In-vitro Efficacy Data on epidermal Keratinocytes

PENTAVITIN® for Skin Care

Effect on the expression level of key genes of epidermal differentiation and skin barrier formation.

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1. Aim

The aim of this *in-vitro* study was to quantify the gene expression in human epidermal keratinocytes of a selection of four genes with key functions in skin barrier formation after treatment with PENTAVITIN®.

2. Introduction

PENTAVITIN® is a based on a 100% natural carbohydrate complex and has shown very positive effects on skin deep hydration and on skin barrier stability. We wanted to demonstrate effects of PENTAVITIN® on key components of skin barrier formation.

We therefore selected for this analysis four genes as key markers with functions related to skin barrier formation and moisturization. The selected genes were filaggrin (FLG), hyaluronan synthase3 (HAS3), loricrin (LOR) and acid sphingomyelinase (SMPD1):

Filaggrin as monomers can become incorporated into the lipid envelope, which is responsible for the skin barrier function in the stratum corneum. Filaggrin undergoes further processing in the upper stratum corneum to release free amino acids to be part of the NMF (natural moisturizing factor) that assist in water retention. Hyaluronan synthase 3 is essential for the synthesis of hyaluronan (hyaluronic acid) in the epidermis that is important for barrier formation and hydration. Loricrin is a major protein component of the cornified cell envelope found in terminally differentiated epidermal cells. Acid sphingomyelinase activity is localized in the epidermal lamellar bodies and is a key enzyme in the generation of ceramides for extracellular lipid lamellae in the stratum corneum as important component of the skin barrier.

3. Material and Methods

*Note: These studies have been performed at the contractor laboratory Bioalternatives SA, 1 bis rue des plantes 86160 Gencay France.*

A) **Test compound and preparation:** PENTAVITIN® Lot 41568601/180-1 has been directly added to the cell assay medium to get the desired final concentration in the cell culture.

B) **Biological model:**

   - Cell type: Normal human epidermal keratinocytes (NHEK), reference K544 used at the 3rd passage
   - Culture conditions: 37°C, 5% CO2
   - Culture medium: Keratinocytes SFM supplemented with epidermal growth factor (EGF) 0.25ng/ml, Pituitary extract (PE) 25ug/ml, Gentamycin 25ug/ml
   - Assay medium: Keratinocyte-SFM supplemented with Gentamycin 25ug/ml

C) **Cytotoxicity preliminary assay (MTT):**

   - NHEK were seeded at 10000cells/well and incubated for 48hours. Cytotoxicity was quantified by the MTT reduction assay and morphological observations were done with a microscope.

D) **Keratinocyte culture treatment:**

   - Keratinocytes were seeded in culture medium and incubated for 24hours, and then the culture medium was replaced by assay medium for 24 hours. Then the assay medium was replaced again with medium containing (or not for the control) the test compound. Cells were then incubated for another 24 hours with the compound. Each experimental condition was performed in triplicates. At the end of the incubation time the supernatants were discarded, the cells were washed in phosphate buffered saline (PBS) solution and immediately frozen at -80°C.
E) Analysis of Gene Expression by quantitative PCR technology:
The expression of four selected genes was analysed using RT-qPCR method on mRNA extracted from the cell monolayers for each treatment. Before RNA extraction the replicas were pooled. The analysis of gene expression was then done in duplicates (n=2). Reverse transcription: Total RNA were extracted from each sample using TriPure solution reagent according to the supplier’s advices. The amount and quality of RNA were evaluated using a lab-on-a-chip Bioanalyzer (Agilent Technologies). Potential contaminant traces of genomic DNA were removed using the DNAfree system (Ambion). The amount of the RNA was evaluated using Nanovue (GE Healthcare). The reverse-transcription of mRNA was conducted in presence of oligo(dT) and Superscript II reverse-transcriptase (Invitrogen). Nanovue was also used for quantification of cDNA and DNA concentration adjustment. Quantitative PCR: The PCRs (Polymerase Chain Reactions) were performed using the Light Cycler system (Roche Molecular Systems Inc.) according to the supplier’s instructions. This system allows rapid and powerful PCRs after determining analysis conditions of the test primers. The reaction mix contains diluted DNA, primers forward and reverse, reagent mix containing taq DNA polymerase, SYBR Green I and MgCl₂. The incorporation of fluorescence in amplified DNA was continuously measured during the PCR cycles. This resulted in a fluorescence intensity versus PCR cycle plot allowing the evaluation of a relative expression (RE) value for each gene. The value selected for RE calculations is the output point (Ct) of the fluorescence curve. For the considered gene the higher the cycle number the lower is the mRNA quantity. The RE value was expressed in arbitrary units (AU) according to the formula: (1/2number of cycles) x 10^6. For additional control we also quantified the expression level of three housekeeping genes namely Ribosomal protein S28, Glyceraldehyde-3-phosphate dehydrogenase and beta-Actin in both untreated control cells and treated cells. The mean relative expression level of the three housekeeping genes was set to 100% and the variation in expression level of individual three genes was within less than 100 ± 50%.

4. Results

4.1. Tolerance of PENTAVITIN® on in-vitro cultured keratinocytes:

<table>
<thead>
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<th>PENTAVITIN® concentration in %</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
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<tr>
<td>viability</td>
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<td>4</td>
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<tr>
<td>Morphological observations</td>
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</tr>
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</table>

Table legend: Viability was quantified based on MTT measurement. Morphological observations, +: normal population, ±: growth reduction, 0: cell mortality
From these MTT measurements we concluded that PENTAVITIN® is well tolerated by the keratinocytes for at least up to 0.37%. We decided to use 0.3% of PENTAVITIN® for the gene expression tests.

4.2. Induction of gene expression in human keratinocytes by 0.3% of PENTAVITIN®:

Data are shown in Figure 1. The relative gene expression level of the untreated control experiment was adjusted to the level of 100% and compared to the expression of cells treated with 0.3% of PENTAVITIN® for 24 hours. As a separate test condition representing a “positive control” for enhanced keratinocyte differentiation we added 1.5mM Ca²⁺ to the assay medium and the expression levels after 24 hours were
763% for filaggrin, approx 200% for acid sphingomyelinase, 166% for loricrin and no stimulated expression for hyaluronan synthase 3 (data not shown in the graph).

![Induction of gene expression in human epidermal keratinocytes by 0.3% Pentavitin at 24 hours incubation](image)

**Fig. 1**

**5. Conclusions:**

Increased expression of key genes for skin barrier function:
Gene expression analysis by quantitative RT-PCR on human keratinocytes revealed that PENTAVITIN® effectively stimulates genes that play a key role in skin barrier improvement and maintenance. Upregulation of the filaggrin and the hyaluronan synthase 3 genes improves skin's hydration ability by boosting NMF and hyaluronan. Increased loricrin gene expression affects the function of the cornified envelope and increased acid sphingomyelinase gene expression stimulates the synthesis of ceramides.