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ORIGINAL PAPERS

Efficacy of Chitin and Keratin Bioactive Fractions in Skin Inflammatory Processes Remission

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Abstract. The development of new therapies in the field of regenerative medicine is a priority area, which integrates, among other fields of research, the use of active molecules from extracts of natural origin with renewable potential. In this context, the biomedical applications of chitin from various natural sources (entomological, marine etc.), as well as those of keratin from leather waste, are included. The studies we initiated show the effectiveness of the Valke fraction of keratin, isolated from sheep's wool, and that of type L (from insects) and R (from crustaceans) chitin preparations, in modulating cellular processes specific to skin tissue degradation: IL6, IL8, IL1α cytokine-directed inflammation, angiogenic repairing processes (extracellular VEGF) and oxidative stress (oxygenated free radicals). The cellular response of keratinocytes from a HaCaT line under induced inflammation (LPS and TNFα) is manifested by increased intracellular levels of oxygenated free radicals, counteracted mainly by chitin. In case of bacterial infections simulated in vitro with LPS polysaccharide, the anti-inflammatory effects induced by Valke keratin on IL6-directed signaling pathways (acute phase cytokine behavior) are noticeable. Moreover, the same effects are reflected through the inhibition of IL8, the chemokine responsible for neutrophil recruitment to the inflammatory site. Nonetheless,

chitin reduces the release of IL6, while strongly stimulating VEGF for "de novo" angiogenesis in the injured tissue. In addition, the pro-irritant cytokine IL1α is inhibited by chitin and keratin, which suggests a reduction in the epidermal irritant potential. The results guide the applications to several etiologies wound therapy. The studies were carried out within 5 PTE Project / 2020 – BIOTEHKER.

Keywords: *keratin, chitin, keratinocytes, TNFα, LPS*

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1. Introduction

The development of new therapies in the field of regenerative medicine is a priority area, which integrates, among other fields of research, the use of active molecules from extracts of natural origin with renewable potential, in order to develop a sustainable bioeconomy, with the full exploitation and conservation of biological resources. In this context are included the biomedical applications of chitin from various natural sources (entomological, marine, etc.), as well as those of keratin from leather waste (e.g. sheep wool).

Due to its size, characteristics and roles, human skin has been the most intensly tested organ for its regenerative capabilities and it has shown promising results. The impact of effective skin tissue regeneration is major, because the skin is affected in the most diverse pathologies, trauma and infections, being our first and main defense organ in the face of the external environment.

Thus, correct and rapid regeneration of the skin can change the trajectory of the disease and the general condition of the patient in the following conditions: bedsores, flame / solar burns, diabetes mellitus, cuts, hemorrhoids, post-operative recovery, extremely diverse dermatological diseases: from dermatitis, to psoriasis and up to epidermolysis bullosa. Rapid regeneration of the skin, assisted through conditions as similar as possible to the physiophysical ones, leads to an increased impliance and an accelerated recovery of the patient, decreasing their vulnerability time to other complications associated with the difficult healing of a wound (risk of infection, depression, aggravation of the wound, amputation, immobility etc.). The regulation of wound healing is critical because inappropriate proinflammatory signaling can result in wounds that heal harder and are at risk of infection [1,2]. Non-healing wounds usually cause distress and require careful management [1]. If the switch to proliferative signaling is not carefully controlled, then repair can result in fibrosis, which implies excessive accumulation of ECM proteins, such as collagen, at the site of injury/damage. Scar formation is the normal end point of mammalian tissue repair, however, excessive scarring can impair normal tissue function [1,3]. Fibrotic skin tissue’s spectrum of severity ranges from flat, pale and relatively static atrophic scars to severe, highly pigmented, rapidly growing, pathological, hypertrophic and keloid scars. Even the

minor normotrophic scar type is dysfunctional because it decreases sensation, can cause discomfort through itchiness and pain, has altered pigmentation and the tightening of the skin can impair movement and have a detrimental impact on the quality of life. Scarring also determines psychological effects on patients, because of the dissatisfaction with the scar appearance and associated stigma [1].

Skin tissue is therefore an important interface for maintaining homeostasis from mechanical, chemical and biological factors [4]. It consists of a complex multicellular network that ensures plastic and dynamic cellular communication in order to maintain several vital processes or to combat disruptive factors. Processes in this category refer to: inflammation, immune response (including tolerance induction and disease prevention), wound healing and angiogenesis [5].

