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Morphological, qualitative and quantitative analysis of transdermal delivery of drugs and biologically active molecules by "Transdermal Therapy"

Introduction

Therapeutic agents and biologically active molecules such as peptides, proteins, oligonucleotides and glycosaminoglycans are characterized by short half-life and low bio-availability; because of such features the correct administration of such drugs is only by parenteral injection, usually performed in clinical environment such as the hospital.

Biological and technological research is now studying new and innovative systems for transdermal drug delivery such as ultrasounds, liposomes, electroporation and iontophoresis; these techniques are able to increase the transdermal delivery of biologically active molecules. This kind of transport is therefore thought to easily deliver drugs and molecules with no significant side effects.

Nevertheless, one of the problems of spontaneous transdermal transport is the low speed of delivery which dramatically reduces its feasibility to few clinical situations. This limit is due to the barrier properties of stratum corneum of skin; this is the outermost skin layer; it is 10-15 μm in thickness and it consists of flat, dead cells, filled with keratin fibers (keratinocytes), surrounded by lipid bilayers. Underlying the stratum corneum there are other layers of cells in different forming the epidermis.

Among the systems commonly used to increase transdermal transport, we can find both systems able to modify the chemical/physical structure of skin and systems, such as ionophoresis and electroporation, which apply an electric field able to drive molecules through the stratum corneum and cells forming the underlying layers. The target of these techniques is to create pores or pathways through the stratum corneum allowing the delivery of molecules which otherwise would cross the skin too slowly or in non significant concentration.

Several studies on transdermal delivery are performed by treating skin biopsies obtained from pig, rat or human cadaver; nevertheless these studies can also be performed on live animal skin by *"in vivo"* systems; these systems consist in performing skin biopsies after the treatment instead of before, creating a situation quite close to that in which a medical operator usually works.

Protocol of the study and methods

In this study we evaluated the effects of transdermal transport by dermoelectroporation on rat skin; in particular in this study we applied bursts of pulsed current delivered by a novel device named "Transderm". The advantage of delivering pulsed currents consists in the significant reduction of degradation, due to electrolysis, of biologically active molecules.
The study is organized in three different sections:

1. morphological microscopic analysis of rat skin after the electric treatment with "Transderm"
2. qualitative analysis of transdermal delivery of type I collagen after the electric treatment with "Transderm"
3. quantitative analysis of transdermal delivery of lidocaine after the electric treatment with "Transderm"

The first part of the study evaluates the integrity and the aspect of histological rat skin structures before and after the electric treatment performed with "Transderm". In fact, it is important evaluate whether the electric treatment creates damages on rat epidermis, dermis and adnexa; in order to rule out this possibility, we compared the histological structures of rat skin treated with "Transderm" with control, untreated, specimens. For this part of the study, we used 2 male Wister rats, age 6-8 month, weight 350 $\pm$ 50 g. Rats were anaesthetized by diethylether in order to correctly perform the electric treatment and the subsequent biopsies. Rats were depilated both by an electric razor and by a manual razor; then a dermoabrasion was performed on selected areas. After the electric treatment, full thickness biopsies were taken both from treated and from control areas. The examination of control, untreated areas is strictly necessary in order to evaluate the possible modifications induced by a 2mA current delivered by "Transderm". Specimens were fixed, embedded and cut; slices were then stained by Haematoxylin-Eosin solution. This staining allows to evidence nucleus (violet) and cytoplasm (pink) of all the cellular population of epidermis and dermis; furthermore, adnexa, such as hair follicles and sebaceous glands, collagen bundles (pink) and fibroblasts (violet) in the dermis are well observed after this staining.

