

**IMPACT OF INJECTION MODE ON
THE ANTI-AGING EFFECT OF MIXTURE COMPOSED
OF HYALURONIC ACID AND MULTIVITAMINS INJECTED
USING THE U225 BIOPHYMED MESOTHERAPY GUN®
INTO HUMAN SKIN MAINTAINED IN SURVIVAL**

Pitié-Salpêtrière Hospital (Paris)

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Groupe de Recherche et d'Évaluation en Dermatologie et Cosmétologie

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Study Sponsor: Jean Louis BARRAU

Study Monitor: Jean Paul BEN

Product tested: mixture of hyaluronic acid and multivitamins

Device tested: **U225 BIOPHYMED®** mesotherapy gun



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INTRODUCTION AND PURPOSE

The purpose of this study was to evaluate the impact of injection mode on the anti-ageing efficacy of mixture of hyaluronic acid and multivitamins injected using the Biophymed U225 mesotherapy gun[®] in the rapid-pulse mode.

Study was carried out on an experimental human skin model in which aging was induced by ultraviolet (UV) irradiation. Evaluation was based on immunohistochemical techniques (mitotic index), histological examination (visualization of collagen and elastic fibres in the dermis) and biochemical assay with stimulation of collagen synthesis by fibroblasts.

MATERIAL AND METHODS

1) Experimental photo-aging and injection a mixture of hyaluronic acid and multivitamins

Specimens of normal skin were collected from 5 donors during the immediate post-operative period. Specimens were immediately utilized to construct an experimental photo-aging model (oxidative stress) in which ultraviolet (UV) A (8 J/cm²) and UV B (2 J/cm²) irradiation was performed in a single session to induce a reduction in fibroblast metabolism and alterations of macromolecules in connective tissue.

After constructing the model of aged skin, a mixture containing hyaluronic acid and multivitamins was injected into the skin using the Biophymed U225 mesotherapy gun[®] either in the normal single-pulse mode (point-by-point 4 pulses per cm²) or rapid-pulse mode (high frequency with 8 to 9 pulses per second i.e. 300 pulses per cm²). Control tests were performed using saline solution injected in the rapid-pulse mode. The following experimental conditions were compared :

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- untreated skin
- skin + UV (control skin)
- skin + UV + injection of mixture of hyaluronic acid and multivitamins in single-pulse mode
- skin + UV + injection of mixture of hyaluronic acid and multivitamins in rapid-pulse mode
- skin + UV + injection of mixture of hyaluronic acid and multivitamins in single-pulse followed by rapid-pulse mode
- skin + UV + injection of saline solution in rapid-pulse mode.

2) Maintenance of human skin in survival

Immediately after injection, skin specimens were deposited on inserts placed over culture wells. Culture medium designed to maintain human skin in survival was added to the bottom of the wells by slow diffusion through a porous membrane (12 µm) between two compartments. The medium was changed 3 times per week.

Skin samples were collected on experimental day 3 for determination of mitotic index and on experimental day 14 for analysis of collagen and elastic fibres content and measurement of collagen synthesis.

3) Assessment techniques

Alterations in epidermis on experimental day 3

a) Histological determination of mitotic index

Epithelial cell replication on day 3 was evaluated by immunohistochemical analysis using anti-Ki67 antibodies (markers of antigens expressed during the M, S, G1 and G2 phases of cell cycle). Immunodetection was achieved using an indirect three-layer immunoperoxidase technique amplified (DAKO kit) and revealed with AEC (3-amino-9-ethyl-carbazole) substrate.

Marked cell count was determined and expressed as a percentage of Ki67-positive cells in relation to basal cell count (between 150 and 200 cells).

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Alterations in epidermis on experimental day 14

a) Histological determination of elastic fibres content by computer-assisted image analysis

Elastic fibres were revealed by (+)-catechine staining and quantified morphometrically using computer-assisted image analysis. The mean percent of total area occupied by the elastic network was determined at the superficial dermis and middle-dermis levels.

b) Histological determination of collagen content by computer-assisted image analysis

Collagen was revealed by Sirius red staining and quantified morphometrically using computer-assisted image analysis. The percentage of total superficial- and middle-dermis area occupied by collagen was determined.

c) Determination of total collagen content by biochemical assay

At the end of the experiment, skin specimens were ground up to allow evaluation of collagen content (ng/ml) using a spectrophotometric assay (Sircol Collagen Assay, Interchim).

To allow comparison of data, collagen content was expressed in relation to total protein content in the specimen. Protein content was determined spectrophotometrically at 562 nm (BCA Assay, Pierce). Results were expressed in μg of collagen/mg of protein.

4) Statistical analysis

Data obtained from treated specimens and specimens that underwent only UV photo-aging were compared.

Mean values were calculated using data obtained from all 5 skin specimens. Statistical analysis was performed using the Student test designated for standard deviation or test for paired samples with a significance level α of 5%.

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RESULTS

1) Immunohistochemical assessment of epithelial cell replication

Data are presented in **Table I** and **Figures 1 to 6**.

Epithelial cell replication was significantly higher after experimental UV photo-aging only (control skin) as compared to untreated skin: 3.9% versus 0.7% ($p = 0.007$). This finding suggests an immediate restorative reaction.

In comparison with control skin, a significant increase in epithelial cell replication was observed after injection of mixture of hyaluronic acid and multivitamins either in the single-pulse mode (12,65%, $p = 0,016$) or in the single-pulse mode followed by rapid-pulse mode (8,45%, $p = 0,028$).

Injection of saline solution in the rapid-pulse mode had no effect on the replication rate.

2) Histological determination of elastic fibres content by computer-assisted image analysis

Data are listed in **Table II**. **Figures 7 to 12** present data obtained at the superficial-dermis level and **figures 13 to 18** present data obtained at the middle-dermis level.

After experimental photo-aging only, a slight trend for lower elastic fibres content was observed with mean elastic fibres values of 2.23% in the superficial-dermis and 7.5% in the middle-dermis ($p=0.03$) versus 5.25% and 7.5% respectively in untreated skin.

At the superficial-dermis level, injection of mixture of hyaluronic acid and multivitamins in the single-pulse mode followed by the rapid-pulse mode led to a significant increase in the percentage of elastic fibres in comparison with control skin submitted to UV photo-aging only: 4.9% versus 2.23% ($p = 0.02$). Injection in the single-pulse mode followed by the rapid-pulse mode led to a significantly greater quantity of elastic fibres than injection by the single-pulse mode alone (2.9%; $p=0.049$).

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Similar results were observed at the middle-dermis level. Injection of mixture of hyaluronic acid and multivitamins in the single-pulse mode followed by the rapid-pulse mode led to a significant increase in the percentage of elastic fibres in comparison with control specimens (UV only): 6.6% versus 3.9% ($p = 0.03$).

Injection in the single-pulse mode followed by the rapid-pulse mode also led to significantly higher elastic fibres content than injection in the single-pulse mode alone (5.5%; $p = 0.02$) or in the rapid-pulse mode alone (4.1%; $p = 0.0035$).

Injection of saline solution in the rapid-pulse mode had no effect on elastic fibres content.

3) Histological determination of elastic fibre content by computer-assisted image analysis

Data are listed in **Table III. Figures 19 to 24** present data from donor n° 4.

Collagen content in both the superficial-dermis and middle-dermis decreased significantly ($p = 0.012$) after UV photo-aging in our experimental model: 60% versus 78.35% in untreated skin specimens.

Injection of mixture of hyaluronic acid and multivitamins had a significant restorative effect on elastic fibres content in experimentally aged skin: 78% after injection in the rapid-pulse mode ($p=0,045$), 78.3 % after injection in the single-pulse mode ($p=0.048$) and 75.94 % after injection in the single-pulse followed by the rapid-pulse mode ($p=0.027$).

Injection of saline solution in the rapid-pulse mode had no effect on collagen content in the dermis.

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4) Biochemical assay of collagen content

Data are presented in **Table IV**.

A statistically significant decrease in collagen synthesis was observed after UV photo-aging in our experimental model: 198.1 µg/mg of protein in control (UV only) versus 235.9µg/mg in untreated skin (p=0.016).

Injection of mixture of hyaluronic acid and multivitamins led to an increase in collagen synthesis in comparison with control skin specimens (UV only) but the difference was not significant thanks to standard deviations. Injection in the single-pulse mode followed by the rapid-pulse mode had the greatest restorative effect on collagen synthesis: 249.3 µg/mg. Injection in the rapid-pulse mode alone also led to an increase: 210 µg/mg.

Injection of mixture of hyaluronic acid and multivitamins in the single-pulse mode and of saline solution in the rapid-pulse mode had no effect on collagen synthesis.

CONCLUSION

The experimental model presented herein, using human skin maintained in survival provided both visual and quantitative evidence that injection using the Biophymed mesotherapy gun in the rapid-pulse (high frequency) mode increases the anti-aging effect of mixture of hyaluronic acid and multivitamins. The enhancement of the anti-aging effect associated with gun injection was documented in terms of a significant increase in the mitotic index in the epithelium (indicative of cell regeneration) and in collagen content (indicative of dermal reconstruction).

Paris, July 24, 2006

Dr S. Boisnic

MC Branchet

The accuracy of unsigned manuscript has been certified by the authors.

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Table I :

Immunohistochemical determination of cell replication

(% of epithelial cells labeled by anti-Ki67 antibodies)

	%
Untreated skin	0,7 ± 0,9
Skin + UV (control skin)	3,9 ± 2,6 * p = 0,007
Skin + UV + injection of mixture of hyaluronic acid and multivitamins in rapid-pulse mode	6,1 ± 3,3
Skin + UV + injection of mixture of hyaluronic acid and multivitamins in single-pulse mode	12,65 ± 8,5 # p = 0,016
Skin + UV + injection of mixture of hyaluronic acid and multivitamins in single-pulse mode followed by rapid-pulse mode	8,45 ± 2,8 # p = 0,028
Skin + UV + injection of saline solution in rapid-pulse mode	4,6 ± 3,3

* : statistically significant difference with untreated skin (Student test for paired data, p < 0.05)

: statistically significant difference with control skin submitted to photo-aging only (Student test for paired data, p < 0.05)

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Table II :

Quantification of percentage of elastic fibres in superficial-dermis and middle-dermis by computer-assisted image analysis

	% in surface derm	% in medium derm
Untreated skin	5,25 ± 2,6	7,5 ± 3,3
Skin + UV (control skin)	2,23 ± 1,4 * p = 0,02	3,9 ± 0,65 * p = 0,03
Skin + UV + injection of mixture of hyaluronic acid and multivitamins in rapid-pulse mode	3,1 ± 1,5	4,1 ± 1,9
Skin + UV + injection of mixture of hyaluronic acid and multivitamins in single-pulse mode	2,9 ± 1,5	5,5 ± 2,95
Skin + UV + injection of mixture of hyaluronic acid and multivitamins in single-pulse mode followed by rapid-pulse mode	4,9 ± 2,36 # p = 0,04	6,6 ± 2,5 # p = 0,04
Skin + UV + injection of saline solution in rapid-pulse mode	2,86 ± 1,2	4,4 ± 1,5

* : statistically significant difference with untreated skin (Student test for paired data, p < 0.05)

: statistically significant difference with control skin submitted to photo-aging only (Student test for paired data, p < 0.05)

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Table III :

Quantification of percentage of collagen content in dermis by computer assisted image analysis

	%
Untreated skin	78,35 ± 7
Skin + UV (control skin)	60 ± 14,35 * p = 0,012
Skin + UV + injection of mixture of hyaluronic acid and multivitamins in rapid-pulse mode	78 ± 6,9 # p = 0,045
Skin + UV + injection of mixture of hyaluronic acid and multivitamins in single-pulse mode	78,3 ± 5,3 # p = 0,048
Skin + UV + injection of mixture of hyaluronic acid and multivitamins in single-pulse mode followed by rapid-pulse mode	75,94 ± 7,4 # p = 0,027
Skin + UV + injection of saline solution in rapid-pulse mode	66,1 ± 9,6

* : statistically significant difference with untreated skin (Student test for paired data, p < 0.05)

: statistically significant difference with control skin submitted to photo-aging only (Student test for paired data, p < 0.05)

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Table IV :

Collagen assay ($\mu\text{g}/\text{mg}$ of protein)

	$\mu\text{g}/\text{mg}$
Untreated skin	235,9 \pm 121,9
Skin + UV (control skin)	198,1 \pm 125 * $p = 0,016$
Skin + UV + injection of mixture of hyaluronic acid and multivitamins in rapid-pulse mode	210 \pm 107,1
Skin + UV + injection of mixture of hyaluronic acid and multivitamins in single-pulse mode	187,55 \pm 93,5
Skin + UV + injection of mixture of hyaluronic acid and multivitamins in single-pulse mode followed by rapid-pulse mode	249,3 \pm 136,1
Skin + UV + injection of saline solution in rapid-pulse mode	189,3 \pm 95,1

* : statistically significant difference with untreated skin (Student test for paired data, $p < 0.05$)