Wound healing is an evolutionarily conserved process that coordinates the efforts of the folowing cell types: keratinocytes, fibroblasts, endothelial cells, macrophages and platelets. They are involved in migration, infiltration, proliferation, and differentiation leading to an inflammatory response, the formation of new tissue and ultimately wound closure. These stages are regulated by an equally complex signaling network: growth factors, cytokines and chemokines. Of particular importance is the epidermal growth factor (EGF) family, transforming growth factor beta (TGF-b) family, fibroblast growth factor (FGF) family, vascular endothelial growth factor (VEGF), granulocyte macrophage colony stimulating factor (GM-CSF), platelet derived growth factor (PDGF), connective tissue growth factor (CTGF), interleukin (IL) family and tumor nerosis factor-α family. These are biologically active polypeptides that act to alter the growth, differentiation and metabolism of a target cell. They have paracrine, autocrine, juxtacrine, or endocrine mechanisms, and influence cell behavior as a consequence of their binding to specific cell surface receptors or ECM proteins. Binding to these receptors triggers a cascade of molecular events. The endpoint of this signaling is the binding of transcription factors to gene promoters that regulate the transcription of proteins controlling the cell cycle, motility, or differentiation patterns [6]. The participation of IL1, IL6, IL8 and VEGF in acute and chronic wound healing can be observed in Table 1.

Table 1. Participation of IL1, IL6, IL8 and VEGF in acute & chronic wound healing – modified after [6]

Cytokine	*Cells*	*Acute Wound*	*Function*	*Chronic Wound*
IL1	Neutrophils Monocytes Macrophages Keratinocytes	Increased levels	Inflammation Reepithelialization	Increased levels
IL6	Neutrophils Macrophages	Increased levels	Inflammation Reepithelialization	Increased levels

IL8[23, 24]	Monocytes Neutrophils Macrophages T-cells Keratinocytes	Increased levels	Inflammation Reepithelialization	Increased levels
VEGF	Platelets Neutrophils Macrophages Endothelial cells Smooth muscle cells Fibroblasts	Increased levels	Granulation tissue formation	Decreased levels

Cytokines regulate immunity and inflammation and thus play an important role in the pathogenesis of various cutaneous disorders [7]. Proinflammatory cytokines, particularly IL-1, IL-6 and TNF-α are up-regulated during the inflammatory phase of wound healing [6]. **IL-1** is produced by neutrophils, monocytes, macrophages, and keratinocytes. Upon wound healing it is immediately released by keratinocytes. In addition to having a paracrine effect, it also works in an autocrine fashion increasing keratinocyte migration and proliferation. Nonetheless, it activates fibroblasts [6]. **IL-6** is produced by neutrophils and monocytes and has been proved to be important in initiating the healing response. It has a mitogenic and proliferative effect on keratinocytes and it is chemoattractive to neutrophils. Its expression is increased after wounding and tends to persist in older wounds [6]. IL-6 plays a central role in acute inflammation and is necessary for the timely resolution of wound healing. Released early in response to injury, it induces the release of proinflammatory cytokines from tissue resident macrophages, keratinocytes, endothelial cells, and stromal cells. IL-6 has also been found to induce chemotaxis of leukocytes into a wound. As inflammation progresses, IL-6 signaling is responsible for the switch to a reparative environment. In normal wound repair, the expression of IL-6 is significantly decreased during the remodeling phase; this is thought to be due to apoptosis of infiltrating inflammatory leukocytes and a subsequent reduction in cytokine signaling [1].

Chemokines are also active participants in the wound healing process by stimulating the migration of multiple cell types in the wound site, particularly inflammatory cells. In addition, the presence of chemokine receptors on resident cells suggests that they also contribute to the regulation of reepithelialization, tissue remodeling, and angiogenesis [6]. **Interleukin-8 (IL-8 or CXCL8)** is a member of the CXC family. Its expression is increased in acute wounds and it has been shown to play a role in reepithelialization by increasing keratinocyte migration and proliferation. It also induces the expression of MMPs in leukocytes, stimulating tissue remodeling. It is, however, a strong chemoattractant for

neutrophils, thus participating in the inflammatory response. High levels of this chemokine accumulate in nonhealing wounds. Moreover, acummulation of IL-8 in high levels decreases keratinocyte proliferation and collagen lattice contraction by fibroblasts. It has been proved that there are relatively low levels of IL-8 in the fetus which may explain the lack of inflammation during the fetal wound healing and contribute to scarless wounds [6].

