Hair Therapy & Transplantation

Boisnic et al., Hair Ther Transplant 2016, 6:2
DOI: 10.4172/2167-0951.1000143

Research Article OMICS International

Miliacin Associated with Polar Lipids: Effect on Growth Factors Excretion and Extracellular Matrix of the Dermal Papilla Hair Follicle Model Maintained in Survival Conditions

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Received date: August 1, 2016; **Accepted date:** September 15, 2016; **Published date:** September 22, 2016

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Abstract

Background: The Dermal Papilla (DP) consists in specialized dermal fibroblasts located at the base of hair follicles and secreting extracellular matrix, especially collagen and glycosaminoglycans. DP cells are responsible for the production of hair fibers, by inducing anagen phase, and by maintaining the hair in this growth phase. Miliacin (contained in millet oil) is known for its healing properties and activity thereof on cellular proliferation. Miliacin has been associated with polar lipids, which are known to act as a vector, for increasing the bioavailability of the active compounds.

Objectives: To explain the cellular hair bulb stimulation by miliacin associated with polar lipids (MPL), we used a model of hair follicle maintained in survival conditions. The modulation of collagen and glycosaminoglycans in the DP, the mitotic index for keratinocytes in the hair bulb and the growth factors excretion (IGF1 and KGF assays) were studied.

Materials and Methods: Human hair scalp fragments were maintained in survival conditions for 7 days and treated by MPL diluted in the culture media.

Results: A significant increase (14.1%) of IGF1 with an important (140%) and significant stimulation of mitotic index (Ki67 positive cells) was observed in the epithelial cells of hair bulb with MPL. A significant increase (20.8%) of collagen thickness was measured in the connective tissue sheath of the hair in contact with MPL.

Conclusion: Miliacin associated with polar lipids acts on dermal papilla where it stimulates IGF1 growth factor production and renewal of keratinocytes in hair bulb. It also increases the thickness of extracellular matrix of the connective tissue sheaths.

Keywords: Human hair; Dermal papilla; Millet oil; IGF; KGF; Mitotic index; Collagen

Introduction

The hair is composed of different compartments, from dermal origin (conjunctive gain, dermal papilla) or from epidermal origin (stem cells in the bulb, sebaceous gland, external and internal epithelial gains). The bulb consists of active dividing keratinocytes forming the air shaft and the inner and outer root sheaths.

Highly vascularized, the dermal papilla at the base of the bulb enables hair irrigation and oxygenation and cellular waste disposal. It consists in fibroblasts secreting an important extracellular matrix, especially collagen and glycosaminoglycans [1,2]. The papilla can be considered as the biological motor of the hair; it is a physical support for follicle growth and it is intrinsically involved in hair life cycle [2,3], comprising of three phases. The anagen phase is the hair growth phase, during which the hair grows from the bulb. Hair matrix cells undergo significant division to form the hair shaft and the inner and outer root sheaths. In the anagen phase, the hair matrix is the site of intense cellular proliferation. Simultaneously, the hair follicle grows in the deep layers of the skin to nourish the hair. This is the longest period of the hair's life cycle, lasting 2 to 5 years on average. The majority of hair is in the anagen phase. The catagen phase (approximately 3 weeks) is a resting phase during which the hair stops growing. Finally, during the telogen phase, the hair is no longer growing, but it remains attached to the hair follicle. At the end of this phase, the old hair falls out, leaving its place for a new follicle in the anagen phase, and the hair life cycle restarts. During this telogen phase, there is little or no cellular proliferation, and no further hair shaft growth is observed. During the hair life cycle, growth factors are expressed in epithelial cells under the influence of dermal papilla. They are produced in the hair follicle, where they promote hair growth by increasing proliferation of keratinocytes [4].

In the skin, IGF1 (insulin-like growth factor-1) has been reported to be exclusively expressed by mesenchymal cells of the dermis and the dermal papilla [5-7]. IGF1 has pleiotropic effects on the skin, including cell proliferation, tissue remodeling, boosting the immune system and having healing powers. IGF1 also controls hair cycle, as well as differentiation within the hair-producing follicle IGF1 and some high-

affinity IGF-binding proteins are present in the hair follicle, suggesting a role of the IGF signaling pathway in follicle biology [5]. IGF1 is a potent mitogen supporting cell growth and survival [8]. It also plays a role in some differentiation processes [9,10]. It stimulates keratinocytes proliferation, guides the follicle development downward and is responsible for hair bulb matrix formation.