Specimens, after staining, were observed by light microscopy (Nikon Instruments S.p.A., Firenze) at different magnifications (4x, 10x, 20x and 40x); thickness, integrity, and staining both of epidermis and dermis were documented by a digital camera connected to the microscope

The second part of the study consists in performing a comparison between spontaneous delivery of type I collagen and delivery of the same molecule after electric treatment performed by "Transderm". This part of the study provides a qualitative evaluation of transdermal transport of type I collagen from the surface of the skin, where the molecule is applied, to the epidermis and into the dermis; in particular, this evaluation allows to investigate the precise area where collagen is preferentially located both after a spontaneous transport and after a transport mediated by the electric treatment performed by "Transderm". For this evaluation, we used 2 male Wister rats, age 6-8 month, weight 350 $\pm$ 50 g. Rats were anaesthetised by diethylether in order to correctly perform the electric treatment and the subsequent biopsies. Rats were depilated both by an electric razor and by a manual razor, then a dermoabrasion was performed on selected areas. After the electric treatment, full thickness biopsies were taken both from treated and control areas. Half of the selected areas was treated by a solution of collagen-fluorescein at final concentration of 2mg/ml, renewed every 2 minutes during the delivery of the current in order to maintain an uniform availability of substrate and a subsequent continuous delivery of collagen through the rat skin. The other selected areas were treated by the same collagen-fluorescein solution, but not followed by the electric treatment. For the evaluation of the distribution of the collagen in rat skin we used bovine type I collagen conjugated to fluorescein (Sigma-Aldrich), and observations were performed by means of a fluorescence confocal microscope. This evaluation provides a very good three-dimensional visualization of the collagen fluorescein in the examined tissue. At the end of the electric treatment, biopsies both from treated skin and from control areas were obtained; specimens were washed with 10 ml of physiological solution in order to remove the surplus of collagen fluorescein solution then biopsies were freezed. Then specimens were cut and slices fixed by 20 minutes of exposure to vapours of a 0,5% solution of para-formaldehyde. Specimens were then observed at different magnifications, by fluorescence confocal microscope.

The third part of the study examined spontaneous transdermal delivery of radioactive lidocaine and delivery of the same drug associated with the electric treatment performed by "Transderm". For this study a solution of lidocaine HCl [carbonyl-14C] (American Radiolabeled Chemicals, Inc. St. Louis, MO) was applied onto rat skin and its delivery was quantitatively evaluated by a liquid scintillation technique. Also for this part of the study we used 2 male Wister rats, age 6-8 month, weight 350 $\pm$ 50 g, and, as previously described, rats were anaesthetised by diethylether in order to correctly perform the electric treatment and the subsequent biopsies. Rats were depilated both by an electric razor and by a manual razor, then a dermoabrasion was performed on selected areas. A 1:10 diluted solution of radioactive lidocaine (0.483 mg/ml) was applied on selected areas before the electric treatment performed by "Transderm"; on control, untreated areas we applied the same solution of radioactive lidocaine not followed by the electric treatment. After the electric treatment, full thickness biopsies were taken both from treated and from control areas. Biopsies were soaked in 7 ml of physiological solution in order to remove non specific radioactivity; then specimens were washed twice (5ml/wash) with physiologic solution. Specimens were then dried and weighed, then each specimen was cut in small pieces by surgical scissors and scalpel to reduce the size of the fragments. This treatment is necessary for the subsequent incubation in collagenase and Trypsin-EDTA solutions. In order to measure the radioactivity emitting from the specimens, the tissue needs to be broken into very small pieces such as a pulp. For its complete demolition the tissue has been incubated for 48 hours in a collagenase10 mg/ml and trypsina-EDTA 25 mM solutions, in continous shaking inside a cell incubator (temperature of 37°C and 5% CO_2). The collagenase solution is necessary for the complete digestion of collagen bundles in the dermis while the trypsina-EDTA solution is used for the dissolution of intercellular bridges and of junctional complexes among cells of epidermis. After 48 hour incubation, specimens were transferred into vials for liquid scintillation counting, each containing 5 ml of scintillation liquid; then radioactivity was measured by liquid scintillation techniques. Data were expressed as counts or disintegrations per minute (cpm or dpm) and a ratio with weight, expressed in grams, for each specimens was done.