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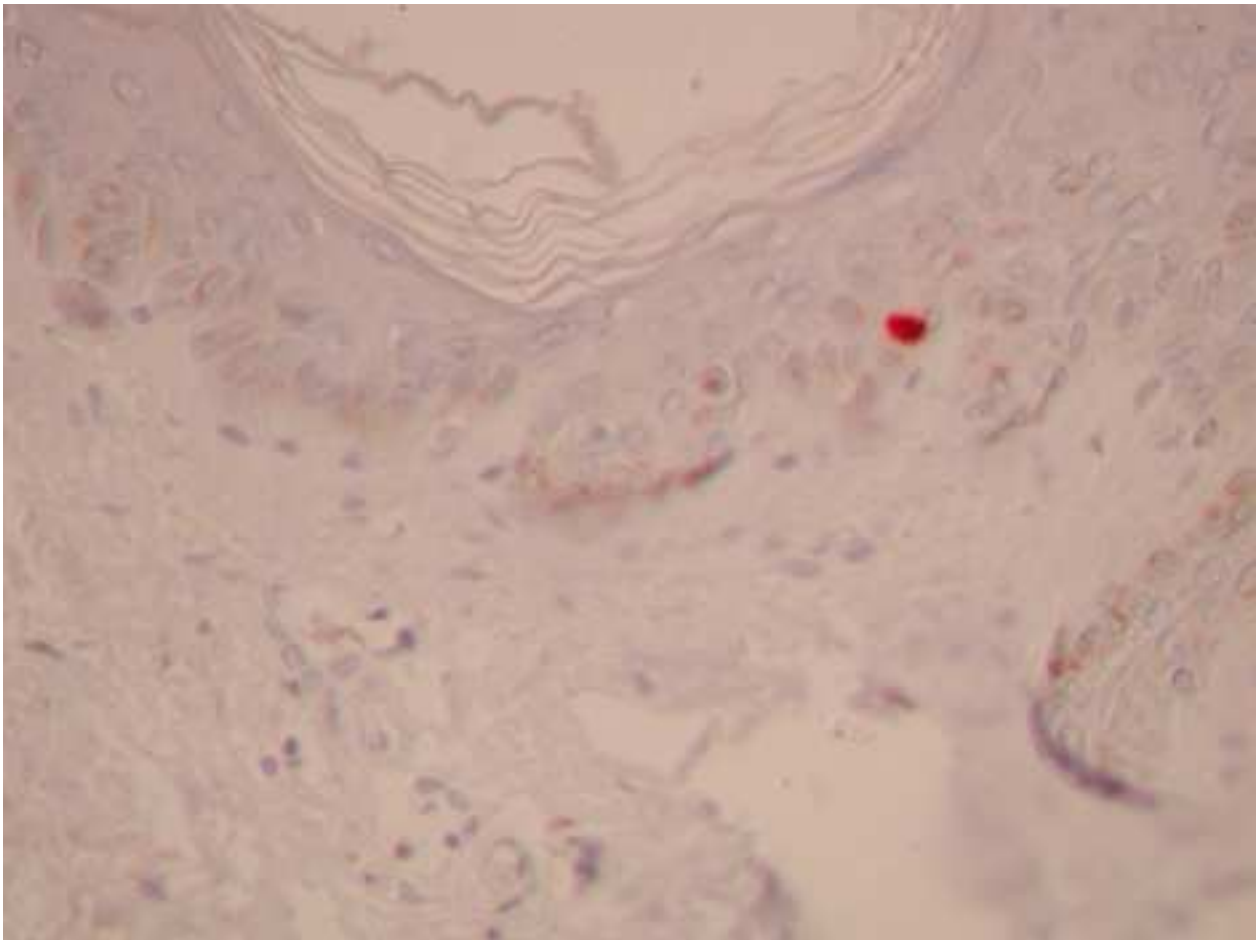
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Figure 1 :

Immunohistochemical determination of mitotic index
in epidermis using Ki67 antibodies

Untreated skin



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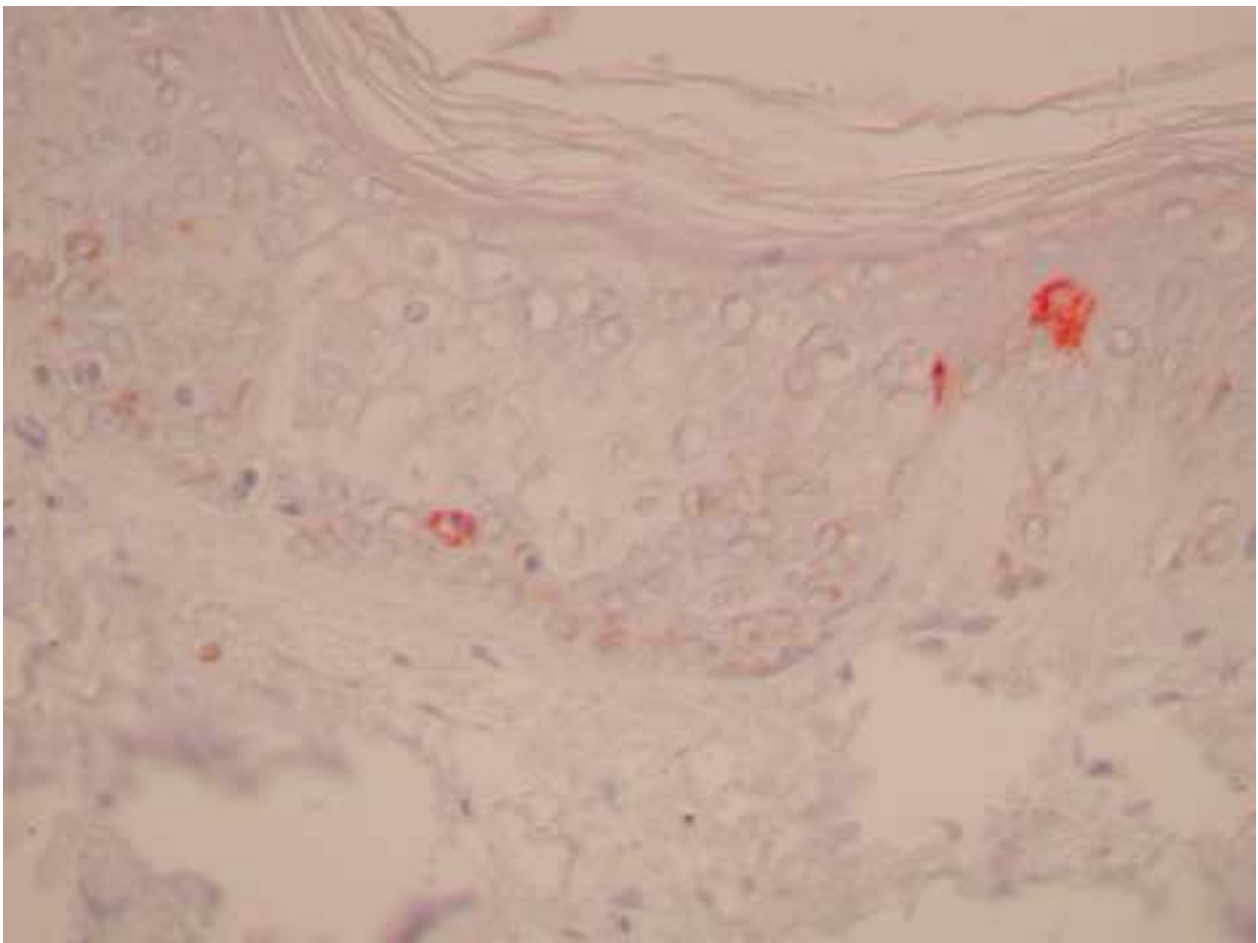
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Figure 2 :

Immunohistochemical determination of mitotic index
in epidermis using Ki67 antibodies

Skin + UV (control skin)



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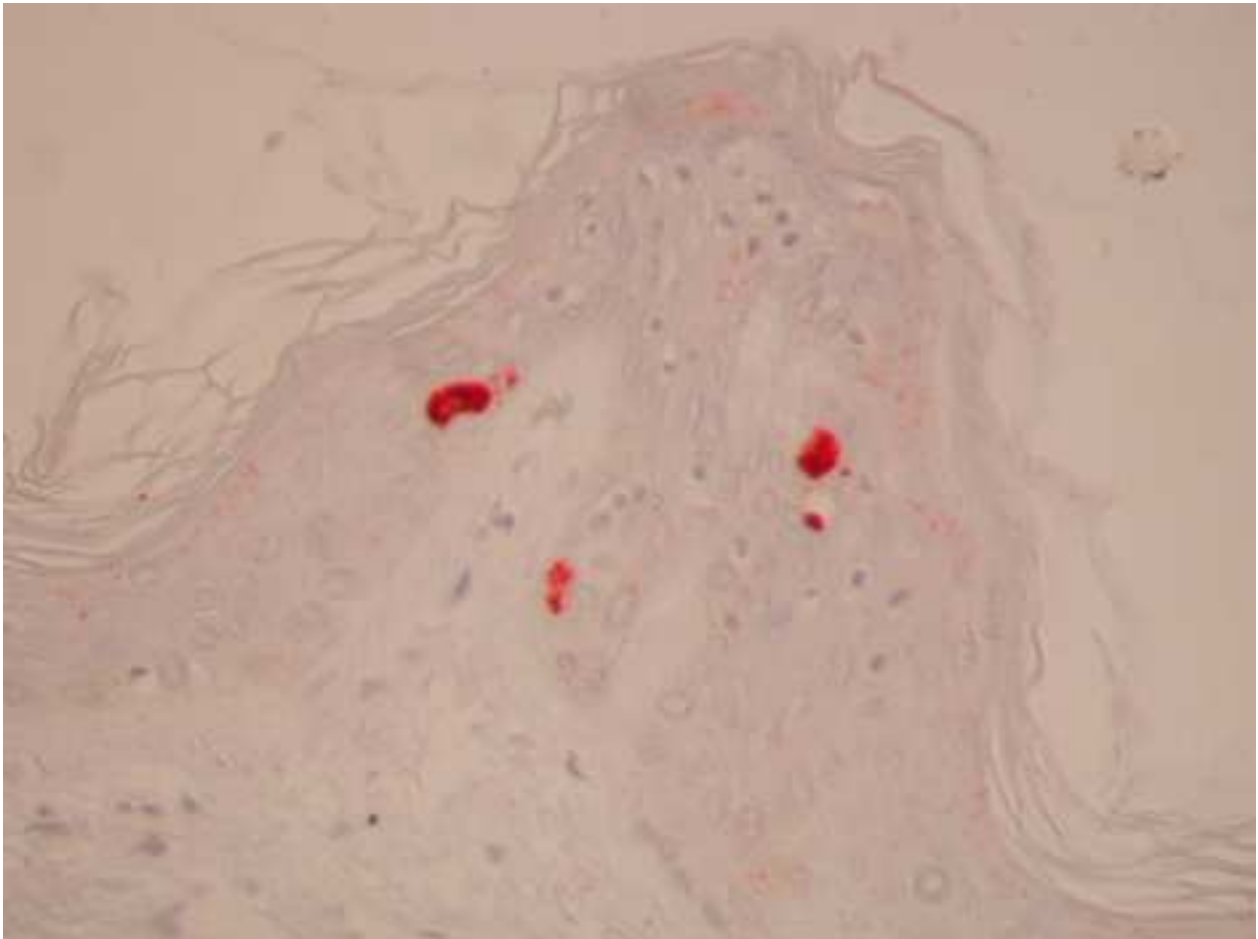
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Figure 3 :

Immunohistochemical determination of mitotic index
in epidermis using Ki67 antibodies

