2 (HC treatment) and G3 (RF treatment). Type III collagen content was likewise significantly higher in G1 than in G2, G3, and G4 ($P < 0.0001$). This reduction was clearly observed in RF-treated groups (G3 and G4) when compared to G2 (HC treatment) and control group.

RF treatment induces angiogenesis

To estimate angiogenesis, we quantified the CD105-positive vessels (newly formed vessels) in the dermal superficial and deep portions by immunohistochemistry. Quantitative analysis of the treated skins showed a general tendency toward a higher microvessel density (MVD) in RF and RF + HC-treated groups compared with control and HC groups (Fig. 3a). In the dermal superficial portion, a significantly higher MVD was observed in G3 and G4 groups ($P < 0.0001$) compared to G1 and G2 groups (Fig. 3b). However, in the dermal deep portions, besides G3 and G4 showing a higher MVD, G2 demonstrated an increased MVD compared to control group (Fig. 3c, $P < 0.0001$). Considering the total MVD, the RF-treated group showed a higher MVD, and the other groups (G2 and G3) demonstrated

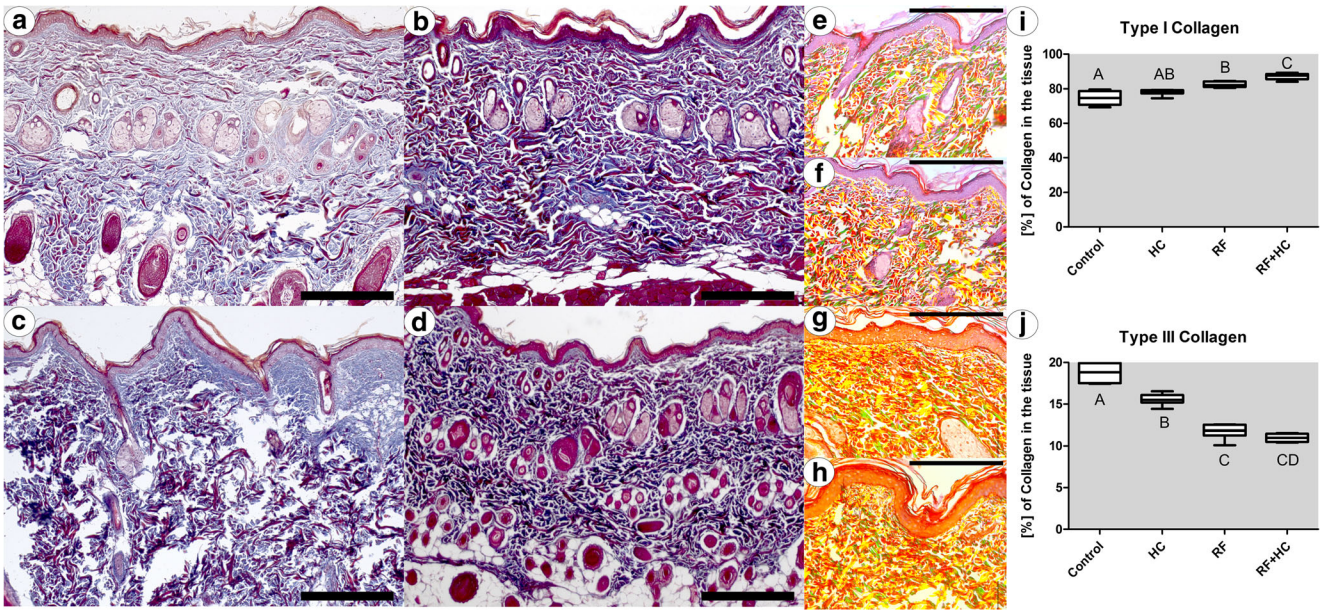

Fig. 2 Photomicrographs and graphic representation of collagenization areas. **a** Sparse collagen fibers and regular distribution parallel of epidermis, control group (G1). **b** Sparse collagen fibers and irregular distribution, HC-treated group (G2). **c** Intense collagen proliferation and disorganized fibers with hair follicle proliferation, RF-treated group (G3). **d** Intense collagen proliferation and disorganized fibers with hair follicle proliferation, RF + HC-treated group (G3). Masson's trichrome stain; the *scale bars* represent 200 μm. **e** Type III collagen were predominant in the control group. **f** Sparse type I and type III collagen fibers were observed in HC-treated group. Type I collagen fibers were observed predominantly in the RF-treated (**g**) and RF + HC-treated (**h**) groups. **i** Graphic representation of type I collagen content in control and treated groups. **j** Graphic representation of type III collagen content in the control and treated groups. $^{**}P < 0.05$, two-way ANOVA. Different *letters* (A, B, C) in *columns* represent statistically significant differences