Results

By light microscopy, after Haematoxilyn-Eosin staining, there are no significant differences between areas treated by “Transderm” and untreated skin areas. Rat skin, as expected, appears quite similar to human skin. On the surface both of treated and of control specimens (Figure 1 and 2) the stratum corneum, partially detached from the other skin layers, is well observed (Fig. 3 e 4). The detachment of the stratum corneum from the underlying layers is a microscopic artefact, commonly observed, which due to the different density of the stratum corneum in comparison to other skin layers. Under the stratum corneum, epidermis is easily observable; it appears such as a strip composed by different layers of cells strictly adherent each other. Epidermis, both in control and in treated specimens (Fig. 5 and 6) has a rippled course, strictly adherent to the dermis below; furthermore it frequently presents, emerging from the dermis, hair follicles and sebaceous glands. Dermis, with its wide thickness, shows both in control and in treated specimens (Fig. 7) a lot of collagen bundles (pink). The bundles both in control and in treated specimens are closely crowed and with different spatial distribution; numerous connective cells such as fibroblasts, commonly present in the dermis, are interspersed among collagen bundles (Fig. 8, 9, 10).

From the observation by fluorescence confocal microscopy of specimens treated by “Transderm”, after application of collagen fluorescein, the following observations are made (Fig. 11 e 12):

- epidermis, with the aspect of a continuous and rippled strip, appears strongly fluorescent (green); in none of the examined field, observed at the different magnifications (10x, 40x, 60x) this structure show any appreciable interruptions or significant modifications.

- under epidermis and in the superficial dermis several molecules of collagen fluorescein are present with their characteristic shape (Fig. 13). The spatial distribution of the fluorescence is not casual; collagen is organized following sloping pathways from the surface to the deeper layers of the dermis. In the inner part of dermis fluorescence is disposed more casually, with no particular, determined spatial orientation (Fig. 11 e 12).

From the observation by fluorescence confocal microscopy of control, untreated specimens, after the application of collagen fluorescein (Fig. 14), the following observations are made:

- epidermis appears such as a continuous, uniform, strip, strongly fluorescent (green).

- both in the outer part of dermis and in the inner part of dermis only a weak, non specific fluorescence is appreciable.

From the quantitative analysis (Graphic 1) of radioactive lidocaine measured by liquid scintillation, after the electric treatment by “Transderm” and after the spontaneous delivery, the following observations are made:

- measurement of radioactive lidocaine, expressed as cpm or dpm/g of tissue, in control untreated specimens, is a value considered as 100.

- measurement of radioactive lidocaine, expressed as cpm o dpm/g of tissue, in specimens treated by “Transderm” delivering a 2 mA current, is a value 3.96 fold higher if compared to the measurement obtained from control specimens.

- measurement of radioactive lidocaine, expressed as cpm o dpm/g of tissue, in specimens treated by “Transderm” delivering a 4 mA current, is a value 4.35 fold higher if compared to the measurement obtained from control specimens.

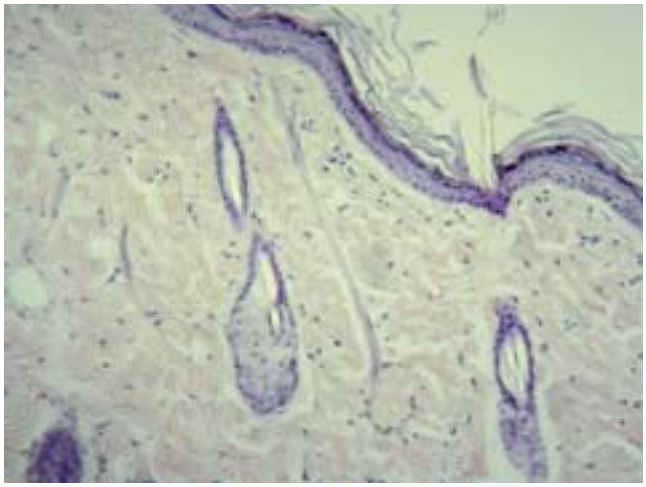
Conclusions

From this study it is clear that the dermoelectroporation treatment performed by "Transderm" does not significantly alter histological structures of rat skin. Both control, untreated areas, and electrically treated areas show well preserved histological structures which normally form the skin. Epidermis does not show any significant modifications at all the different magnifications used; its size, consistency, and staining are normal. Dermis appears dense and composed by numerous collagen bundles differently orientated in the space and among the bundles a lot connectival cells are interspersed; adnexa such as hair follicles and sebaceous glands are normal for shape, distribution and aspect.