Among growth factors, **VEGF** is one of the primary stimulators of neovascularization. Vascularization is an important component of wound healing, and if this process is impaired, then wound closure is delayed. However, the dysregulation of vascularization, potentially through the expression VEGF, may be a component of fibrotic disease pathogenesis, as many fibrotic diseases, much like proliferative neoplasms, feature increased vascularization [1,6].

Taking into account the complexity of the wound healing and skin regeneration processes, as well as the fact that many people exhibit skin hypersensitivity to cosmetics and textiles, the development of materials without inflammatory activity is essential. Natural polymers are widely used in the field of regenerative medicine, for dressings customed for wounds and burns, due to biocompatibility, biodegradability and similarity with ECM. Inducing and stimulating the wound healing process, natural polymers are involved in repairing damaged tissues and, consequently, in the regeneration of the skin [8]. Chitins (polysaccharides) and keratins (proteins) fall into the category of natural molecules, with self-similar structures, that stimulate cutaneous regeneration.

Chitin is a major component of the exoskeleton and peritrophic matrix of arthropods (crustaceans, insects, arachnids, centipedes etc.), being the second most abundant biopolymer, after cellulose. It is a linear polysaccharide polymer composed of N-acethyl-β-D-glucosamine units (GlcNAc) bound in the β – 1,4 position by glycoside groups. Chitin from natural sources is a heteropolymer made up of remnants of GlcNAc and glucosamine (GlcN) in different proportions. [9,10].

The main biochemical activities of chitin and chitosan-based materials proved until now in wound healing are activation of polymorphonuclear cells, activation of fibroblasts, production of cytokines, migration and stimulation of type IV collagen synthesis gigantic cells. The increasing interest in chitin and its deacetilated forms comes from the biological activity resulting from its susceptibility to degradation under the influence of enzymes present in bodily fluids, such as lysozyme and N-acethylglucosoaminidase. Degradation products, called chito-oligomers, are able to stimulate macrophages and positively influence collagen deposition, thus accelerating the wound healing process. The monomeric unit of chitin, N-acethylglucosamine, is part of hyaluronic acid, a molecule that is important in wound healing. Therefore, chitin possesses characteristics favorable to rapid skin regeneration and accelerated wound healing [11]. Chitin is also used

as an excipient and vehicle in films, gels or powders formulated for applications involving mucoadesivity [12].

Keratin is a widespread protein, with an exceptional structural role, that is unfortunately insufficiently valued. In the epidermis, it has two main functions: to adhere cells to each other and to form a protective, waterproof layer on the outside of the skin. Along with collagen and elastin, gives skin its strength [13].

Numerous studies have been dedicated to the use of keratin extracted from sheep wool for wound healing, tissue engineering, controlled release drugs or cosmetics. The efficiency of biomaterials made from keratin extracted from wool or hair on cell proliferation and adhesiveness is due to the presence of amino acid sequences: leucine – aspartic acid - valine (LDV) and glutamic acid - aspartic acid - serine (EDS), self-assembly properties and biocompatibility [14-18]. Clinical tests with keratin-based products (keragel, keramatrix) have shown improvements in tissue surgery [19], foot ulcer [20] and bullous epidermolysis, a genetic disorder [21]. Various types of keratin-based materials studied to date: films, nanowires, sponges, gels have shown great potential for biomedical applications [22].

Taking into account all these aspects, the focus of our studies was the proper definition of chitin and keratin involvement in oxidative and inflammatory processes directed by intracellular oxygenated radicals and inflammation signaling factors (IL6, IL8, VEGF, IL1α) in order to straight forward the identification of mechanisms of wound healing modulated by chitin and keratin fractions, biotechnologically obtained from leather wastes.

2. Materials and methods

2.1. Materials

Tested active principles:

- **Keratin fragments extracted from wool** – Valke hydrolysate - water soluble lyophilised powder – **named Che III Valke**
- **Chitin hydrolysate from entomological waste (Lepidoptera sp.) - named Chitin L**; the assay was performed compared to a commercial reference (**Chitin R**) - alkaline hydrolysis in potassium hydroxide.
- **Standardized cell line**:
  Keratinocyte (HaCaT) - immortalized epithelial cells, with a pronounced regenerative potential, which undergo a differentiation process during their migration from the germinal basal layer to the corneous, scaly layer. HaCaT cells were cultured in DMEM medium containing 10% SFB, 1% Antibiotic/Antifungal, supplemented with glucose (0.135 gr/100 ml).
  Keratinocytes are the major source of cytokines in the epidermis. Normally, keratinocytes do not actively secrete cytokines; however, a number of agents mediate keratinocyte cytokine production, including