**Skin + UV + mixture of hyaluronic acid and multivitamins
injected in rapid-pulse mode**



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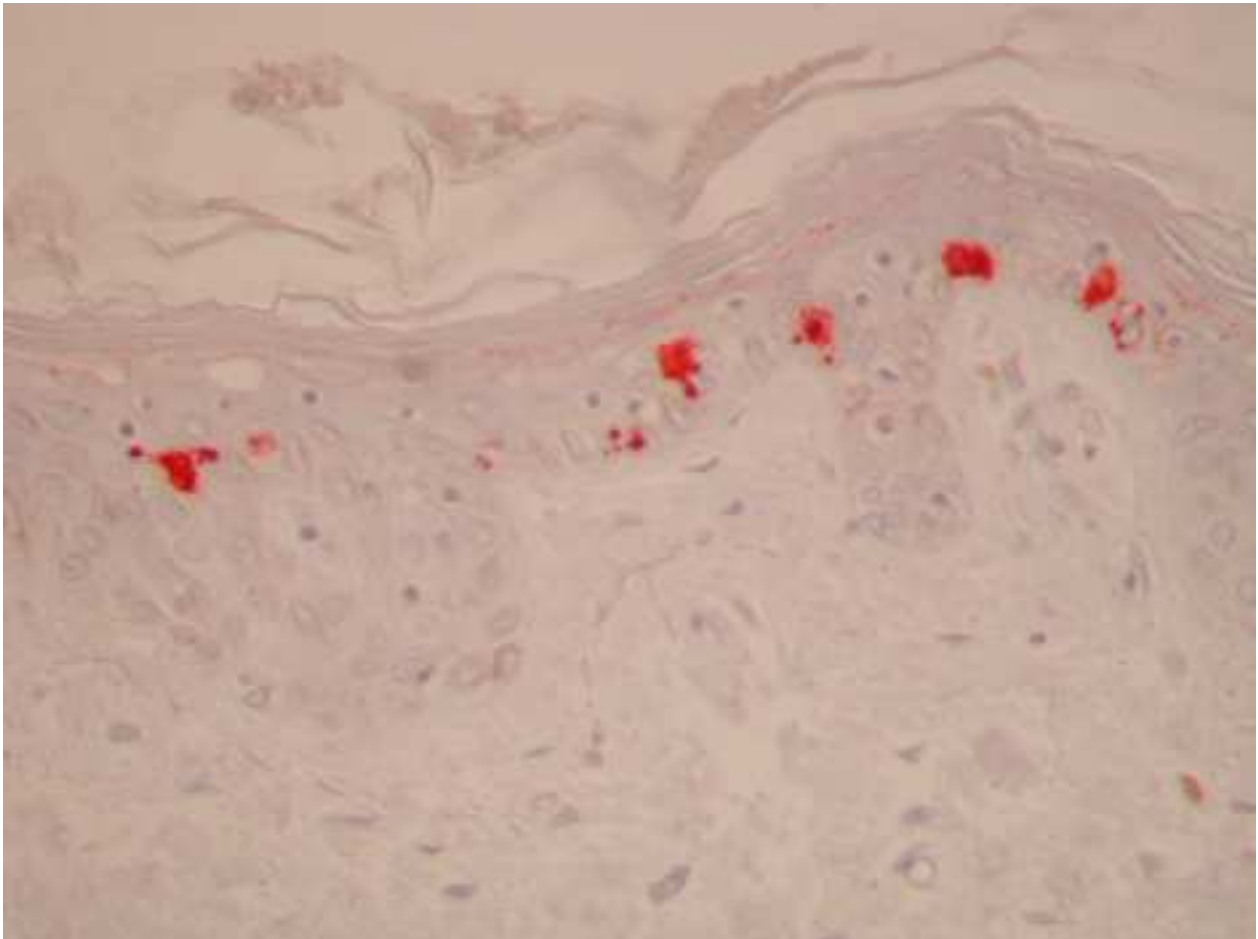
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Figure 4 :

Immunohistochemical determination of mitotic index in epidermis
using Ki67 antibodies

**Skin + UV + mixture of hyaluronic acid and multivitamins
injected **single-pulse mode****



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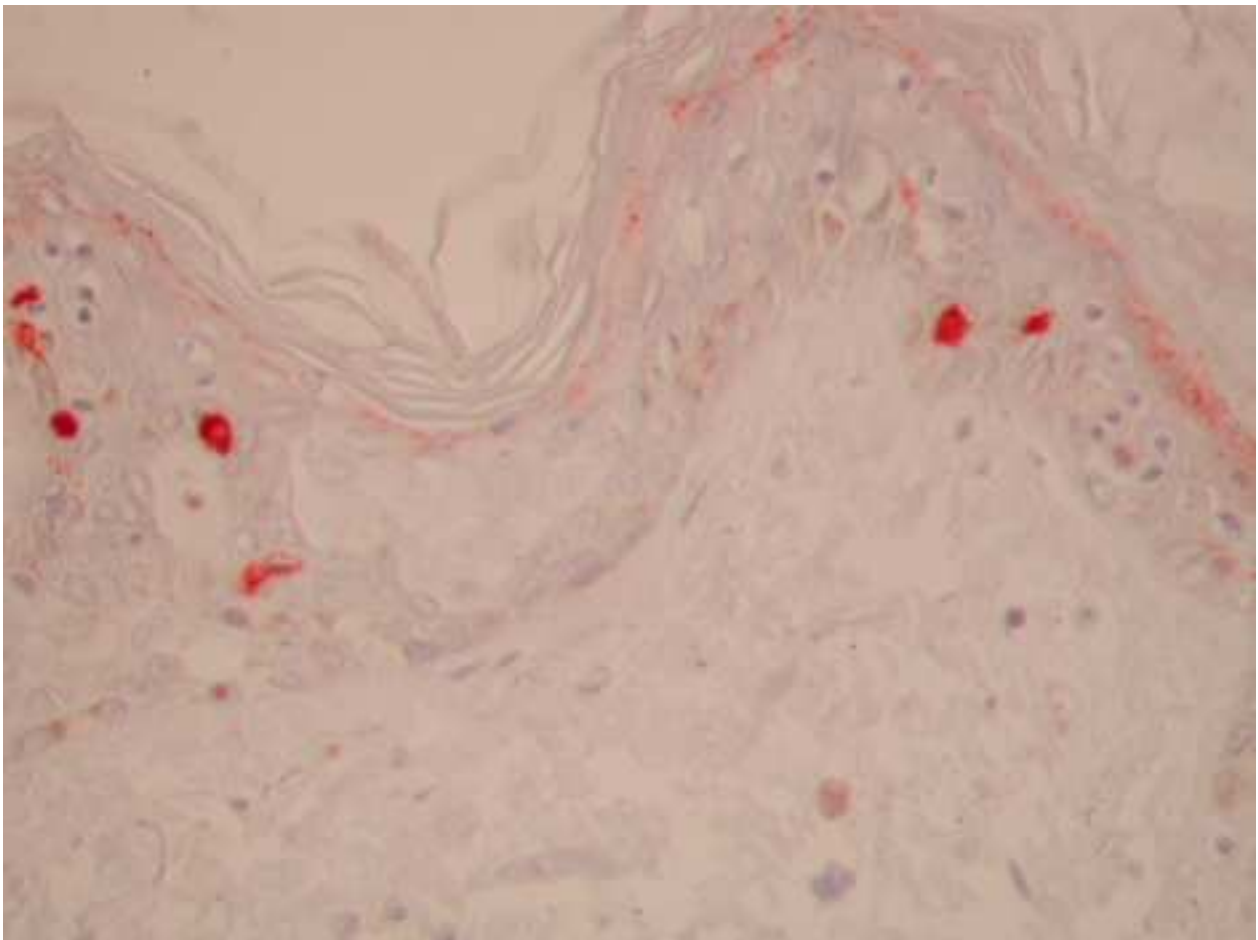
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Figure 5 :

Immunohistochemical determination of mitotic index in epidermis
using Ki67 antibodies

**Skin + UV + mixture of hyaluronic acid and multivitamins
injected single-pulse mode followed by rapid-pulse mode**



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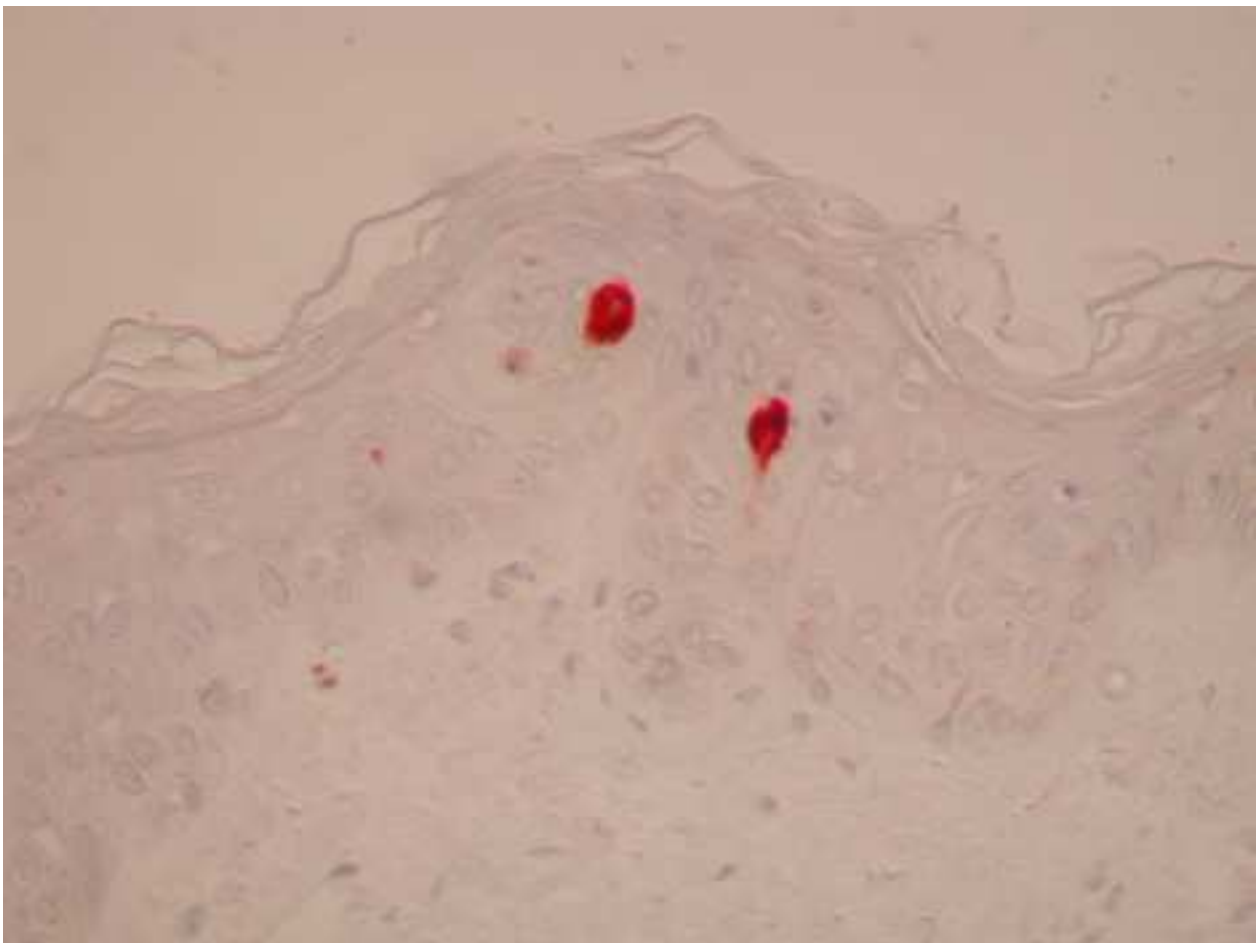
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Figure 6 :

Immunohistochemical determination of mitotic index in epidermis
using Ki67 antibodies

Skin + UV + saline solution injected in rapid-pulse mode



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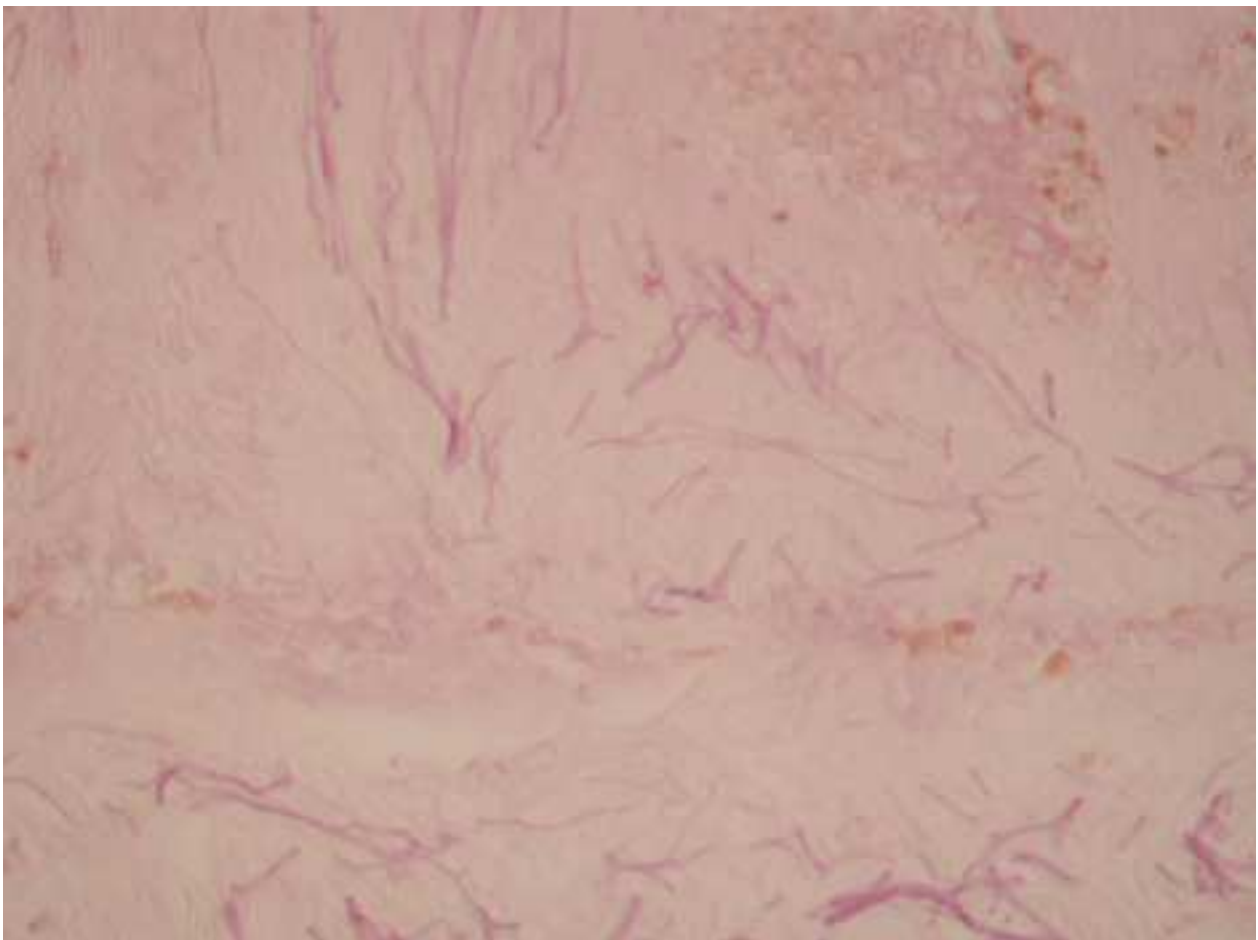
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Figure 7 :