Furthermore the dermoelectroporation induced by "Transderm" for the transdermal delivery of molecules such as type I collagen does not cause any significant alteration of the treated tissue even after observation by fluorescence confocal microscopy.

The electric treatment performed by "Transderm" increases the transdermal transport of type I collagen conjugated to fluorescein; collagen fluorescein molecules as single molecules, or more frequently, as clusters, appear in the tissue characterized by a distribution which changes from the surface to the depth of dermis. In the outer part of dermis fluorescence appears having a sloping pathway while going toward the inner part of the dermis distribution shows a less precise organization with fluorescence interspersing among the numerous collagen bundles forming the inner part of the dermis. In control, untreated specimens, there is no significant fluorescence; collagen fluorescein is observable only on the surface of the skin where the solution was applied while no fluorescence is present into the dermis.

Furthermore from this study emerges also that the transferral delivery of local anaesthetic commonly used such as lidocaine is strongly increased after the dermoelectroporation delivered by "Transderm" in comparison to the spontaneous delivery. The amount of lidocaine measured in the tissue after the electric treatment is about 4 fold higher than the amount measured in control untreated specimens. Then, even though more studies are necessary to better clarify this aspect, we observed a sort of dose-response curve which shows a progressive increase of the transdermal delivery of lidocaine with the increasing of the intensity of current applied (2 mA and 4 mA).



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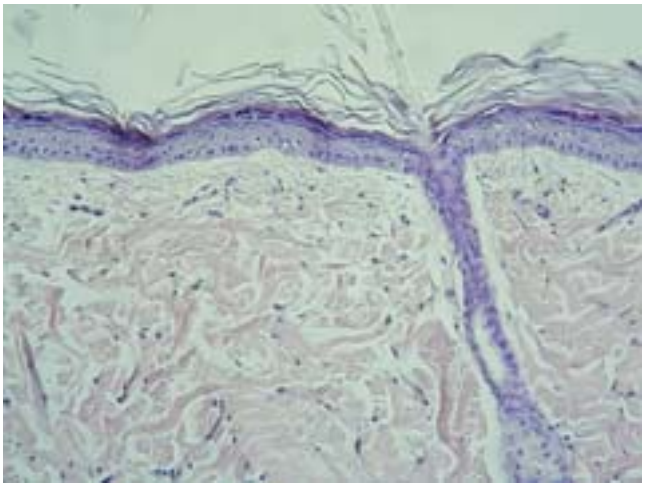

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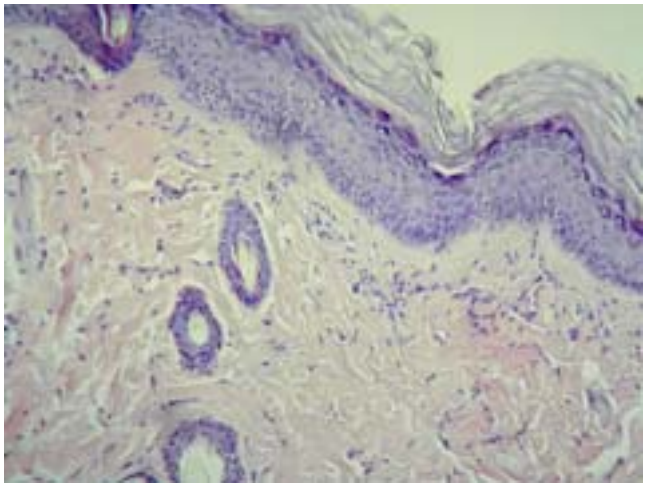

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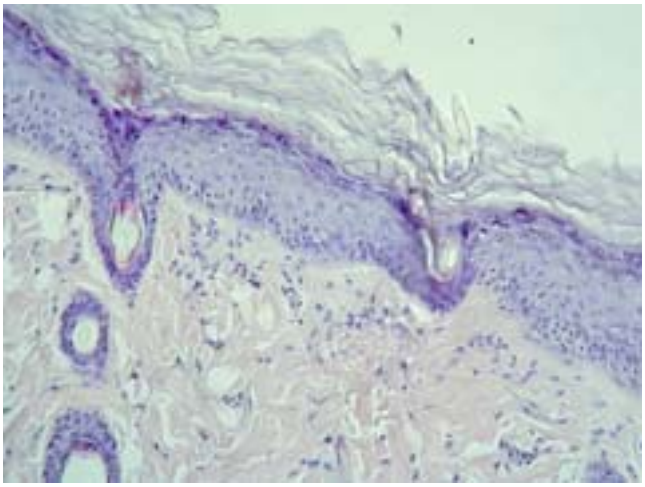