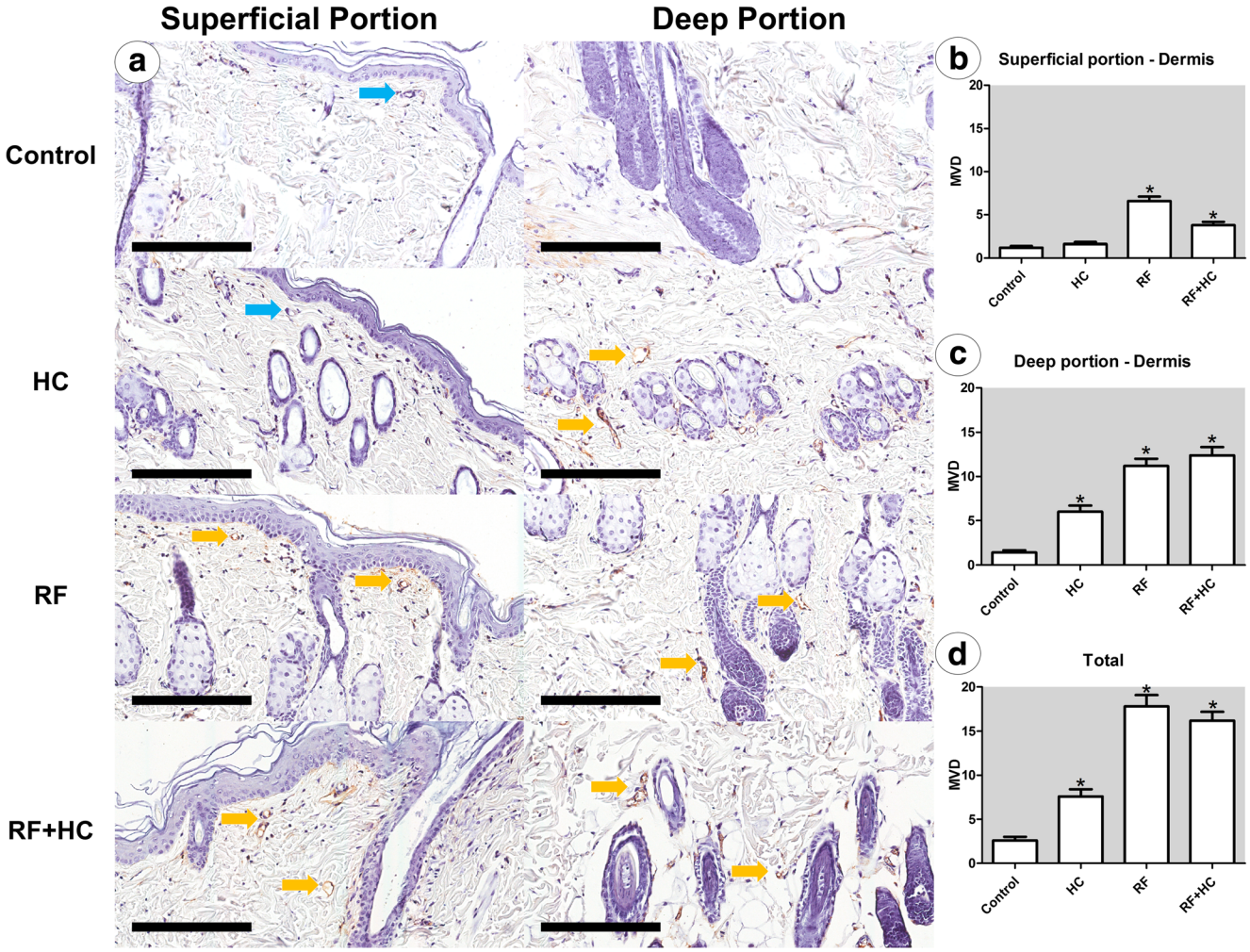

Fig. 3 IHC for CD105 and microvessel density (MVD) index. Positive staining for CD105 was observed on endothelial membranes of the newly formed vessels. **a** Photomicrographs of representative areas in control and treated groups. Control and HC-treated groups demonstrated sparse CD105-positive vessels in the superficial and deep portions. RF- and RF + HC-treated groups showed a high number of CD105-positive vessels in the superficial and deep portions. The *scale bars* represent 200 μm. *Blue* and *yellow arrows* represent negative and positive vessels, respectively. **b** Graphic representation of the mean of CD105-positive vessels in the superficial portion. *Error bar*: standard deviation. **c** Graphic representation of the mean of CD105-positive vessels in the deep portion. *Error bar*: standard deviation. **d** Graphic representation of mean of CD105-positive vessels in the superficial and deep portions. *Error bar*: standard deviation. *Asterisks* (*) represent significant difference with the control group, $P < 0.05$, two-way ANOVA

a significant increase in MVD compared to the control group (Fig. 3d).

RF treatment modulates the FGF2 and COX-2 expression

