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Study Report

The evaluation of Hyaluronic acid (HA) skin penetration in several cream formulations In an ex vivo pig skin

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Run details:	**Name - RBK and FBE**	**Date -17-10-2017**	**Signature - RBK**

REVISION HISTORY		
Version No.	**Date**	**Reason for Issue**
01	19-11-2017	Initial Issue
02	21-11-2017	Final
03		
04		

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Report	**November 19, 2017**
Name of Report:	**The evaluation of Hyaluronic acid (HA) skin penetration in several cream formulations**
Number of OP:	**DTB10875**
Number of Price quote:	**1719**

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1. Goal

This Report was submitted further to a request of Professor Rachel Lubart from Bar Ilan University to assess the penetration of a compound into pig skin by IHC.

2. Background and study principal

This document is submitted on the basis that it remains the property of Da-Ta Biotech Ltd. and its contents are confidential to Zingboim. This R&D study was non GMP neither GLP study but conducted under scientific best practice. In this first run, the selected IHC method for HA tissue content evaluation was estimated for the first time and evaluated for further use. Professor Lubart sent cream formulations. The laboratory team was blinded as to the identity of the different formulations. The cream was used in a thin layer to cover the surface of small pieces of pig skin and left for 5 hours. Skin was fixed and samples were prepared on histological slides. IHC analysis was performed using a first "anti-HA" antibody and a second layer of fluorescent antibody against the first "anti-HA" antibody.

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3. **Materials and Methods**

I. Sample preparation and treatment – Day 1:

2cmx2cm fresh pig skin pieces (from a control cadaver) were used for the experiment. The pieces were laid on a PBS soaked dipper. Coded cream samples were used for the study (Table 1). An amount of 0.2gr from each sample was spread on each piece for 2 minutes and then rubbed into the piece gently for 30 seconds. The left over, thin layer, was left for 5 hours (picture 1 – study set up) and the samples were wiped using a paper and inserted into formalin.