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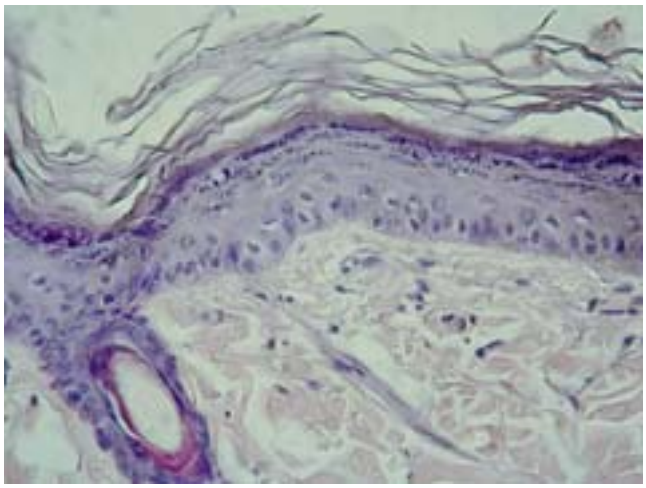

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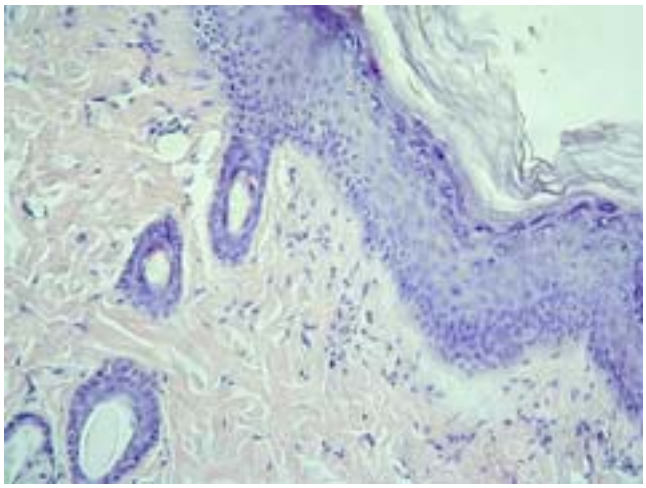

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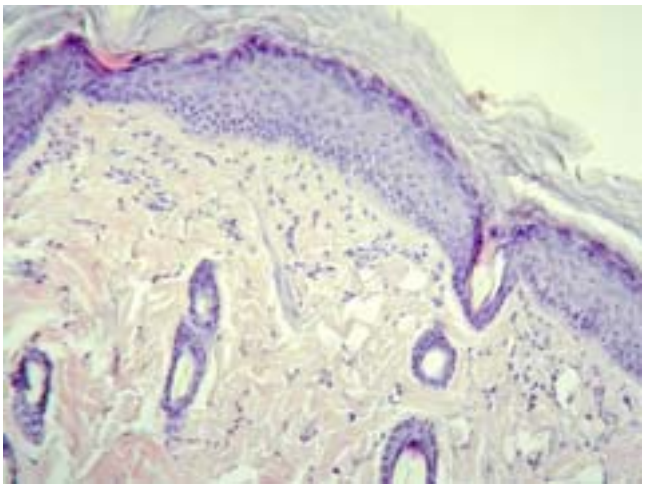