We stained skin tissue sections with FGF2 and COX-2 to assess the role of these markers in RF-treated tissues. FGF2 demonstrated a cytoplasmic expression in keratinocytes, fibroblasts, and some vessels (Fig. 4a). Similarly, the pattern of COX-2 expression was cytoplasmic in fibroblasts, inflammatory and endothelial cells, and in keratinocytes.

A quantitative analysis revealed that epidermal FGF2 expression was significantly higher in the RF and RF + HC-treated groups compared with the control and HC groups ($P < 0.0001$, Figure 4b).. Interestingly, in RF-treated skins, the epidermal FGF2 expression was significantly increased (90.01% positive cells) compared with RF + HC-treated skins (84.53% positive cells). No differences were noted between the control group (4.68% positive cells) and the HC-treated group (4.73% positive cells). Dermal FGF2 expression was higher in all treated groups compared with the control group ($p < 0.0001$); however, the RF-treated group (G3) demonstrated a higher FGF2 expression (43.66% positive cells) than in HC- (35.60% positive cells) and RF + HC-treated groups (36.89% positive cells) (Fig. 4c)

Quantitative epidermal COX-2 expression analysis showed that this marker was expressed similarly in all groups (Fig. 4d), and no statistically significant variations between the groups were registered ($P > 0.05$). Moreover, dermal COX-2 expression was significantly higher in RF- and RF + HC-treated group (G3 and G4), demonstrating 81.51 and 21.47% positivity,