Picture 1 – study set up

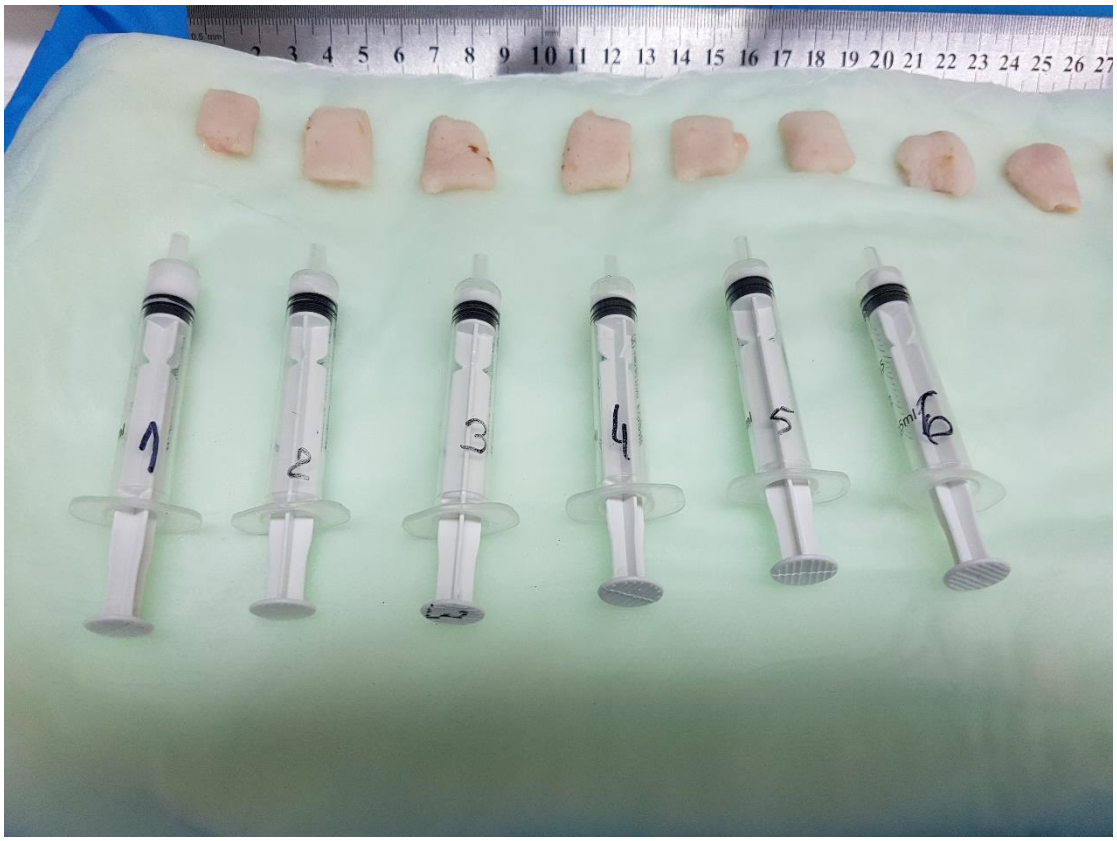

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Table 1- Sample List

Skin treated with Test article 1
Skin treated with Test article 3
Skin treated with Test article 5
Skin treated with Test article 7
Skin treated with Test article 9
Non treated control - skin

II. Sample preparation and treatment – Day 2 - end:

Samples were transferred to 70% Ethanol (after 24 hr. in Formalin). And paraffin blocks were prepared. Slides were cut and stained using a 1:2000 dilution of anti-Hyaluronic acid antibody primary antibody overnight at 4°C followed by an Alexa fluor® marked Secondary antibody for 1 hour.

4. Results

The Leica SP8 Confocal Microscope was used for data collection (Table 2). First the negative control was used for background evaluation and threshold settings.

This skin with no cream (#11) was stained at the same protocol with both antibodies (Picture 2). High background was demonstrated and this slide was used for setting the thresholds to enable more specific readings. This picture demonstrates that there is an unspecific Attachment of one of the antibodies used. It can be seen that

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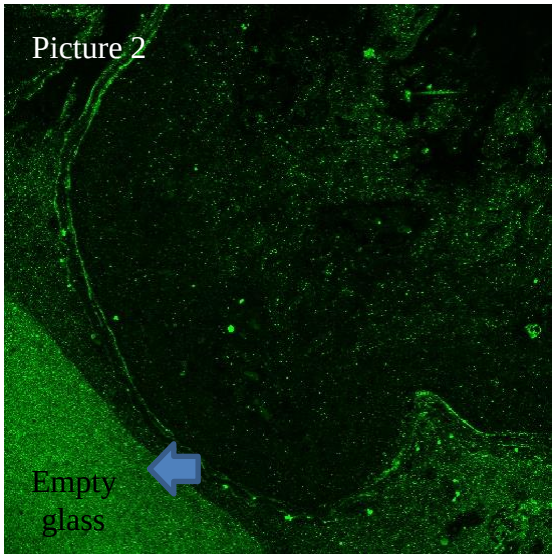
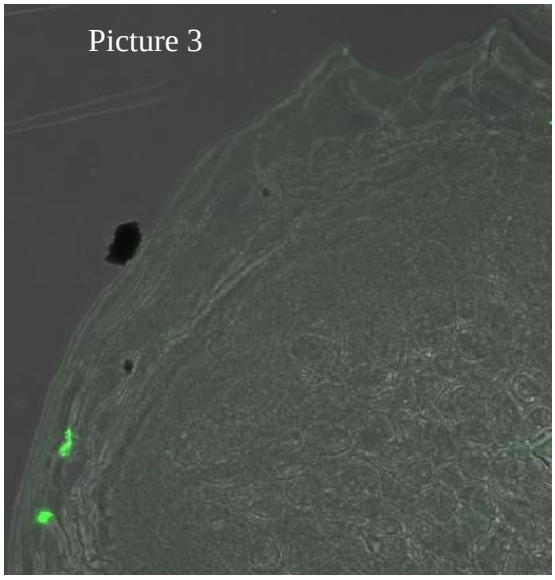
even and mostly the glass caught the antibodies (ARROW HEAD).



Using untreated skin stained only with the 2nd antibody, the responsibility to the background was placed on the 1st antibody (Picture 3).