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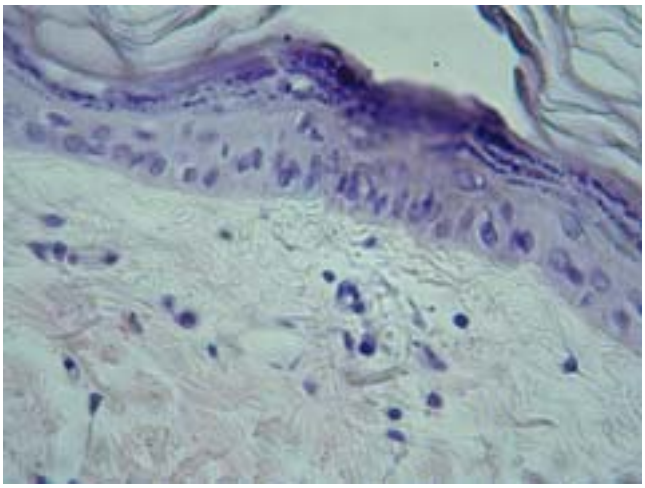

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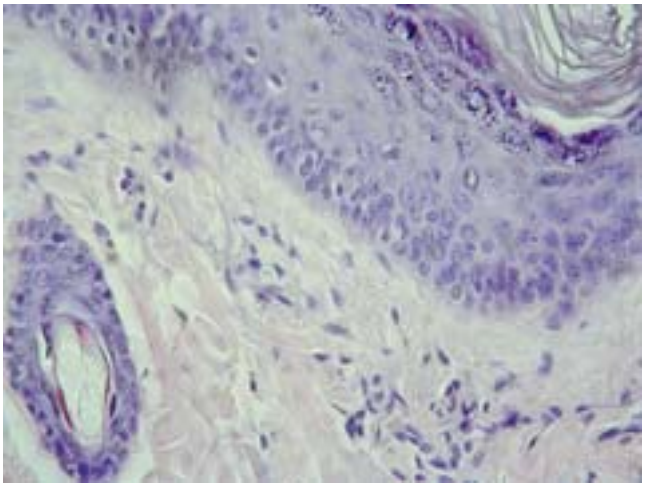

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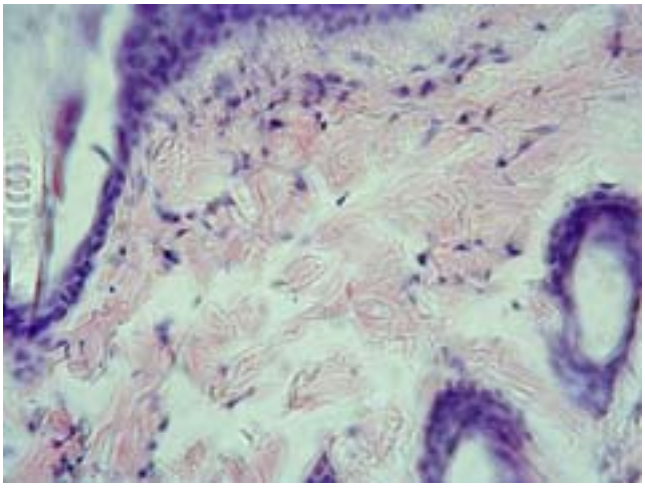

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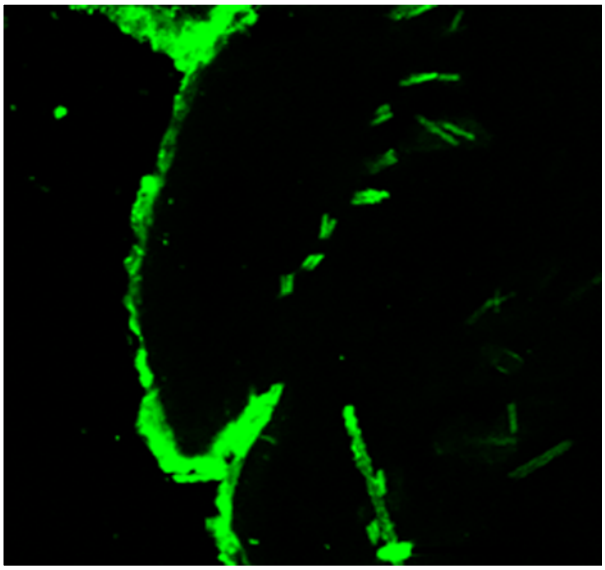