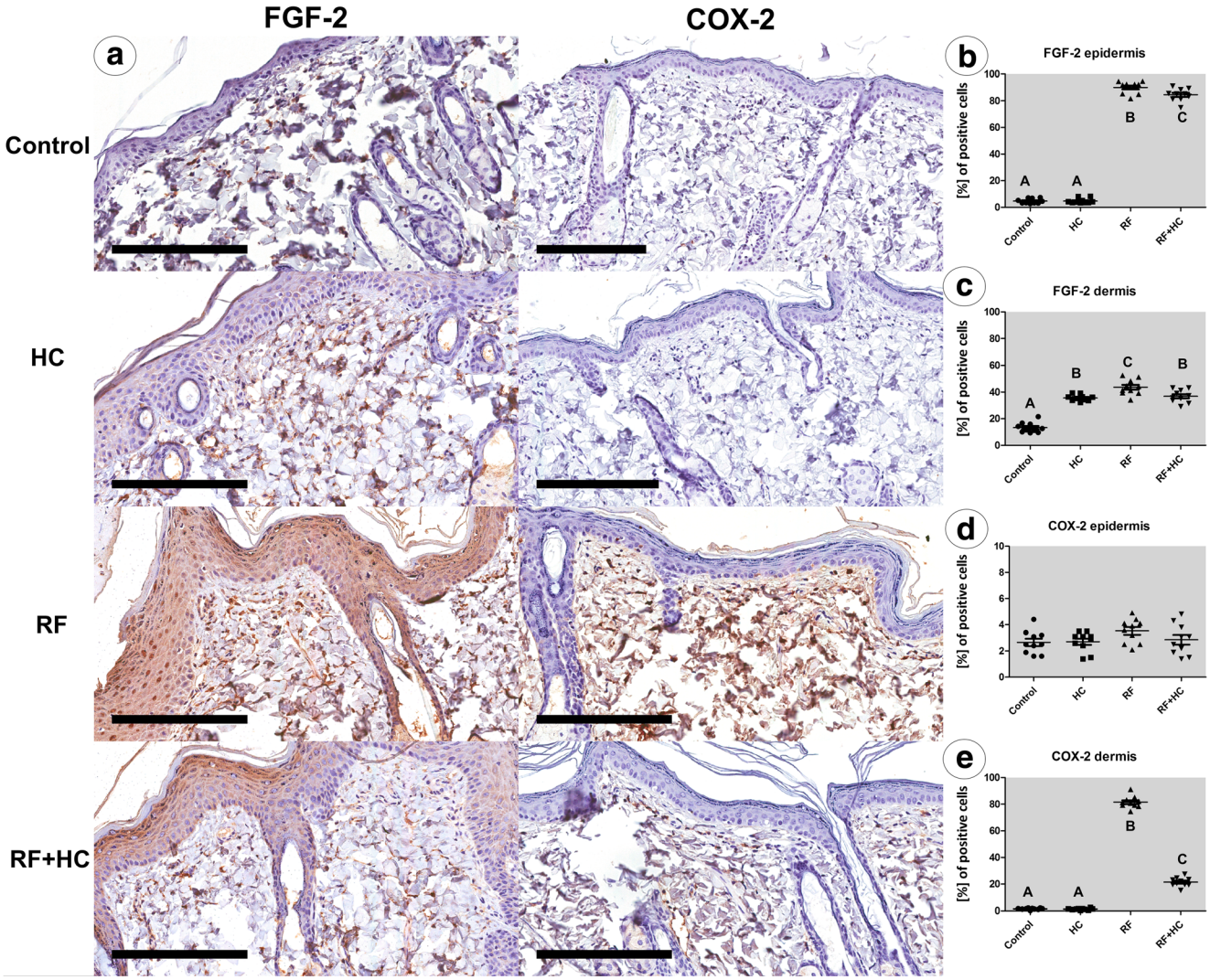

Fig. 4 IHC for FGF2 and COX-2 markers. **a** Photomicrographs of representative areas in the control and treated groups. Positive staining for FGF2 was observed on epidermis and dermis. In the control group, weak expression was observed in the epidermis and dermis. HC-treated group demonstrated a significant increase in the dermal FGF2 expression, while the RF- and RF + HC-treated groups showed a higher epidermal and dermal FGF2 expression. COX-2 was expressed significantly in RF- and RF + HC-treated groups; however, RF-treated group showed a significantly higher expression compared to the RF + HC-treated group. The *scale bars* represent 200 μm. **b** Graphic representation of mean of FGF2-positive cells in the epidermis. *Error bar*: standard deviation. **c** Graphic representation of mean of FGF2-positive cells in the dermis. **d** Graphic representation of the mean of COX-2-positive cells in the epidermis. **e** Graphic representation of mean of COX-2-positive cells in the dermis. $^{**}P < 0.05$, two-way ANOVA. Different *letters* (A, B, C) in *columns* represent statistically significant differences