KGF (Keratinocyte growth factor) is a 28-kDa heparin binding member of the fibroblast growth factor (FGF) family (alternative designation FGF-7). KGF is expressed only by mesenchymal cells, such as fibroblasts and hair follicular dermal papilla cells. KGF is a potent epithelial cell-specific growth factor that stimulates both proliferation and differentiation of keratinocytes in hair follicles [11-13].

The hair life cycle may be subject to abnormal changes. The causes of such changes may be particularly linked to the seasons, fatigue, stress, hormonal imbalance, and hair treatment, medication having an effect on hair, pollution or ageing. Hair loss has a great impact on quality of life and should be considered as an important subject in science. In alopecia, changes were detected in dermal papilla cells which were less able to synthesize regulatory factors [14]. Nevertheless, whatever the alopecia cause, an anti-hair loss product has to have one, several or all of the following properties [15,16]:

- Inhibit hair loss more rapidly than the natural growth evolution.
- Induce hair growth more rapidly than the natural growth evolution.
- Increase the growth speed of stem cells.

Traditional plant remedies have been used for centuries in the treatment of hair loss, but only a few have been scientifically evaluated.

Millacin triterpenoid is a natural component of millet (*Panicum miliaceum*) oil for which no toxic properties have been reported. Pure miliacin is a white odorless crystal with two distinct geometric forms showing different optical characteristics [17]. Miliacin was first studied through millet oil effect for its healing properties and activity thereof on cellular proliferation [18].

Polar lipids are a separate lipid class since they have a hydrophilic part enabling them to play a predominant role at interfaces, in living organisms or in dispersed systems. In particular, they are the main constituents of biological membranes.

Polar lipids play major biological roles in the structure of the epidermis and hair. They ensure intercellular cohesion and protect hair and skin from the entry of undesirable compounds. They also have a structural role and provide the mechanical properties of the hair [19,20].

A further property of polar lipids and phospholipids in particular, relates to their ability to act as vectors or substrates. Indeed, phospholipids are recognized and used for their ability to form vesicles. These vesicles may enhance the bio-availability of some active substances by facilitating intestinal passage [21-23].

Thanks to polar lipids, miliacin efficacy should be multiplied. The optical microscopic examination of a mixture containing polar lipids, miliacin in crystal form and water, clearly indicates vesicle formation. These relatively rigid structures are between 5 and 25 µm diameter. This system is only observed in the presence of miliacin, which plays a key role at the interface of liposomes which stiffen to form actual capsules. The liposomes formed may enhance the bioavailability of miliacin by promoting intestinal passage, as previously described for other molecules (Curcumin, Naringenin) [22,23].

Previous work showed that miliacin (3.5 to 4.0 mg/day, during 3 months) alone is efficient to fight against hair loss in women. A double blind, placebo-controlled study was conducted on 60 female subjects. The method used to determine the percentage of hair in the telogen and anagen phase was the Trichogram with Trichoscan® (unpublished data).

The results obtained with miliacin alone were very promising. To improve miliacin bioavailability, which considering its structure could be low, miliacin was associated to polar lipids. A second double blind, placebo-controlled study was conducted on 65 women to demonstrate the efficacy of a lower dose of miliacin when associated with polar lipids. A significant decrease of the number and the percentage of telogen hair were observed (about 50%) at W12 compared to W0 (unpublished data).

To shed light on the underlying mechanism of action of miliacin associated with polar lipids, we used a model of hair follicle maintained in survival condition to study the modulation on collagen and glycosaminoglycans. Moreover, cellular bulb stimulation by MPL was studied through the measure of mitotic index in hair bulb and growth factors excretion (IGF1 and KGF assays).

Materials and Methods

MPL preparation

Polar lipids are obtained thanks to an ethanolic extraction from wheat grain. Millet oil, containing miliacin, is extracted using the supercritical CO_2 extraction technique. MPL may be obtained by mixing these two ingredients as follows:

Injection of the polar lipids in the millet oil during CO_2 extraction circuit, in an oxygen-free, supercritical medium.