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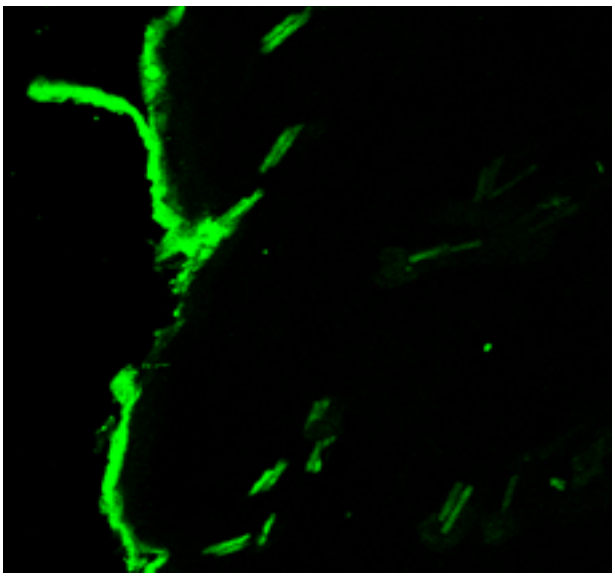

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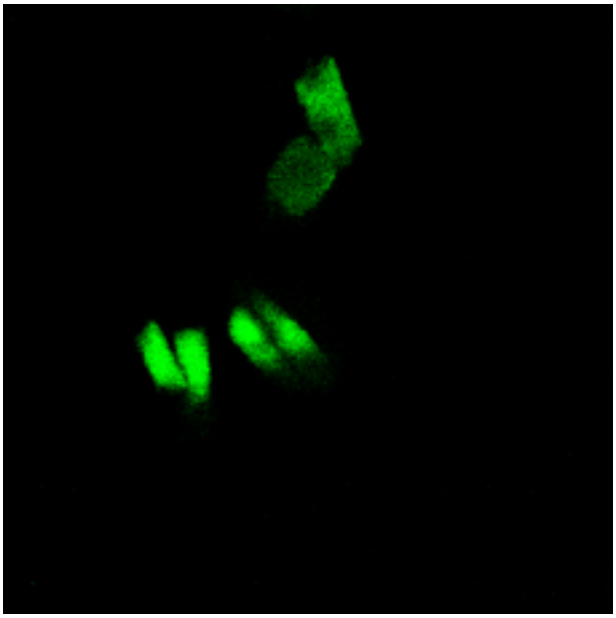

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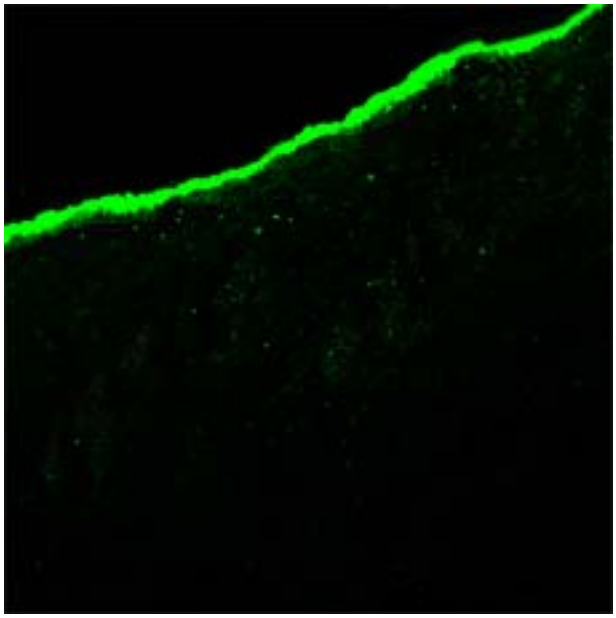

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Legend to Pictures

Pictures 1 and 2
Untreated rat skin (control). Stratum corneum, partially raised and detached from the under laying layers, epidermis and dermis with adnexa are evident. 100x and 200x.