Histological determination of elastic fibres
in epidermis using (+)-catechine

Untreated skin



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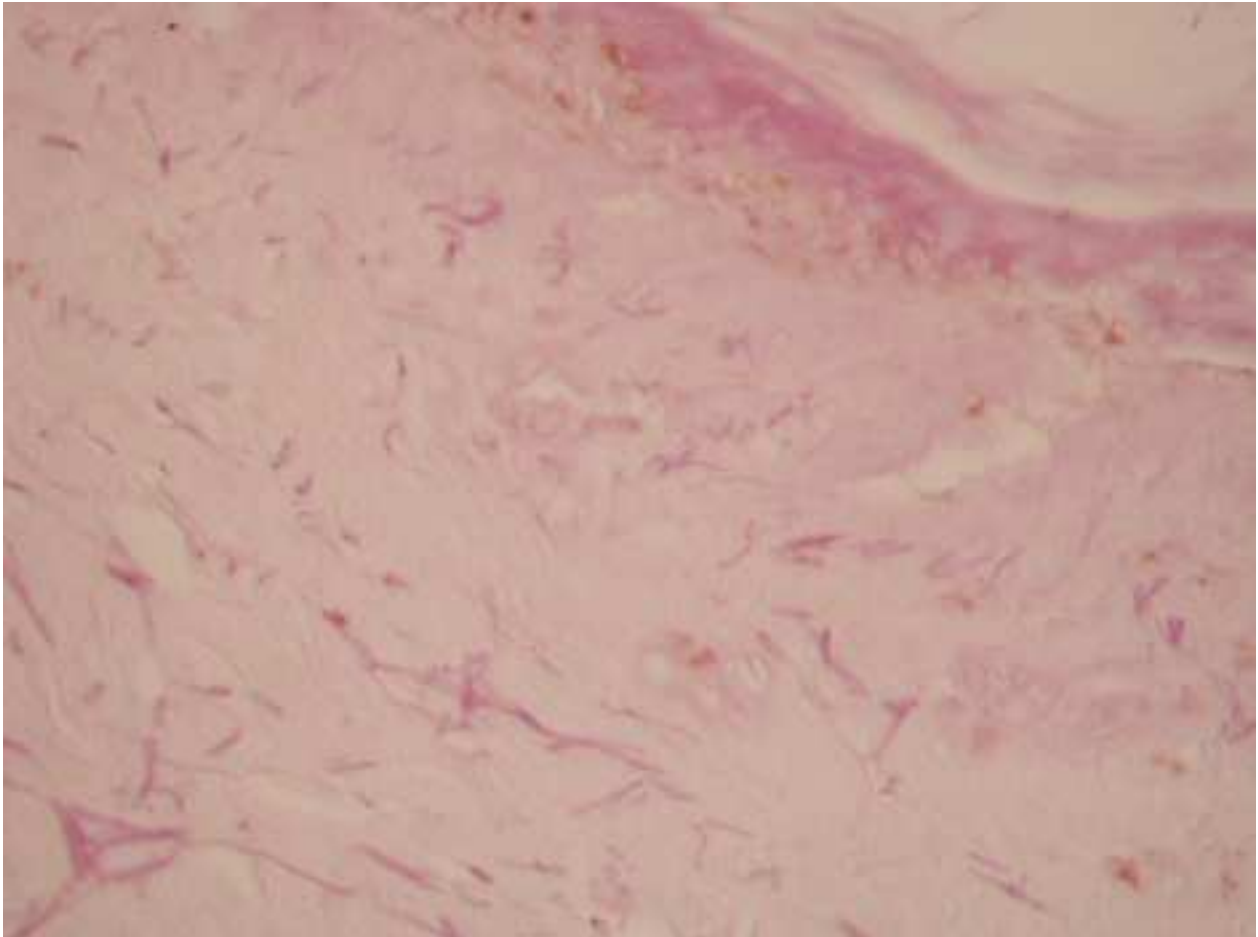
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Figure 8 :

Histological determination of elastic fibres
in epidermis using (+)-catechine:
decrease in extent of network

Skin + UV



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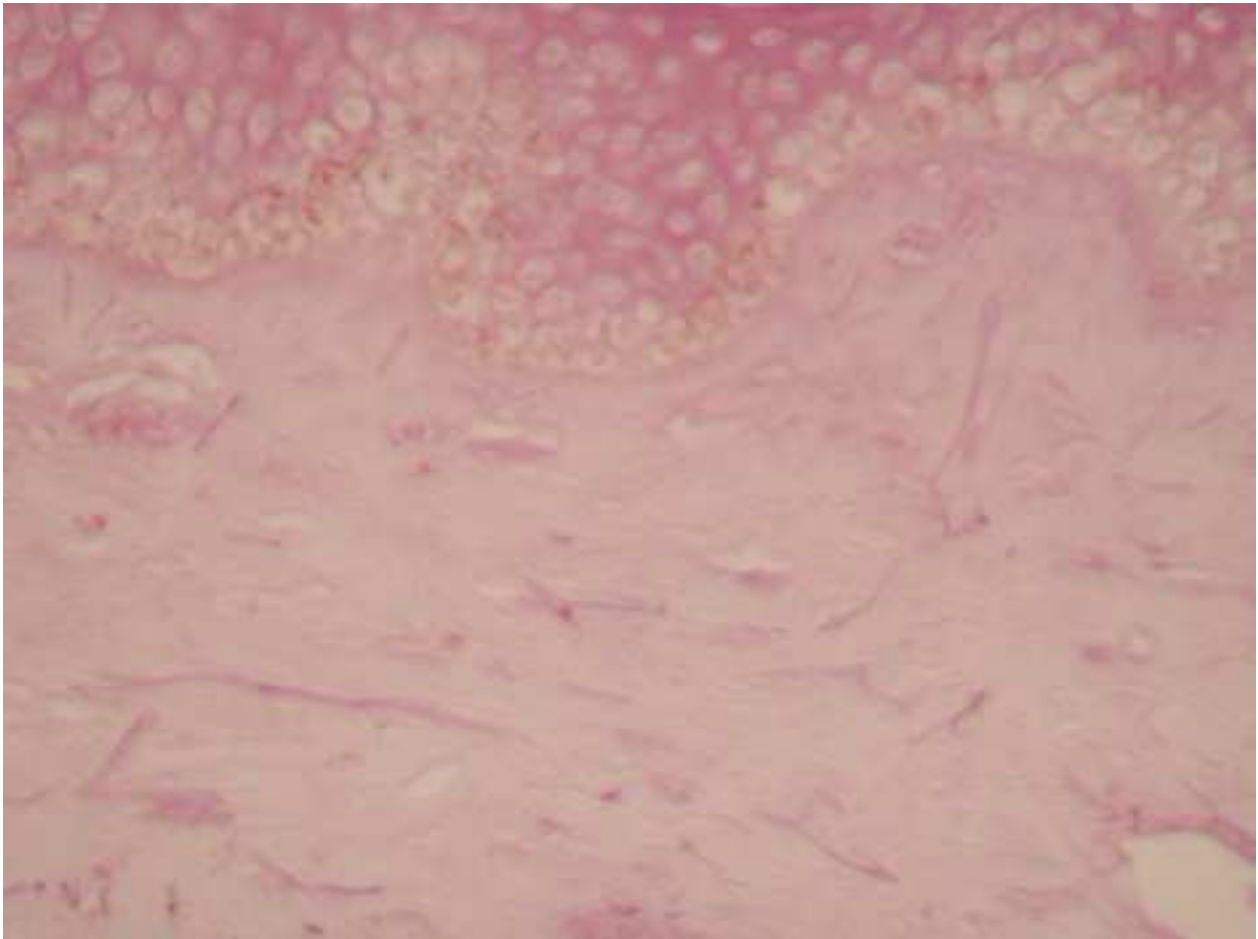
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Figure 9 :

Histological determination of elastic fibres in epidermis using (+)-catechine:
restoration of network

**Skin+ UV + mixture of hyaluronic acid and multivitamins
injected in rapid-pulse mode**



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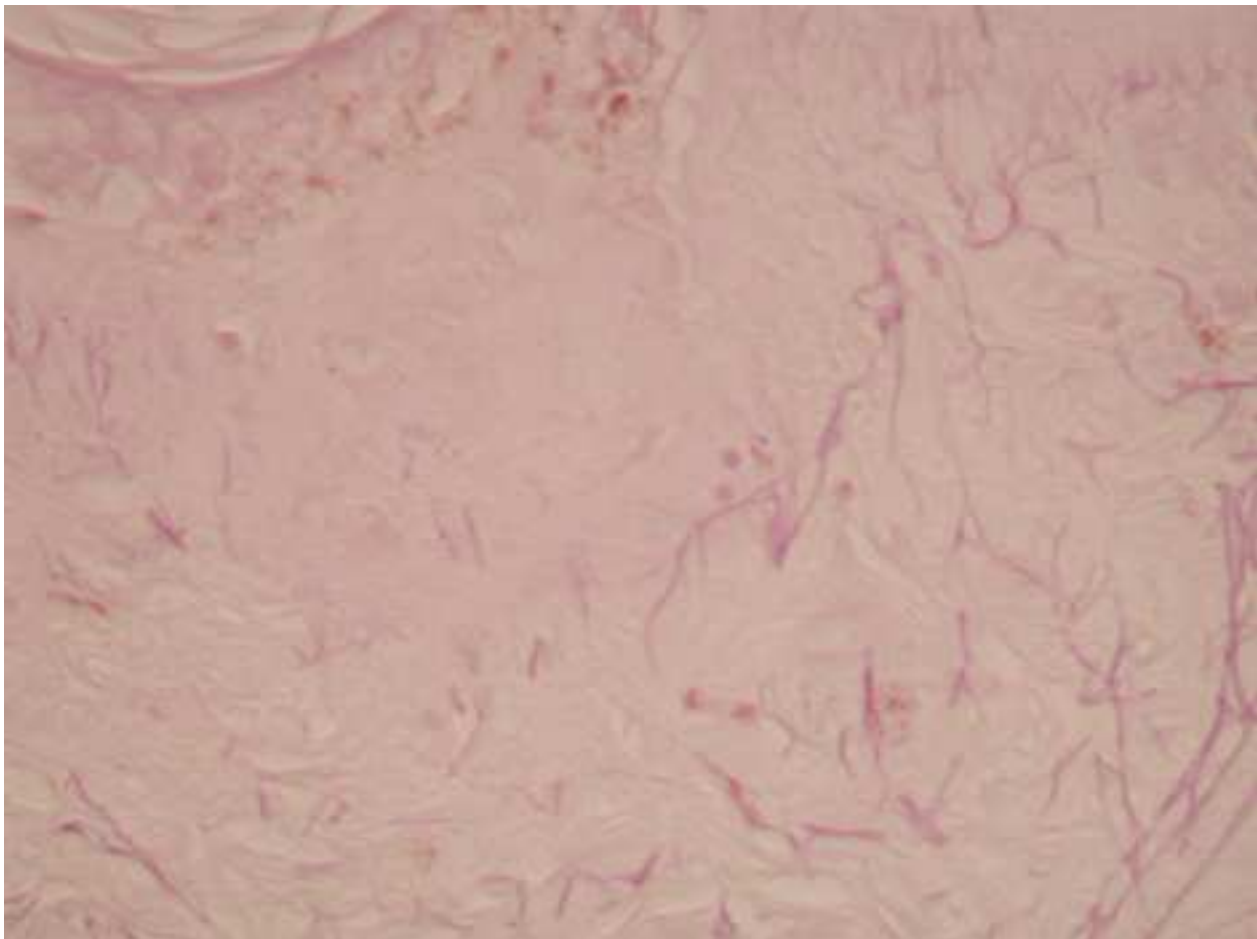
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Figure 10 :