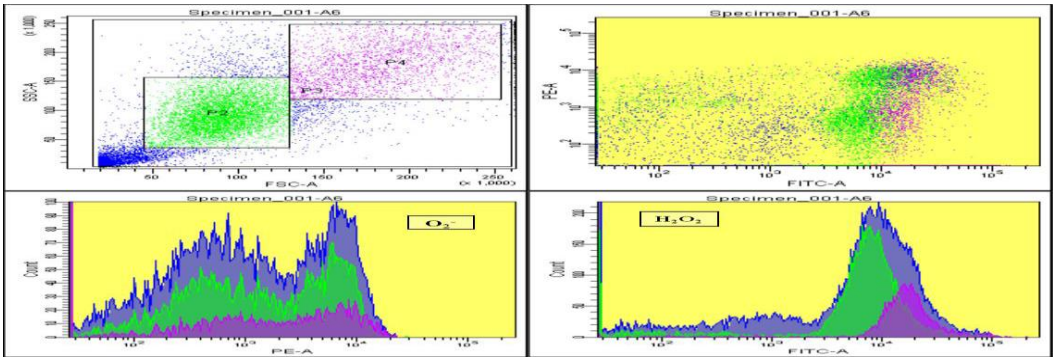
cytokines themselves. IL-1α, IL-6, IL-8 play an important role in the normal regulation of the epidermis [25].

2.2. Methods

2.2.1 Simultaneous identification of intracellular oxygenated radicals (superoxide anion and oxygenated water) by flow cytometry

The oxidation of 2'-7' dichlorodihydrofluorescein (H2DCF) to 2'-7'dichlorofluorescein (DCF) has been used quite extensively for the quantitation of H_2O_2. The diacetate form, H_2DCFDA and its acetomethyl ester H_2DCFDA-AM are taken up by cells where non specific cellular esterases act upon them to cleave off the lipophilic groups, resulting in a charged compound believed to be trapped inside the cell. Oxidation of H_2DCF by ROS converts the molecule to 2', 7' dichlorofluorescein (DCF), which is highly fluorescent. The reported wavelengths for the measurement of DCF fluorescence are 498 nm for excitation and 522 nm for emission. The production of green fluorescence is proportional to the amount of hydrogen peroxide generated [26, 27]. Cellular production of superoxide anion can be visualized by dihydroethidium, also referred to as hydroethidine (HE). This compound exhibits a blue fluorescence in cytosol until oxidized primarily by the superoxide anion and to a much lesser extent by other reactive oxygen or reactive nitrogen species. Oxidation of dihydroethidium results in hydroxylation at the 2-position forming 2-hydroxyethidium. With oxidation the compound intercalated with cellular DNA, staining the nucleus a bright fluorescent red with reported excitation and emission wavelengths of 620 nm(PE-A) [28, 29, 30]. The highlighting, in FSC / SSC coordinates of the normal cell population, then of the subpopulations corresponding to the cellular activation of the hydrogen peroxide (FITC –A), respectively of the superoxide anion (PE-A), is represented in the below figure:

Fig. 1. Flow cytometry representation of different cell populations' response to oxidative stress (PE- fluorescence of O_2^-; FITC – fluorescence of H_2O_2)



There are 2 categories of cellular response regarding the intracellular formation of the superoxide anion and hydrogen peroxide, which lead to the differentiation of 2 subpopulations: one of them characterized only by the presence of the superoxide anion, with a low granularity (blue events in the dot point), another one, more granular in morphology and with simultaneous formation of hydrogen peroxide and superoxide anion (blue events in the point-by-point representation)

2.2.2 Assessment of inflammatory status: IL-6, IL-8, IL-1α cytokines and VEGF determination

Flow cytometry is an analysis tool that allows discrimination of different particles based on size and color (fluorescent signal). Multiplexing is the simultaneous analysis of several analyzes in a single sample. CBA - kit uses a series of particles with discrete fluorescence intensities for the simultaneous detection of several soluble analytes (inflammatory cytokines). Each particle (beads) in the kit has a capture surface for a specific protein. Capture beads, conjugate detection antibodies and recombinant standards or test specimens are incubated together to form a sandwich complex that is visualized in APC-A / PE-A coordinates following the acquisition of flow cytometry. The kits use particles with different fluorescence intensities to detect soluble analytes [31, 32, 33]. Capture beads of a certain fluorescence intensity are bound to specific antibodies for a certain soluble protein (in our case IL6, IL8, IL1α and VEGF). The evaluation method consisted in the detection by flow cytometry of soluble proteins by capture with fluorescent beads. Results are expressed in pg/ml protein, after interpolation on a calibration curve with FACS Express software. The following kits were used:

- Human IL6 Single Plex Flex Set (BD Pharmingen)
- Human IL8 Single Plex Flex Set (BD Pharmingen)
- Human IL1α Single Plex Flex Set (BD Pharmingen)
- Human VEGF Single Plex Flex Set (BD Pharmingen)
- Human Soluble Protein Master Buffer Kit (BD Pharmingen)

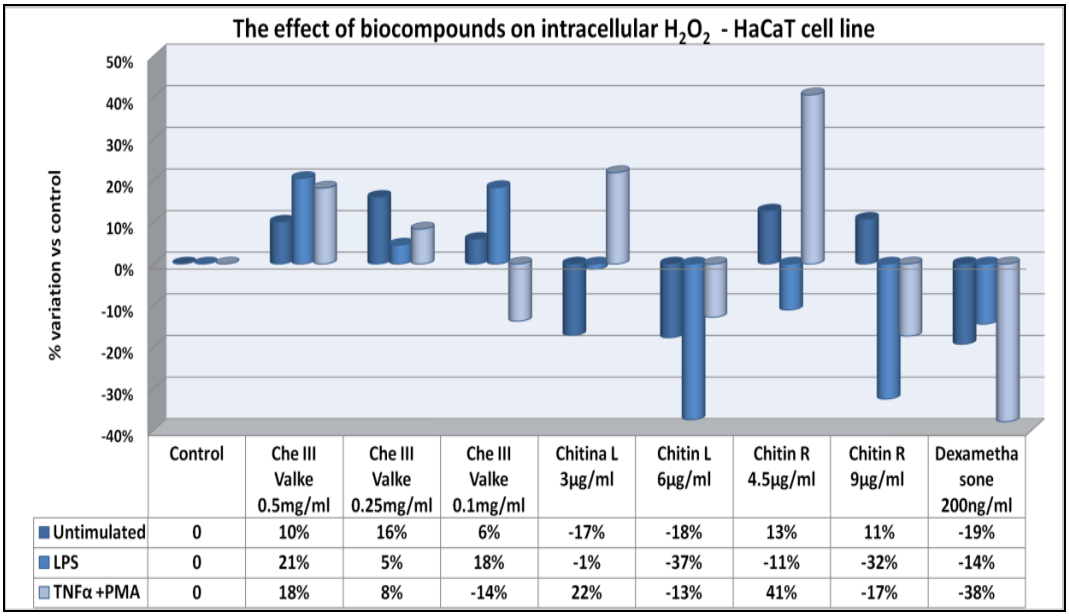
3. Results and discussions

The reactive oxygen species generate a cascade of molecular events leading to cellular damage. Organisms possess inner systems to counteract this decline, but a pro-inflammatory condition emphasizes tissues disruption. In this context, the experimental design applied in our studies concern the pro-inflammatory status of keratinocytes induced by relevant factors for *"in situ"* pathology-related conditions: TNF α – a systemic powerful stimulus, associated with PMA (phorbole miristat acetate, a pro-oxidative one) and LPS – a bacterial polysaccharide that mimics the cutaneous infection.

OXIDATIVE STRESS - intracellular oxygenated free radicals

Oxidative balance is very fragile especially on inflammatory conditions. As a consequence, we focus on it as a first step in finding skin healing solutions.