Pictures 3 and 4
Rat skin treated by “Transderm”. Stratum corneum, partially detached from the other layers of epidermis, is well evident. Epidermis has a uniform thickness with no observable interruptions; numerous collagen bundles, spatially well organized, compose the dermis; various connective cells such as fibroblasts are interspersed among the collagen bundles; adnexa appear unaffected. 100x and 200x.

Picture 5
Untreated rat skin (control). Epidermis, dermis and adnexa appear normal. 250x.

Picture 6
Rat skin treated by “Transderm” . Epidermis, dermis and adnexa appear normal. 200x.

Picture 7
Rat skin treated by “Transderm”. Epidermis, dermis and adnexa appear unaffected. 200x.

Picture 8
Rat skin treated by “Transderm”. Epidermis, dermis and adnexa appear unaffected by the electric treatment. 250x.

Picture 9
Rat skin treated by “Transderm”. Epidermis, dermis and adnexa appear completely normal. 400x.

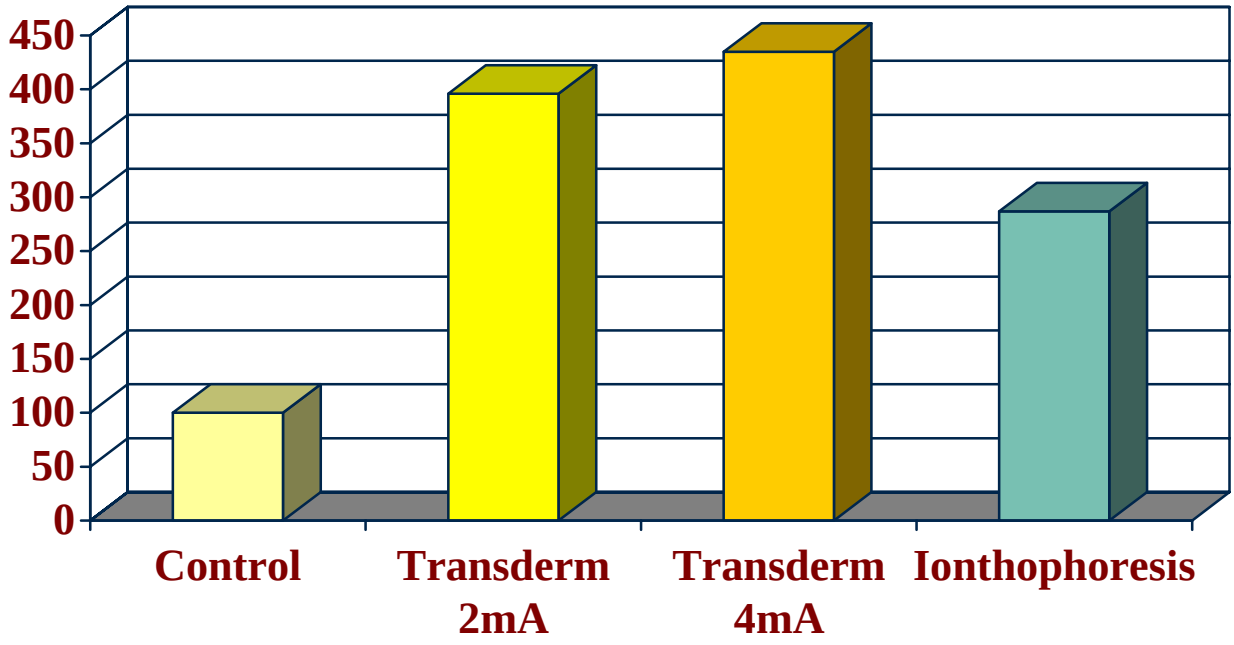
Picture 10
Rat skin treated by “Transderm”. Numerous collagen bundles and various connective cells compose the dermis. 450x.

Pictures 11 and 12
Rat skin treated by “Transderm”. The skin surface appears uniformly covered by fluorescence; numerous molecules of fluorescent collagen are observable from the outermost part to the inner part of the dermis. 150x.

Picture 13
Higher magnification of some molecules of bovine type I fluorescent collagen. 400x.

Picture 14
Untreated rat skin; the skin surface appears uniformly covered by a fluorescent stratum while no fluorescence is present in the dermis. 150x.

Relative value of radioactive Lidocaine delivery



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