Mixing the two ingredients, at atmospheric pressure in particular.

Scalp model in survival conditions

Human scalp fragments were obtained from plastic surgery (9 donors aged 42 to 75 years old; mean 53.8 ± 13) at the edge of the scalp (face lift) or at the occipital crown (pre-transplant scalp). Fragments were put, with the epidermis uppermost, at an air/liquid interface, in inserts (Costar, VWR, and France) which were then set on a culture 12 well plate. A specific culture medium especially adapted to survival conditions for hair follicle and dermal papilla (Follicle Dermal Papilla Cell Basal Medium®, PromoCell, Germany), without SVF, without red phenol with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin, Gibco, BRL) was added into the wells. A slow diffusion between the two compartments was performed through a porous membrane (filter pore size 0.45 µm). Scalp fragments were maintained in survival conditions at 37°C in air/CO_2 5% during 7 days [24-26]. Culture media were changed daily.

MPL was added at 150 µg/ml (0.015%) in the culture medium (systemic condition) daily for 7 days, to facilitate contact with hair bulbs.

At D7, fragments were formalin-fixed and paraffin-embedded for immunohistochemical and histological analysis. Culture media were frozen at the end for the dosage of growth factors.

Growth factors dosage

IGF1 and KGF dosages were carried out from supernatants by enzyme immunoassay ELISA with spectrophotometric lecture of the concentration (pg/ml) at 450 nm (Quantikine® Colorimetric Sandwich ELISAs). Growth factors dosage was performed in duplicate.

Immunohistochemical analysis of mitotic index in hair bulb

Epithelial proliferation was analyzed by immunohistochemistry with anti-Ki67 antibodies (monoclonal murine antibodies MIB1, dilution 1/300, DAKO) which is a marker of mitotic index (cells in phases M, S, G1 and G2). The immunodetection was performed with an indirect immunoperoxidase technique (CsA kit, DAKO, France) and revealed in red with an AEC solution (3-amino-9-ethylcarbazole). The number of marked hair bulb cells was evaluated on an approximately total of 100 cells

Histological quantification of dermal collagen and glycosaminoglycans by computerized image analysis

Serial sections of 4 µm thickness were obtained and specifically stained with a picric acid solution containing 0.1% sirius red to reveal collagen. Glycosaminoglycans (GAG's) study was undertaken on histological sections of 6 µm colored with Hale colorant. The stained slides were examined by a microscope (Leitz, magnification X400) connected with a camera unit (QImaging Retiga 2000R) and with a microprocessor (Dell, Image Pro Plus). The thickness (µm) of collagen bundles and GAG's around the bulb was measured.

Statistical analysis

Mean values and standard deviations were calculated for quantitative variables. The statistical significance of changes recorded with the measured parameters was determined with Student's t-test on paired samples, with an alpha risk at 5%.

Results

Effect of miliacin associated with polar lipids on growth factors excretion.

MPL seems to have a direct influence on IGF1 excretion but no action on KGF.

Indeed, a significant ($p<0.01$) increase (+11.5%) of IGF1 excretion was measured in the scalps with MPL (506.46 pg/ml) in comparison with control scalps (454.26 pg/ml) (Figure 1).

A non-significant increase (+14.2%) of KGF excretion was measured in the scalps with MPL (33.00 pg/ml) compared to control scalps (28.90 pg/ml) (Figure 2). There is important inter-individual variability on KGF concentration which leads to non-significant results.

Effect of miliacin associated with polar lipids on mitotic index

An important and significant stimulation of mitotic index was observed in the keratinocytes of hair bulb. With MPL, the number of cells in mitosis was about 21% compared to 8.8% for the control scalp ($p<0.007$).

So, MPL at a concentration of 150 µg/ml increase of about 140% the hair bulb cells proliferation (Figures 3-5).