Histological determination of elastic fibres in epidermis using (+)-catechine:
restoration of network

**Skin + UV + mixture of hyaluronic acid and multivitamins
injected in single-pulse mode**



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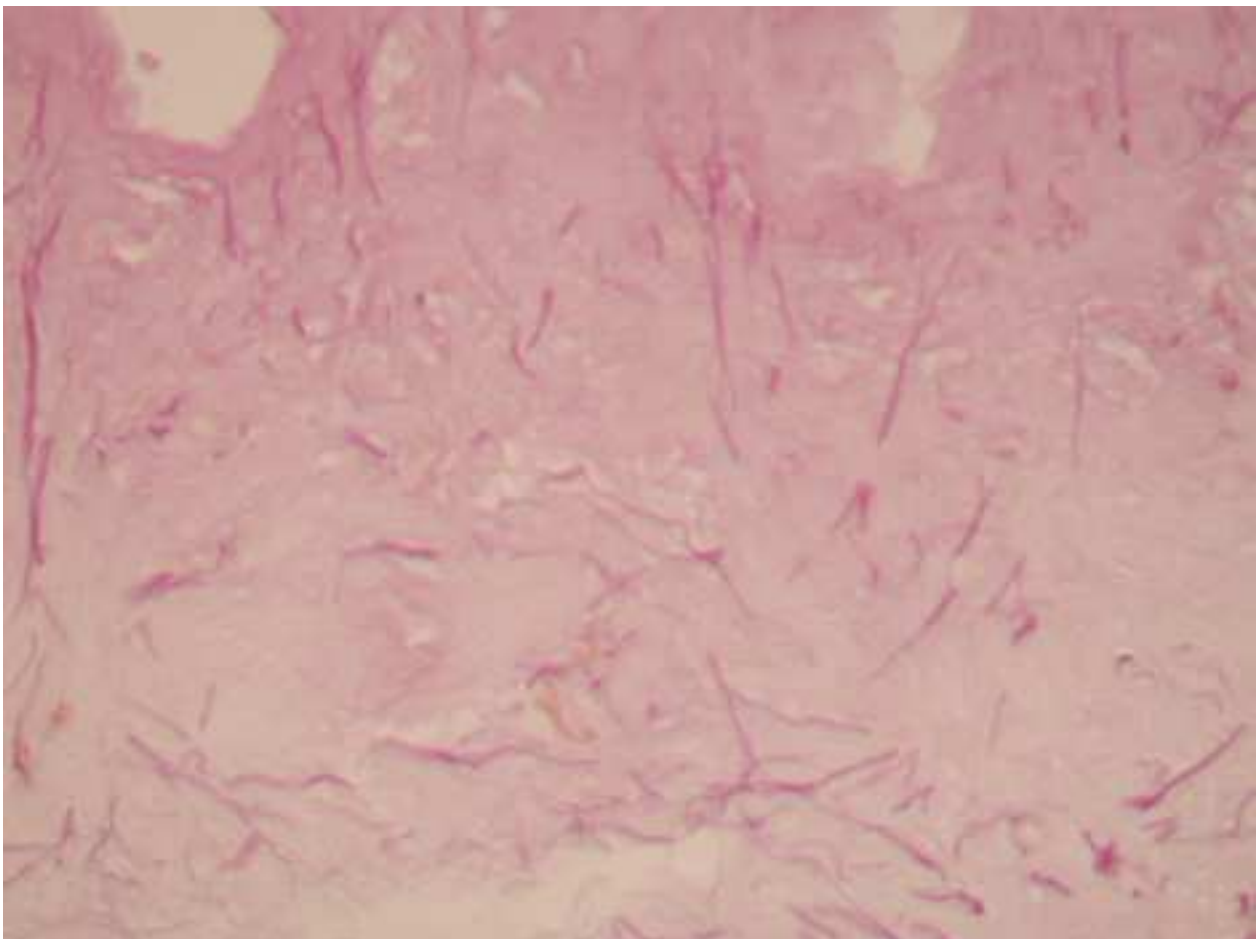
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Figure 11 :

Histological determination of elastic fibres in epidermis using (+)-catechine:
restoration of network

**Skin + UV + mixture of hyaluronic acid and multivitamins
injected in single-pulse mode followed by rapid-pulse mode**



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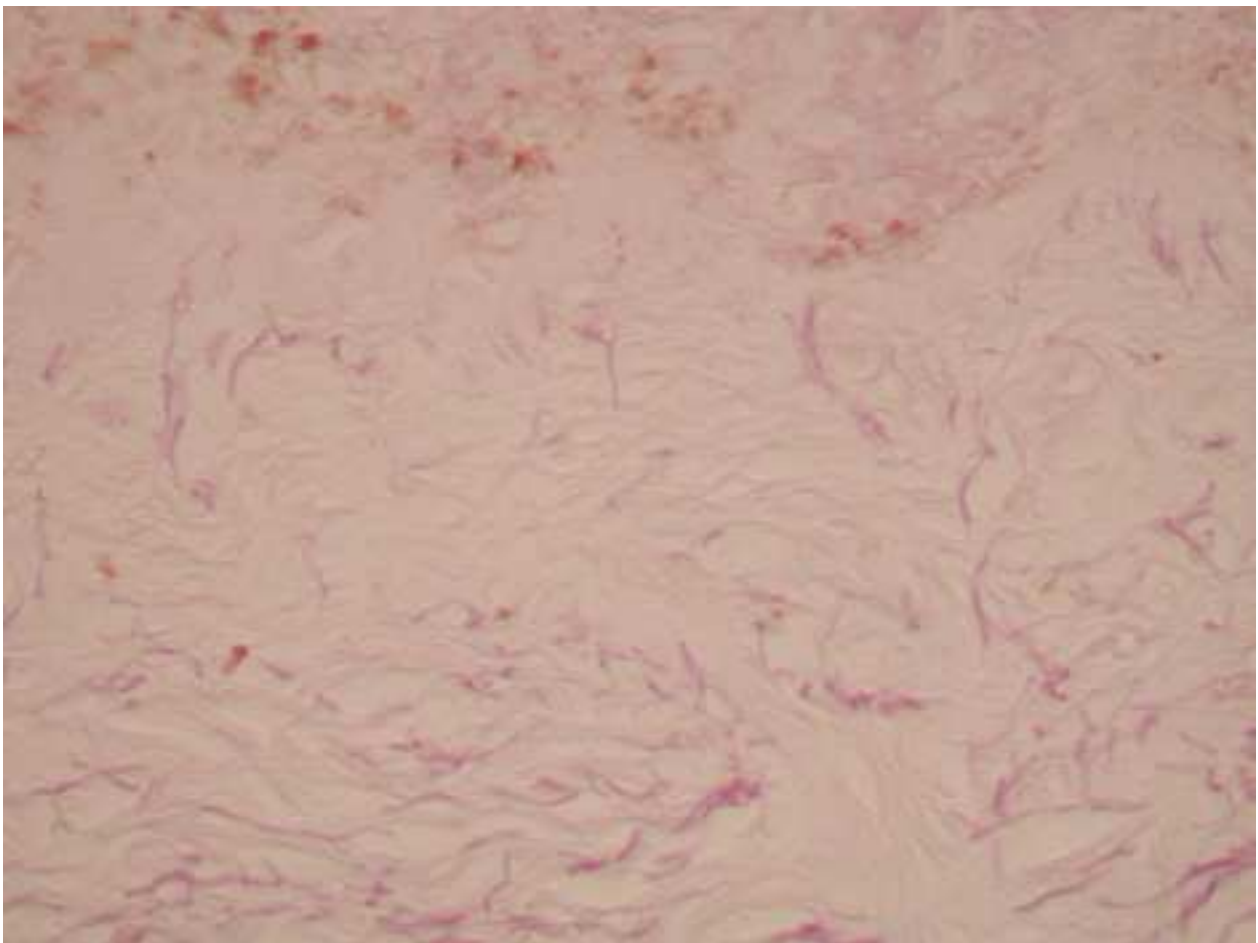
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Figure 12 :

Histological determination of oxytalan-elaunine elastic fibres in epidermis using (+)-catechine

Skin + UV + saline solution injected in rapid-pulse mode



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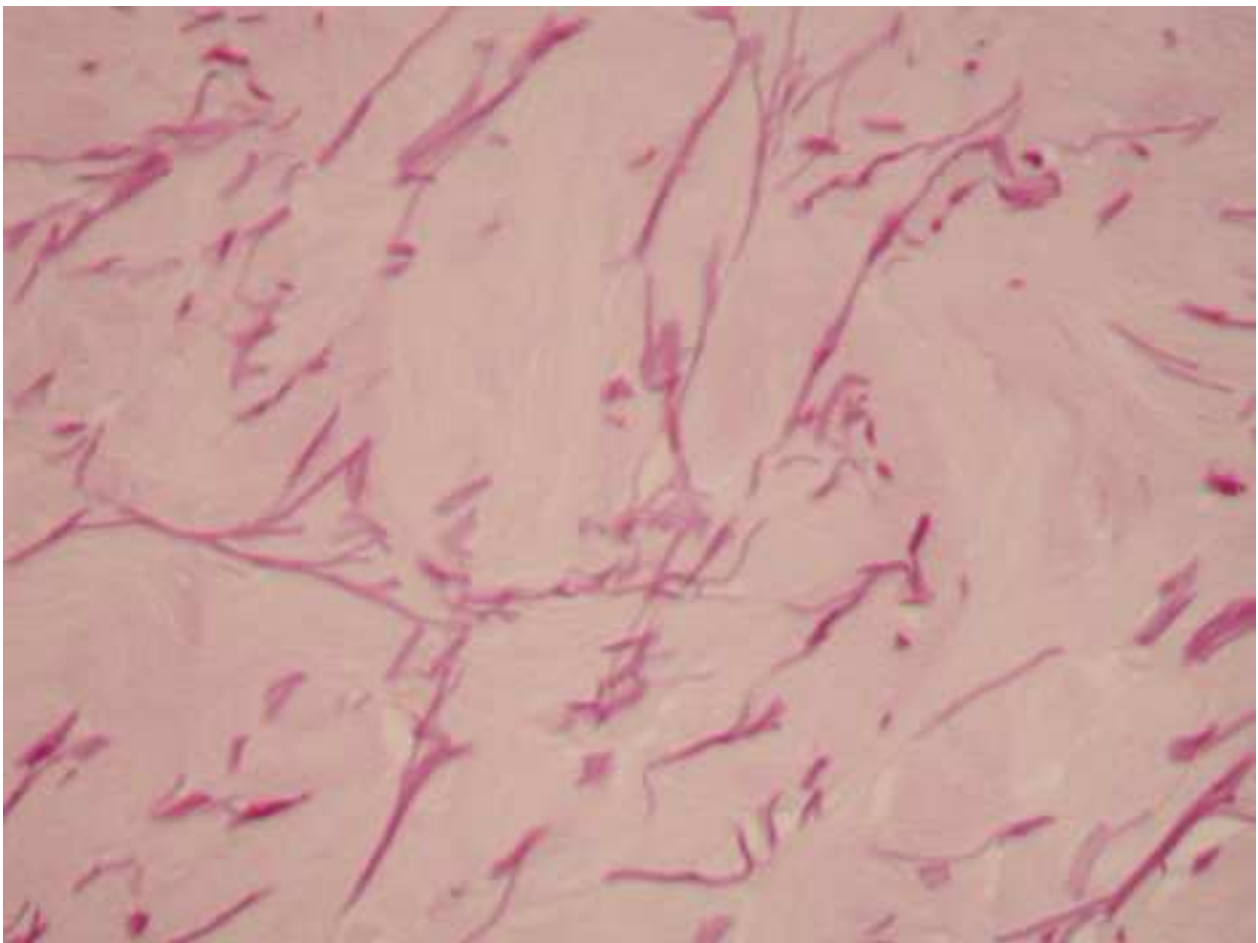
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Figure 13 :

Histological determination of mature elastic fibres
in dermis using (+)-catechine