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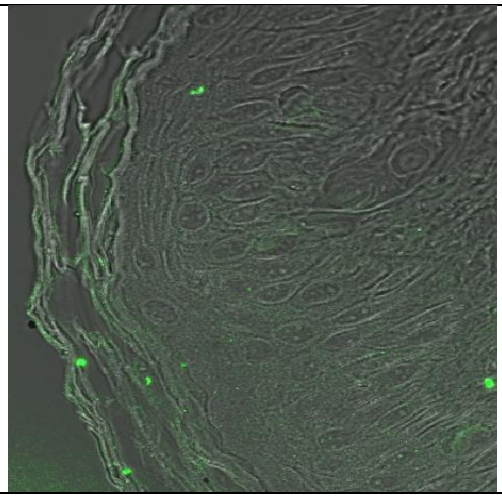
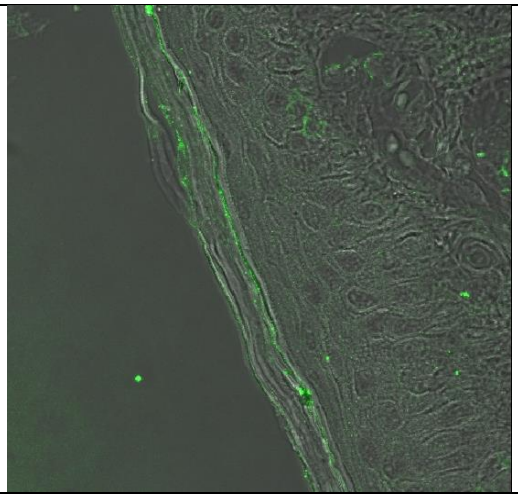
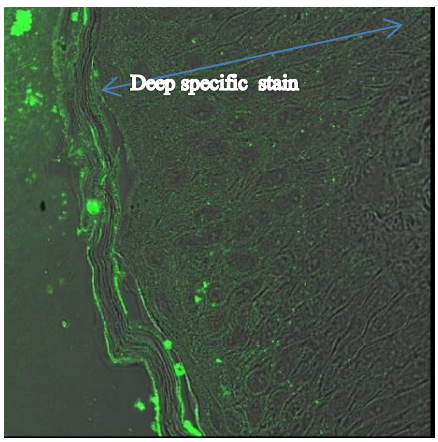
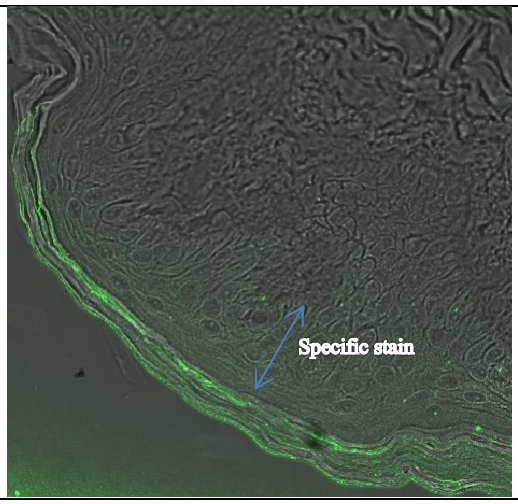
Following that parameter set up, the technicality of the IHC method revealed the need for improvement in regards to blocking stages to be added in order to get better pictures.

Results can be analyzed as all nest pictures were taken under the understanding of background presence and intensity. Background was reduced as much as possible for the following pictures (Table 2).