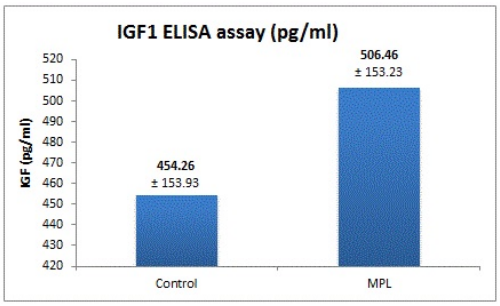

Figure 1: Comparison of IGF1 excretion between control scalps and scalps with MPL.

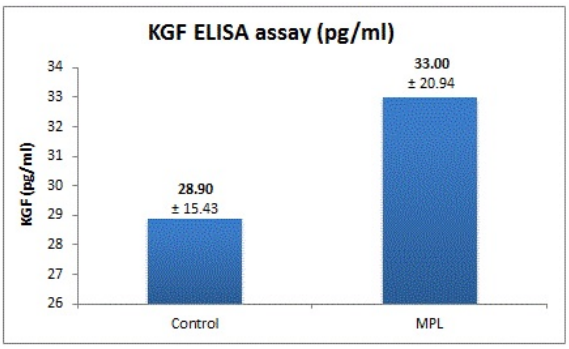

Figure 2: Comparison of KGF excretion between control scalps and MPL scalps.

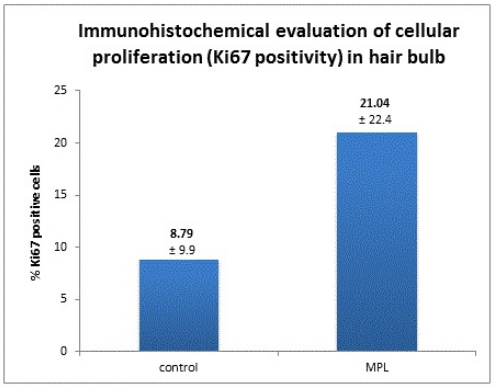

Figure 3: Immunohistochemical evaluation of cellular proliferation (Ki67 positivity) in hair bulb.

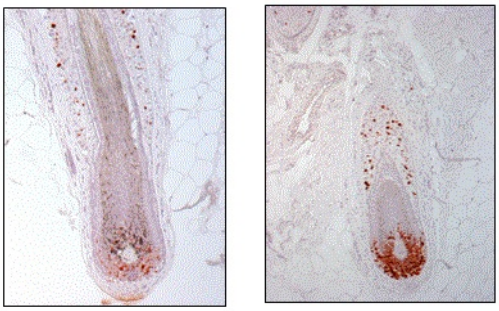

Figure 4: Immunohistochemical analysis of mitotic index (Ki67 expression) in keratinocytes hair bulb (magnification X100).

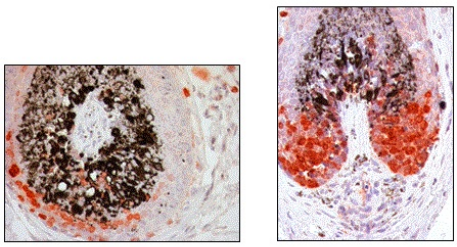

Figure 5: Immunohistochemical analysis of mitotic index (Ki67 expression) in keratinocytes hair bulb (magnification X400).

Effect of miliacin associated with polar lipids on collagen

A significant ($p<0.01$) increase (+20.8%) of collagen thickness was measured in the connective tissue sheath of the hair with MPL (22.95 µm) in comparison with control scalps (19.0 µm) (Figures 6 and 7).

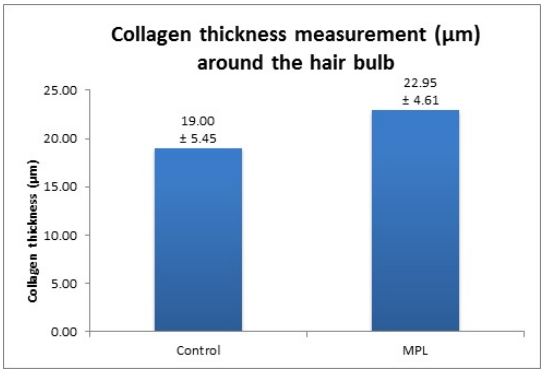

Figure 6: Collagen thickness measurement (µm) around the hair bulb.