Untreated skin



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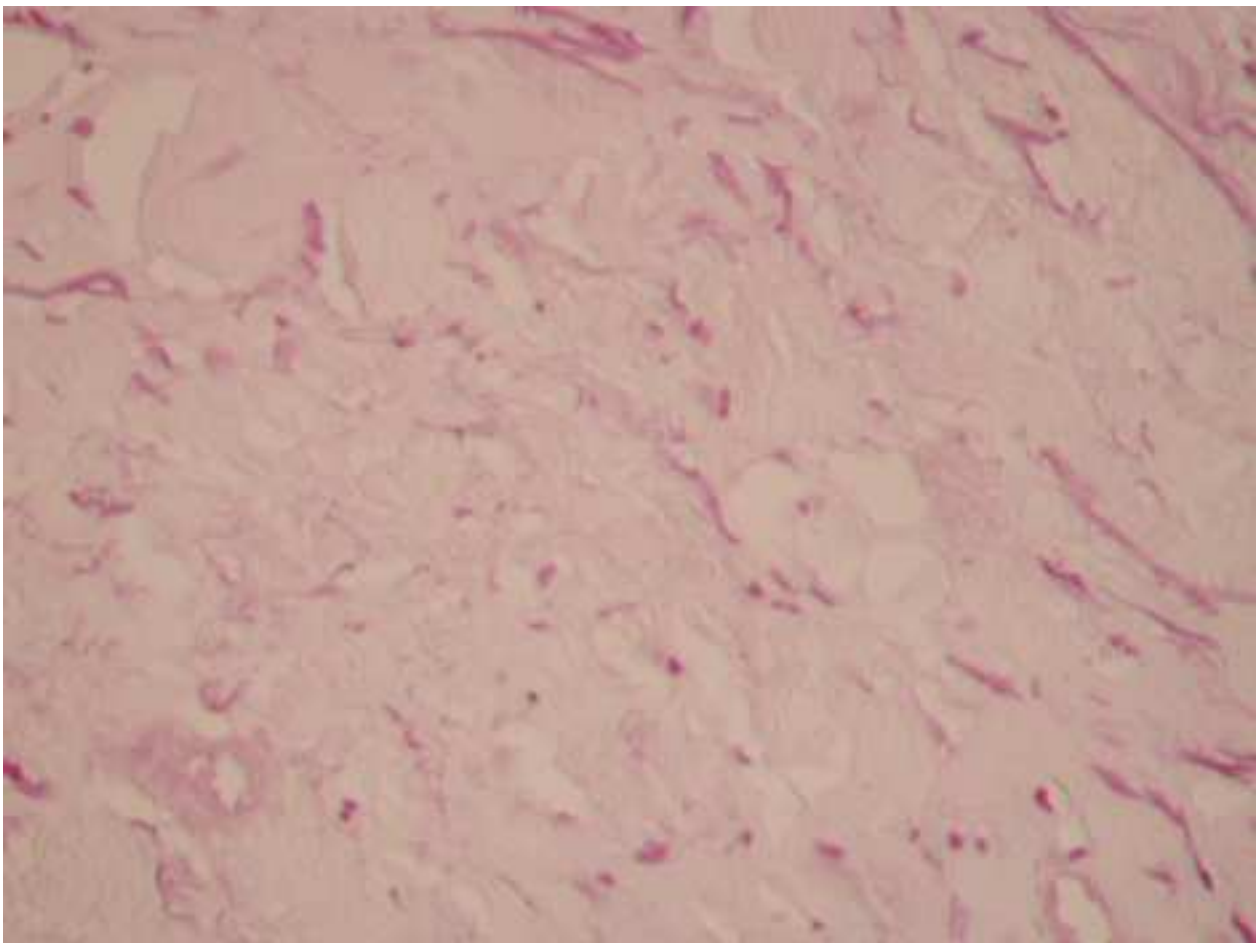
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Figure 14 :

Histological determination of mature elastic fibres in dermis using (+)-catechine:
extensive fragmentation of fibres

Skin + UV



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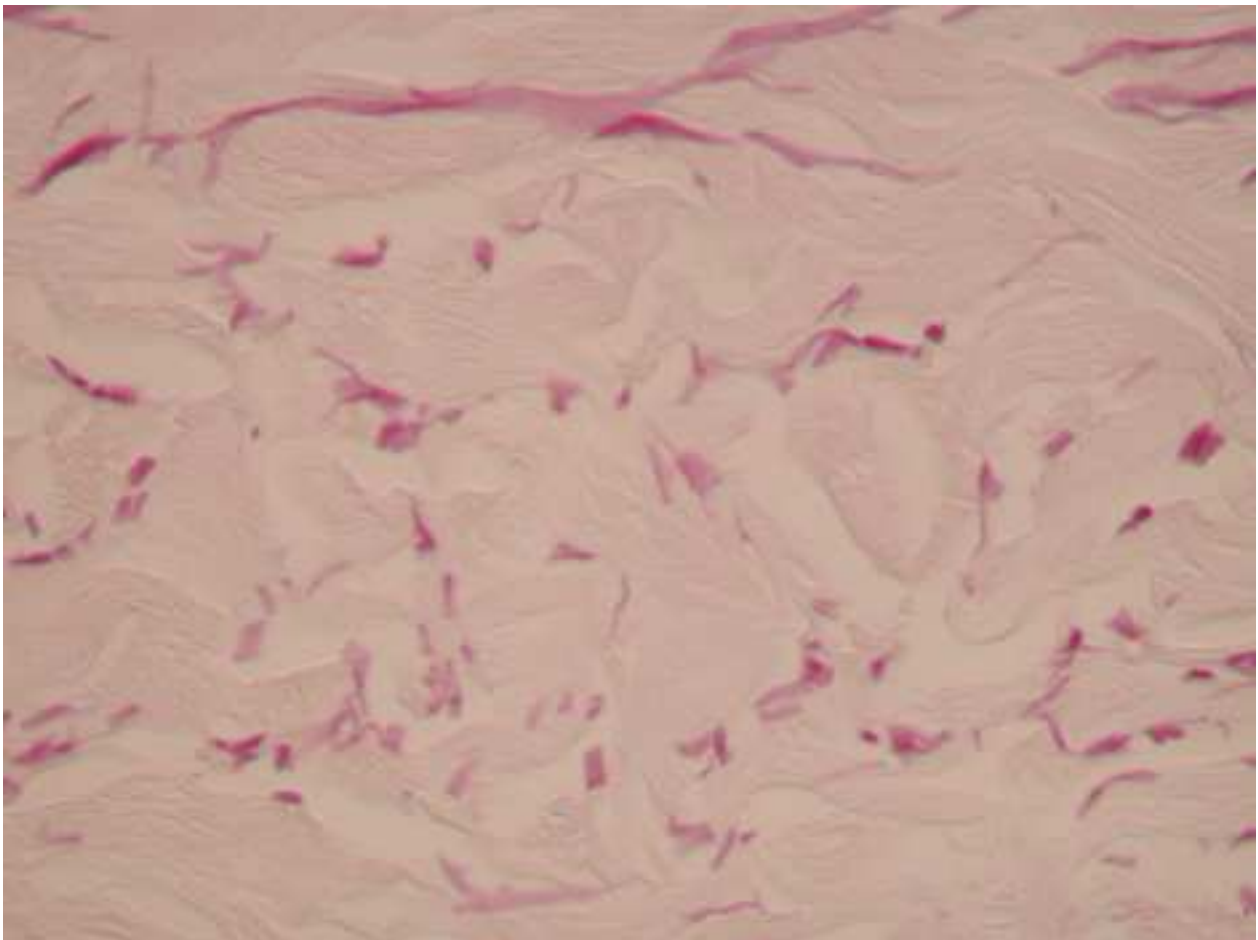
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Figure 15 :

Histological determination of mature elastic fibres in dermis using (+)-catechine

**Skin + UV + mixture of hyaluronic acid and multivitamins
injected in rapid-pulse mode**



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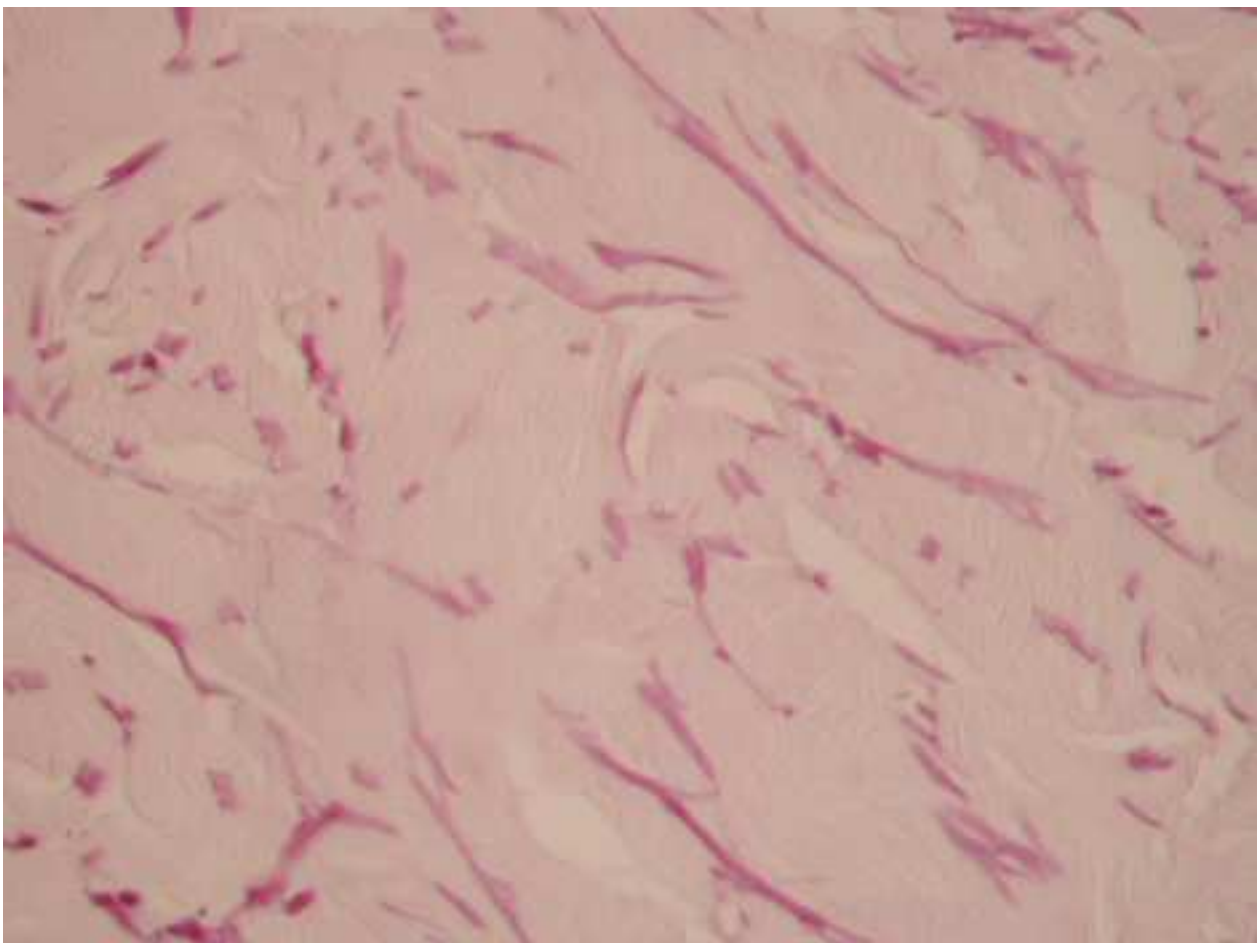
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Figure 16 :

Histological determination of mature elastic fibres in dermis using (+)-catechine:
restoration of network