respectively. The control and HC-treated group showed 1.47 and 1.25% positivity, respectively (Fig. 4e). In addition, the dermal COX-2 expression was significantly higher in the RF-treated group than in the RF + HC-treated group.

Discussion

Some non-invasive techniques serve to assist skin rejuvenation. These techniques have been widely studied, among which are chemical peels, dermabrasion, and different types of laser radiofrequency (RF) [20, 21]. RF is a technique based on electric current, which produces heat generated by resistance by the components of the dermis and subcutaneous tissues. This hyperthermia stimulates an increase in the expression of TGF-beta-1 (transforming factor beta-1 growth) as a response and stimulates HSP 47, causing fibroblasts to respond by increasing collagen production [24, 25]. Another common and effective therapy is the use of hydrolyzed collagen (HC) [23]. Despite clinical studies and the indication of RF in cosmetic medicine for the treatment of facial wrinkles and nasolabial creases, deep knowledge of its effects at the microscopic and molecular level is still limited [26–28]. A

thorough microscopic description of the RF effects in the dermis and epidermis of subjects was the chosen method for this study. Moreover, the aim of the present study was to extend our knowledge on the expression and action of FGF2, CD105, and COX-2 in skin tissue of rats treated with RF, and RF associated with HC. To this end, we evaluated the immunohistochemical expression of these markers.

During the experimental period, subjects did not have adverse or side effects, which probably occurred owing to longer periods between applications. In other studies, erythema was observed in the areas treated with RF [29]. Even with the long period between applications, beneficial effects described in similar studies were found.

Qualitative analysis run on HE technique showed normal epidermal and dermal tissues in the samples. Nevertheless, epidermal hyperplasia was observed in groups treated with RF (G3 and G4). In some cases, granular layer seemed more evident, indicating more cellular activity. The dermis was measured on its extension (papillary and reticular dermis). RF-treated subjects evidenced increased dermis and added fibroblast proliferation and increased number of mononuclear cells. The HC associated with RF did not alter such finding. According to Serrated et al. [23] the increase in dermis is the cause of edema and vascular congestion. However, vascular events were not regarded. The increment in dermis was accredited to increase in intercellular substance and the population of resident cells (fibroblasts, histiocytes).

Qualitative analysis G1 individuals revealed that collagen fiber arrangement occurred in tissues with quiescent cells. Fibroblasts had pyknotic nuclei, in opposition to what occurred in the RF and RF + HC groups wherein the spindle fibroblasts displayed prominent nuclei and intense collagen deposition on the dermis. Hydrolyzed collagen did not significantly alter treatment with RF. Morphological changes in the RF + HC group were discreet. It is probable that its beneficial activity is inhibitory and not stimulatory. Hydrolyzed collagen reduces circulating levels of matrix metalloproteinase [30]. However, the stimulatory effects of RF with the inhibitory effect of HC were effective for increasing the dermis.

Previous studies have demonstrated that type I collagen content in normal skin indicates stages of collagen remodeling [31, 32]. In this study, we demonstrated that treated groups showed increased amounts of type I collagen and decreased amounts of type III collagen. These results suggest that RF showed beneficial effects in collagen remodeling and consequently skin rejuvenation.