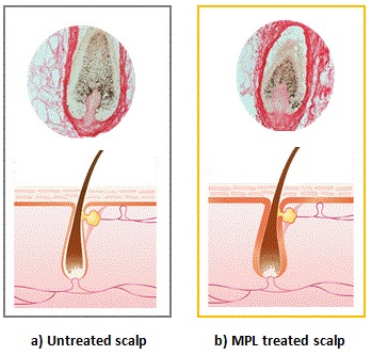

Figure 7: Histological evaluation of collagen around hair bulb after Sirius red staining (magnification X200).

Effect of miliacin associated with polar lipids on Glycosaminoglycans

There was no modification of GAG's thickness with MPL. Indeed, after treatment, Glycosaminoglycans thickness around the hair bulb was 22.59 ± 9.64 in comparison with untreated scalp where the thickness was 22.73 ± 11.53 µm.

Discussion

It has been known that the follicle's DP, which is of mesenchymal origin, plays an important role in the control of all aspects of hair follicle morphogenesis and life. In the anagen phase, cells at the base of the follicle start to proliferate, which results in downward growth of the follicle and envelopment of the DP. During the catagen phase, the epithelial cells at the base of the follicle undergo apoptosis whereas the volume of the extracellular matrix is reduced to become almost indiscernible when the telogen phase is reached [27]. Another study shows that fibronectin, a proteoglycan from the extracellular matrix, was found to be minimal when hair is in its telogen phase while, in the anagen phase, a great increase of fibronectin around the DP was observed [28]. The present work shows the collagen increase, around the hair bulb in the DP after the MPL treatment, with a significant (20.8%) increase of collagen thickness. In the same way, a significant increase (140%) of keratinocytes mitotic index was shown in hair bulb.

Paracrine growth factors such as KGF and IGF have been linked with hair growth stimulatory activity [4]. A significant IGF1 increase of 11.5% has been observed in this study after hair bulbs treatment with MPL. A non-significant increase of KGF was also measured.

Previous studies on mice and rats indicated roles of growth factors in morphogenesis of epithelium, re-epithelization of wounds and hair development. Indeed, KGF elevated levels have been reported in case of full-thickness excision in rats. These observations were supported by an increase of differentiation in cultured keratinocytes treated with KGF. In addition, an increase cell proliferation in the follicle and a differentiation of keratinocytes were detected within the dermis adjacent to wound treated with topical KGF [11,12]. Previous works have already shown that natural extracts have the capacity to modulate the mRNA expression level of various growth factors including IGF1 and this leading to an effect on hair growth [29,30].

Growth factors, which are produced in the hair follicle under the influence of dermal papilla, promote hair growth by increasing proliferation of keratinocytes. In this study, we demonstrate the significant increase of keratinocytes mitotic index in hair bulb after treatment by MPL. Previous studies have shown that millet oil seems to improve the healing process on animal and human skins, especially on patients suffering from diabetic wounds [18,31] and it has a strong anti-inflammatory and anti-microbial activity.

A more recent *in vitro* test [32] studied the influence of miliacin on human keratinocytes. It was done on human keratinocyte cultures exposed to different miliacin concentrations. Mitochondrial dehydrogenase activity was used to measure metabolic activity and BrdU-uptake to measure cell proliferation. Miliacin (6 mg/ml) increased metabolic activity by 162% and stimulated cell proliferation by 215%. In another preliminary work (results not published) we have shown an increase of 92% of cell proliferation after hair bulb treatment with miliacin alone (1.7 µg/ml). In this study, a potentiated effect has been demonstrated, using the similar miliacin concentration associated with polar lipids. Indeed, with MPL cell proliferation was increased by 140%. These studies confirm the role of millet oil and especially miliacin to improve the healing process and cellular renewal in the hair bulb.

We demonstrate that MPL acts on dermal papilla where it stimulates growth factors production like IGF1 and increases the thickness of extracellular matrix of the connective tissue sheaths. Moreover, it stimulates the renewal of keratinocytes in the hair bulb. These can explain the anti-hair loss effects observed in the previous two clinical studies. For the first time, we have clarified miliacin mechanism of action, which can account for the anti-hair loss effect and wound healing properties, previously reported.

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