**Skin + UV + mixture of hyaluronic acid and multivitamins
injected in single-pulse mode**



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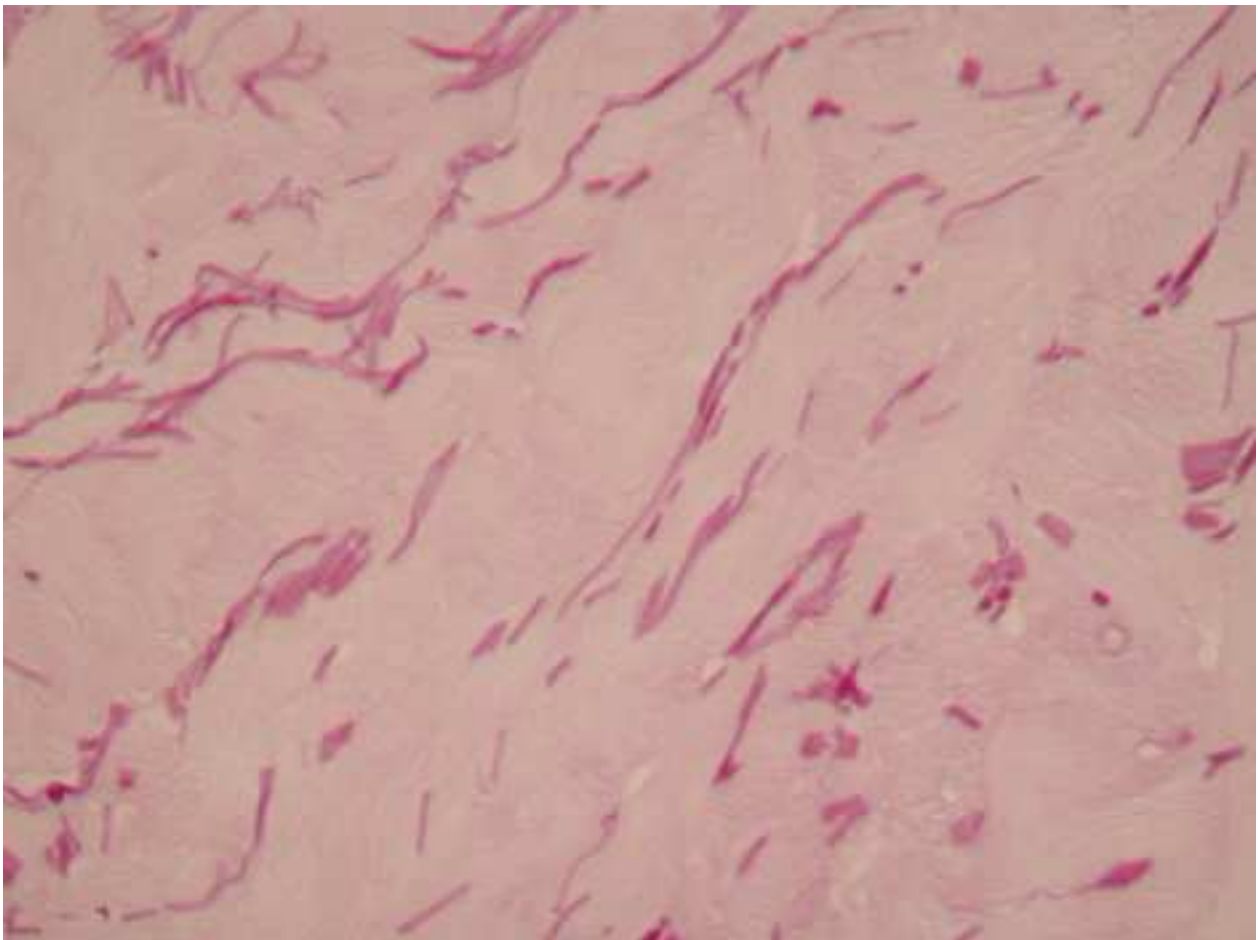
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Figure 17 :

Histological determination of mature elastic fibres in dermis using (+)-catechine:
restoration of network

**Skin + UV + mixture of hyaluronic acid and multivitamins
injected in single pulse mode followed by rapid-pulse mode**



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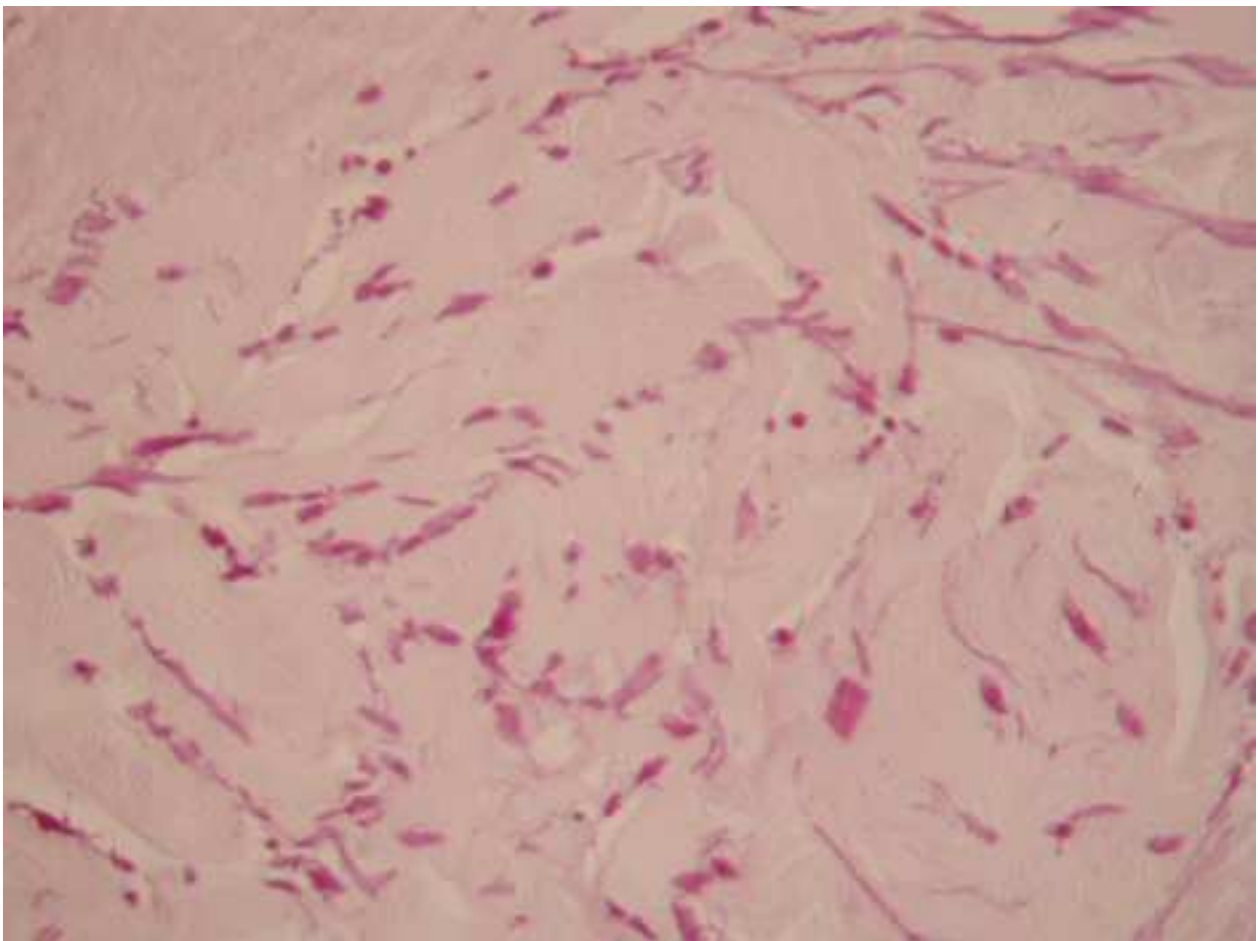
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Figure 18 :

Histological determination of mature elastic fibres in dermis using (+)-catechine

Skin + UV + saline solution injected in rapid-pulse mode



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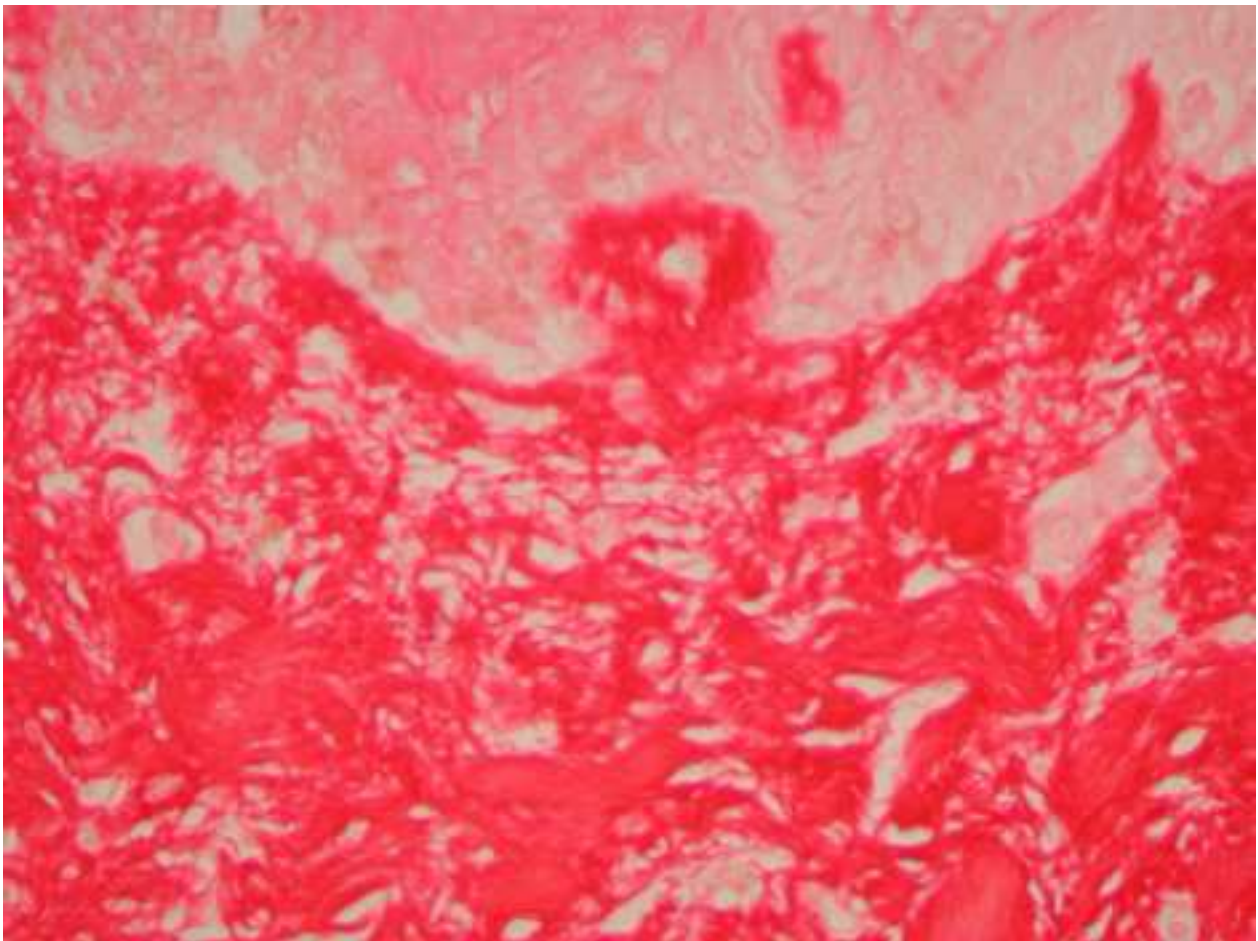
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Figure 19 :

Histological determination of collagen content using sirius red

Untreated skin



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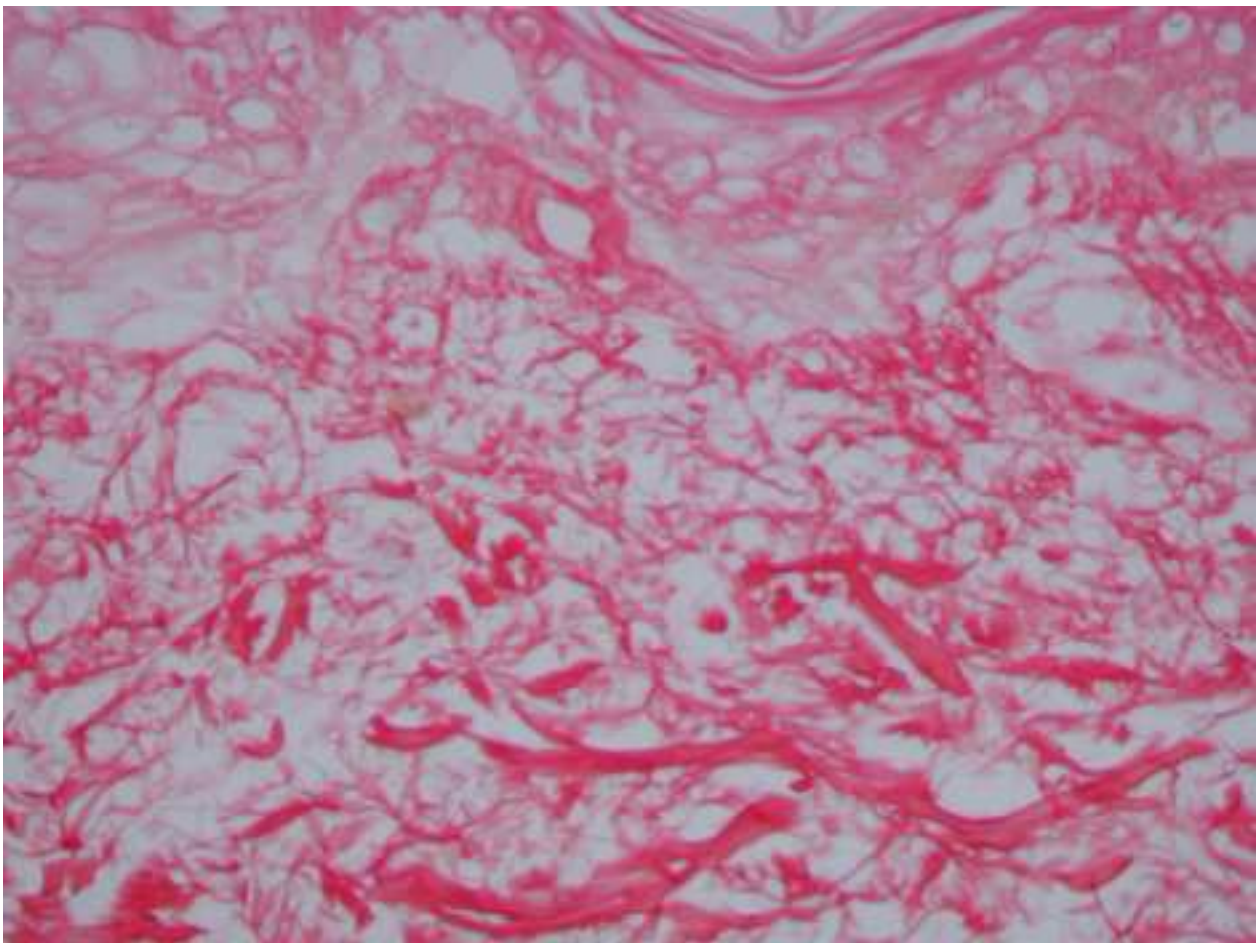
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Figure 20 :