Table 2 – Results

1	3
	
5	7
	

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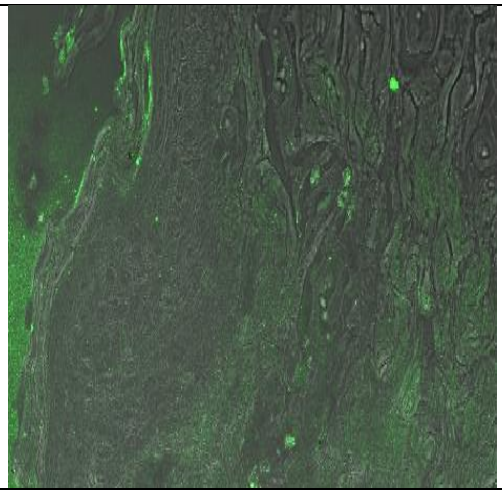
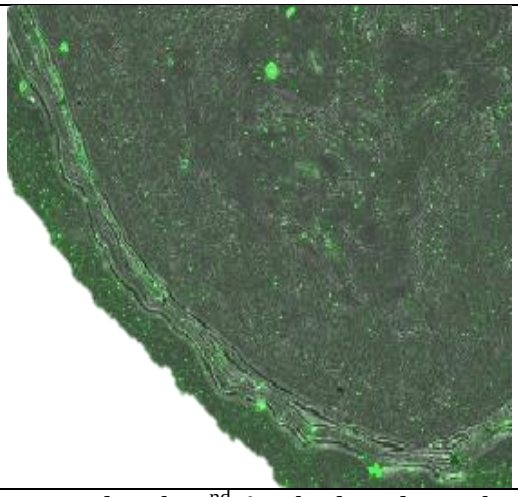
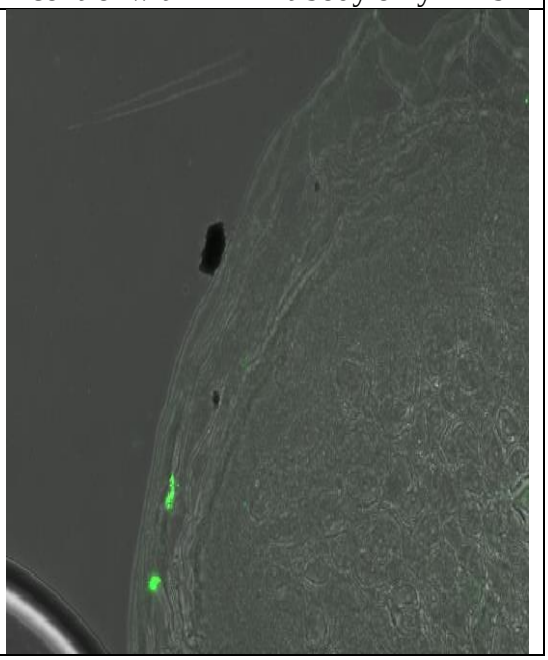
9	control with 1st & 2nd Antibodies – 11a
	
	control with 2nd Antibody only –11b
	

Subjective grading of staining intensity (Table 3) was performed based on background reduction, staining intensity of specific layers of the skin and Histological patterns revealed by specific staining.

At that stage we've received the identity of samples 1, 3 and 5 and included them in the table.

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Table 3 – Estimated grading (0/ 1/ 2/ 3) of specific staining in the samples and skin layers

Sample number		Stratum corneum	Deep epidermis - dermis
1	**HMW (High molecular weight) HA water solution, inserted in formulated Prophecy cream (20%)**	**0 (smeared green background)**	**0 (smeared green background)**
3	**LMW (Low molecular weight, 50kDa) HA water solution, inserted in formulated Prophecy cream (20%)**	**1 (Partial continuous green lines)**	**0 (smeared green background)**
5	**Treated (using Bar Ilan University and Hava Zingboim's new technology) HA water solution, inserted in formulated Prophecy cream (20%)**	**3 (Long continuous green lines outer rims and inside)**	**3 (Wide area of green inter-cellular marking. Cells are not stained. Green in a clear histological pattern)**
7	Experimental formulation	**2 (Long continuous light green lines outer rims and inside)**	**1 (Thin area of green inter-cellular marking. Cells are not stained. Green in a clear histological pattern)**
9	**Treated (using Bar Ilan University and Hava Zingboim's new technology) HA water solution, inserted in formulated Prophecy cream** low concentration	**1 (smeared green background)**	**0 (smeared green background)**
11	**None treated control**	**1** (high beck ground - smear)	**1** (high beck ground - smear)
11	**None treated control** stained with 2nd AB only	**0**	**0**

5. Conclusions

Analyzing the penetration results based on a selective analysis of Immuno-Histo-Chemistry (IHC) results following 5 hours of ex vivo pig skin exposure to 5 cream

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formulations and untreated control, reveals differences in penetration of Hyaluronic acid (HA) into the stratum corneum and deeper towards the dermis.

Following the subtraction of the high background that might be overcome in the next sessions by Blocking, the technicality could be rest aside.

Noticing carefully the continuous staining along the stratum corneum and the histological pattern of the cells in the deeper skin layers, enabled the subjective estimation of relative specificity and intensity of the staining (Table 3).

The results indicated that sample #5 had the highest intensity of specific staining of HA in the Stratum corneum and the deep layers towards the dermis. This #5 as stated by Professor Lubart was the PROPHECY CREAM (Laboratory team was blinded for the duration of the experiment).

The "none treated" controls were included in order to show that the smeared staining could be seen and avoided.

Better pictures that should enable conclusive results are expected in case that blocking stage is included. The performance of 3 independent repeats on the study will enable scientifically based results if background is reduced. Apart from that unique requirement, the method chosen seemed to be adequate.

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