An additional finding was the greater number of hair follicles in subjects who were exposed to RF. This occurred in a lesser frequency than in RF + HC group. According to Yoon et al. [33], 1763 MHz RF exposure stimulates hair growth, based on stimulation via galvanic pulse, which promoted electrical stimulus, inducing cell migration and wound repair through a rise in protein and DNA synthesis. In contrast, Kim et al. [34] suggest that 27-MHz RF causes significant damage to hair follicles. They concluded that different energy stimuli could induce molecular responses in a specific manner. It is substantial to ensure the effectiveness of treatments, as subjects evidenced lack of systemic change, keeping satisfactory health conditions throughout the experimental period.

Neoangiogenesis is a step necessary for skin health because this biologic process can facilitate nutrient supply and waste removal from the skin, resulting in improvement of collagen remodeling and skin rejuvenation [35]. In this study, we evaluated the neoangiogenesis process based on quantification of CD105-positive vessels (microvessel density—MVD). We observed that MVD was significantly higher in RF-treated groups than in HC-treated groups, especially in superficial portions of the dermis, indicating that the direct effect of RF is preponderant for neoangiogenesis.

It has been reported that RF alters the expression of stress-related genes, including TNFRSF11B, FGF2, TGFB2, ITGA2, BRIP1, EXO1, and MCM10 [36]. However, no studies have evaluated the expression of these markers in skin tissue through in vivo investigations. This study was performed only in normal human lung fibroblast cells, which limits the comparison with our results, and the study of RF effects in epidermal cells should be a subject of further studies.

In the present study, we have shown that FGF2 is expressed in normal and stimulated skin tissues (epidermis and dermis). When treated with RF or RF + HC, the skin tissue demonstrated an increased expression of FGF2; this is consistent with other events observed in treated animals, neocollagenesis and neoangiogenesis, and highlights a possible mechanism of action of RF. The epidermal FGF2 expression was more evident than the dermal expression in RF-treated skins (G3 and G4), suggesting that the RF effects directly affect the epidermis, causing hyperplasia and cell proliferation. On the other hand, dermal FGF2 expression was also more evident in RF-treated skins; however, the RF + HC-treated group revealed a lower expression compared with RF-treated group. Taken together, this data confirms that RF causes epidermal and dermal stimulation.

Surprisingly, some differences between the RF- and RF + HC-treated groups were observed. RF-treated group demonstrated a significantly higher dermal FGF2 and COX-2 (more evident) expression than in RF + HC-treated group. One possible explanation could be that HC exerts a protective thermal effect on the dermis, decreasing the levels of FGF2 and COX-2 expression. Similarly, the MVD in the superficial portion of the dermis was higher in the RF-treated group than in the RF + HC-treated group; this data corroborates with the hypothesis that RF exerts a direct effect on superficial dermis [16].

Our study highlights a mechanism of action of RF in the skin tissue. We demonstrated that RF associated or not with HC causes morphological changes in the epidermis and

dermis tissues, resulting in increased thickness dermis, number of hair follicles, and inflammatory cells. Finally, concomitant expression of two potent angiogenic molecules, FGF2 and CD105, in RF-treated animals indicates that RF treatment stimulates neoangiogenesis and increases the source of nutrition and oxygen for skin tissue, and these events are strictly associated with neocollagenesis and improvement in tissue rejuvenation and restoration.

Conclusion

In this study, we demonstrated that radiofrequency (RF) treatment, isolated or associated with hydrolyzed collagen, increases and improves neocollagenesis, neoangiogenesis, and dermis thickness. These findings confirm that RF treatment exerts a significant effect on the fibroblast proliferation, number of hair follicles, and epithelial thickness. RF treatment also modulates COX-2 expression, overregulates FGF2 expression, and induces neoangiogenesis. Taken together, the observations of the current study highlight the mechanism of action of RF on the skin tissues and warrant further studies to confirm the molecular and microscopic effects of RF treatment.

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Compliance with ethical standards This experimental protocol followed the guidelines of the Animal Experimentation Code of Ethics and Brazilian College of Animal Experimentation and was duly approved by the Ethics Committee of Potiguar University Laureate International Universities (protocol number 013/2013).

Conflict of interest The authors declare that they have no conflicts of interest.

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