Histological determination of collagen content using sirius red: significantly damaged collagen with fragmentation and decreased coloration of the network

Skin + UV (control skin)



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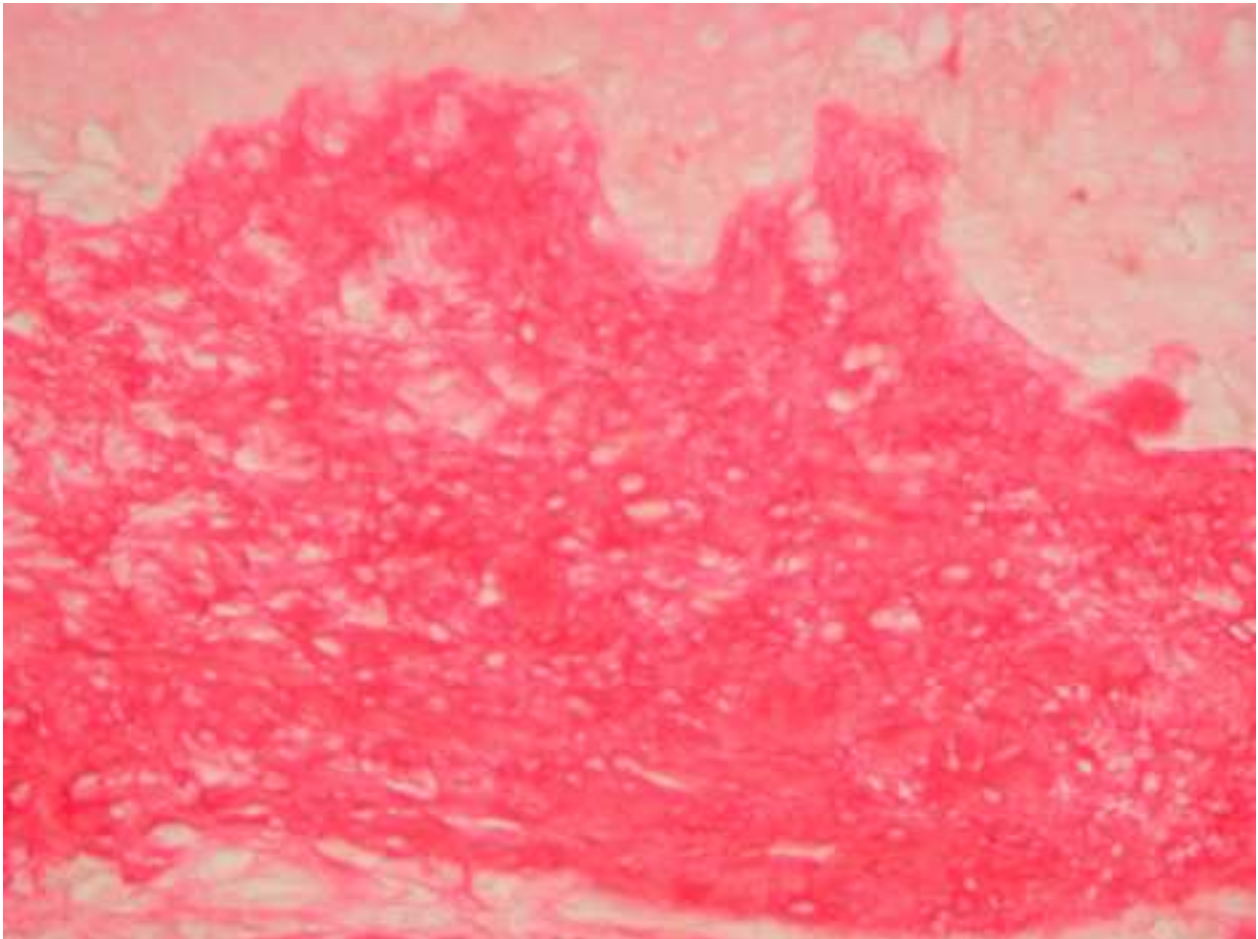
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Figure 21 :

Histological determination of collagen content using sirius red:
restoration of collagen network

**Skin + UV + mixture of hyaluronic acid and multivitamins
injected in rapid pulse mode**



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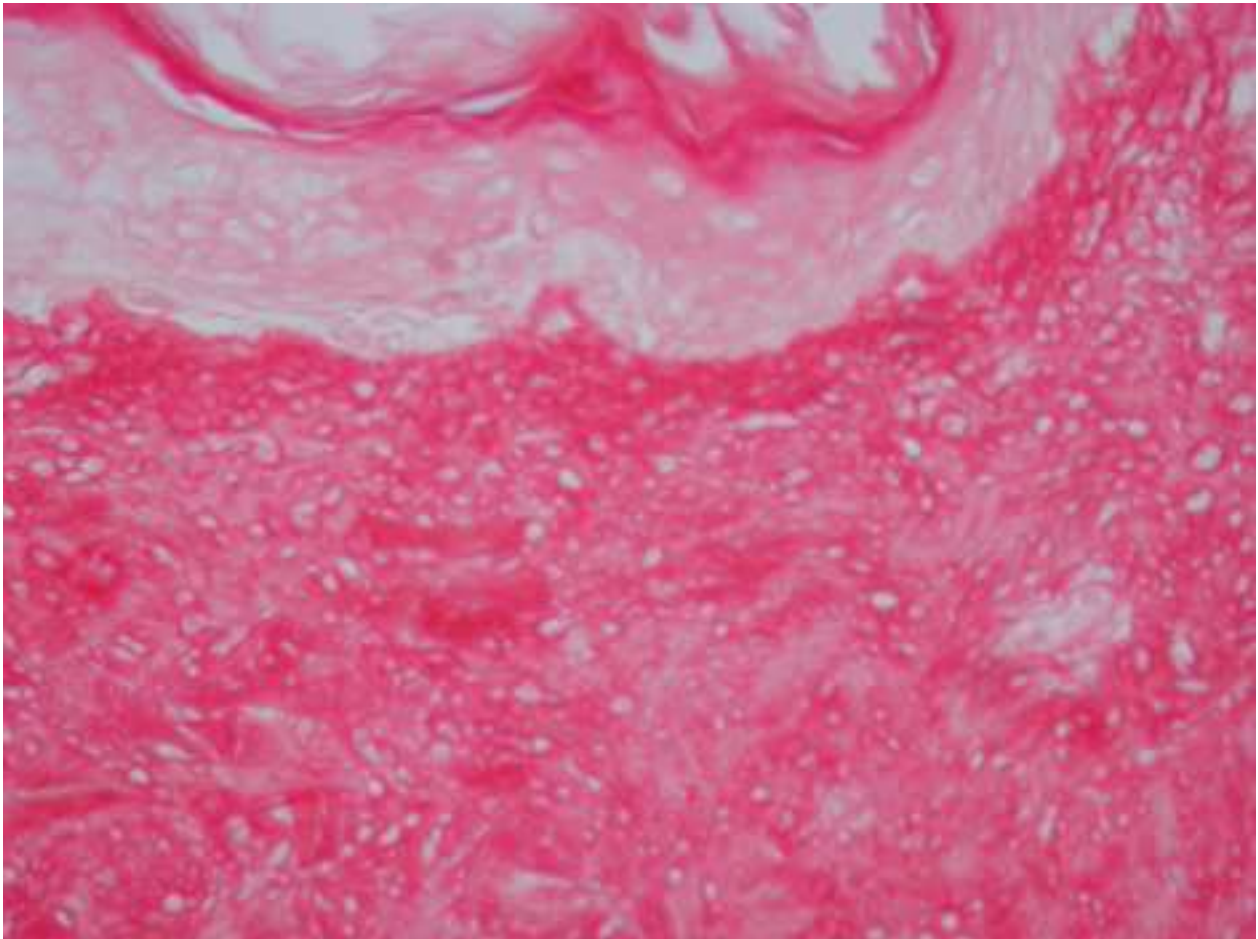
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Figure 22 :

Histological determination of collagen content using sirius red:
restoration of collagen network

**Skin + UV + mixture of hyaluronic acid and multivitamins
injected in single-pulse mode**



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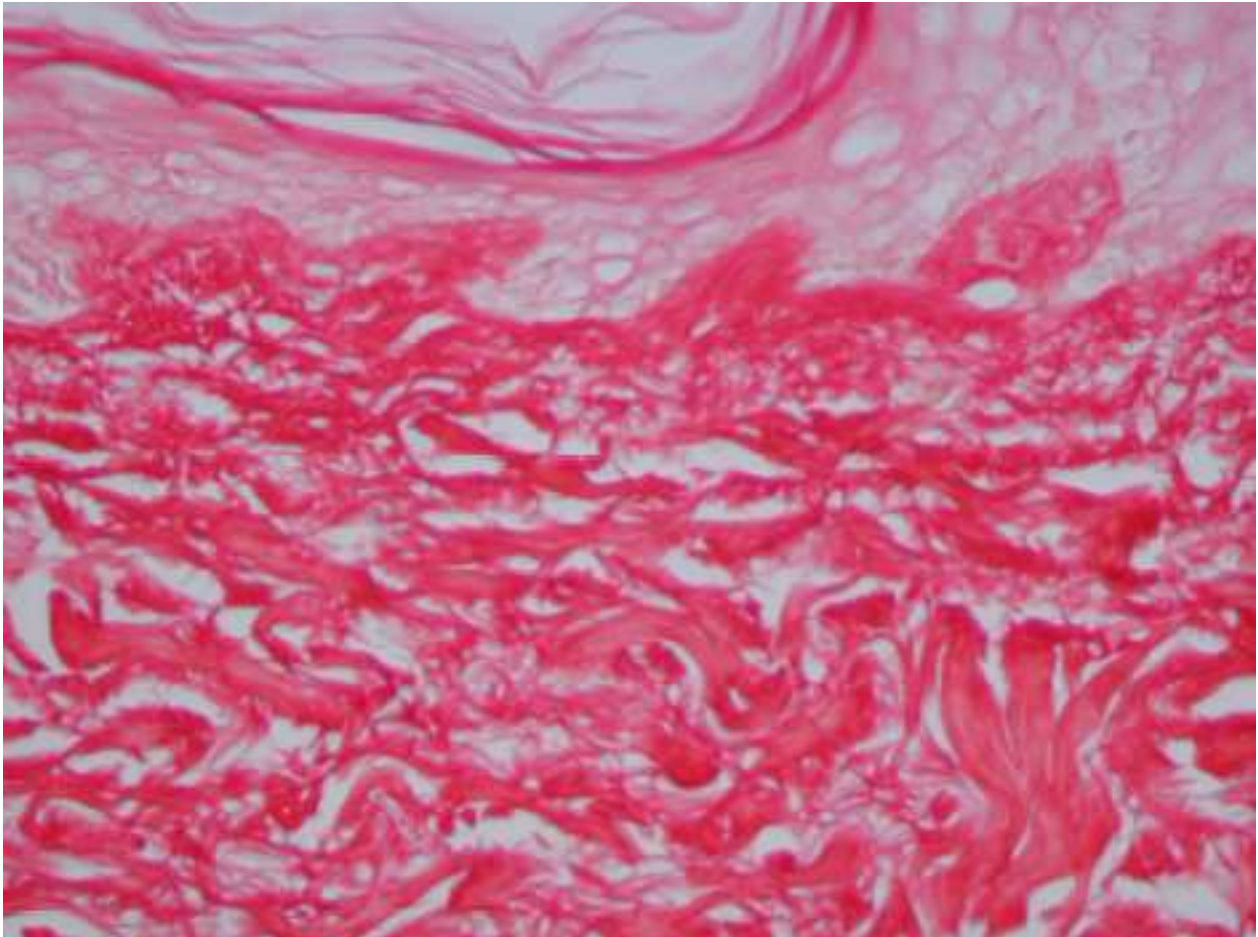
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Figure 23 :

Histological determination of collagen content using sirius red:
restoration of collagen network

**Skin+ UV + mixture of hyaluronic acid and multivitamins
injected in single pulse mode followed by rapid-pulse mode**



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Figure 24 :

Histological determination of collagen content using sirius red

Skin + UV + saline solution injected in rapid-pulse mode