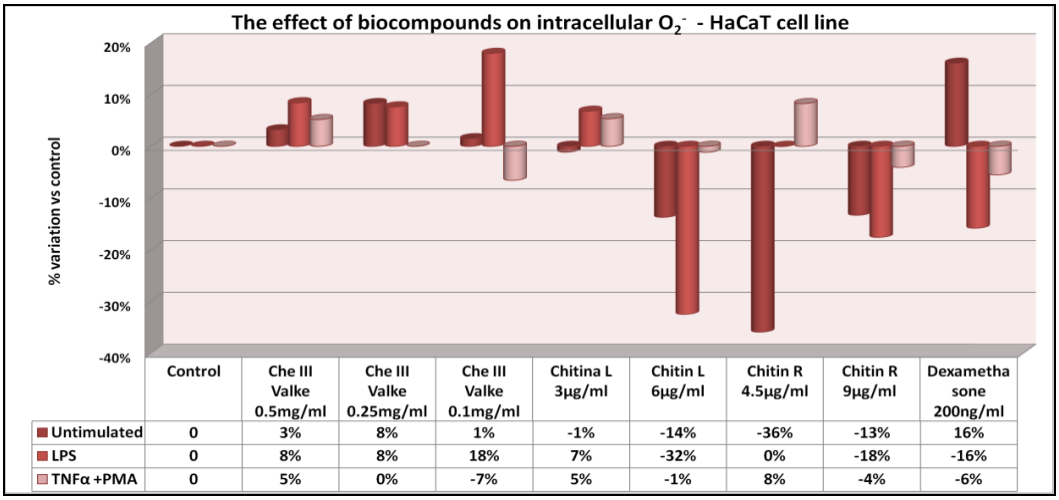
Keratinocytes were cultivated 24h, followed by treatment with test substances for 48h, from which 24 h stimulation with LPS1µg/ml and TNF α 15ng/ml+PMA0.1µM respectively. After this experimental time, cells were detached, stained for intracellular reactive species detection and flow cytometry analyzed. Results are presented in the figures below, expressed as % of variation compared with the control cells.



	Control	Che III Valke 0.5mg/ml	Che III Valke 0.25mg/ml	Che III Valke 0.1mg/ml	Chitina L 3µg/ml	Chitin L 6µg/ml	Chitin R 4.5µg/ml	Chitin R 9µg/ml	Dexametha sone 200ng/ml
Untimulated	0	10%	16%	6%	-17%	-18%	13%	11%	-19%
LPS	0	21%	5%	18%	-1%	-37%	-11%	-32%	-14%
TNFα +PMA	0	18%	8%	-14%	22%	-13%	41%	-17%	-38%

Fig. 2. Keratinocytes' response in terms of intracellular H_2O_2 to pro-inflammatory stimulation

Keratin fractions act mainly on H_2O_2 and O_2^- generated by pro-inflammatory systemic stimulation, Che 0.1mg/ml inhibiting the hydrogen peroxide with 14% and superoxide anion with 7%. Chitin is more active, especially those isolated from Laepidoptera sp., reducing the reactive oxygen species with 32-37%, but only on inflammatory conditions genereted by LPS. Keratin fraction Valke increase the reactive oxygen species, especially on LPS stimulation, suggesting caution on its use on infected wounds.

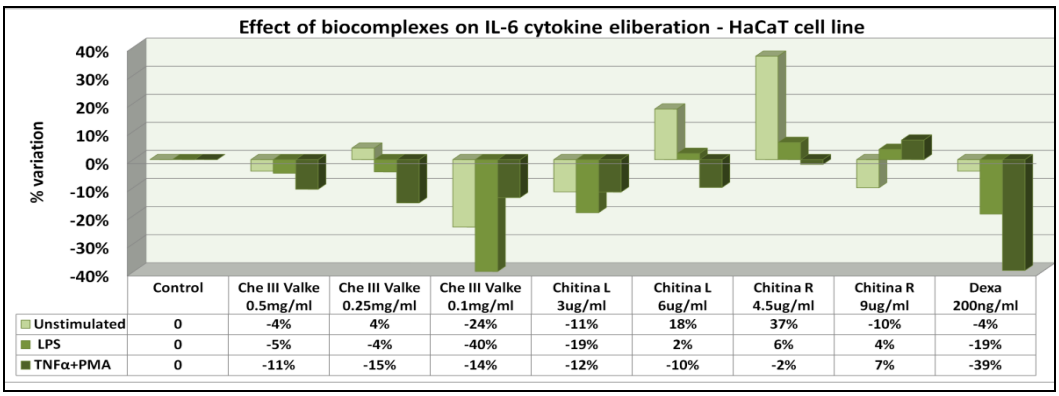


	Control	Che III Valke 0.5mg/ml	Che III Valke 0.25mg/ml	Che III Valke 0.1mg/ml	Chitina L 3µg/ml	Chitin L 6µg/ml	Chitin R 4.5µg/ml	Chitin R 9µg/ml	Dexametha sone 200ng/ml
Untimulated	0	3%	8%	1%	-1%	-14%	-36%	-13%	16%
LPS	0	8%	8%	18%	7%	-32%	0%	-18%	-16%
TNFα +PMA	0	5%	0%	-7%	5%	-1%	8%	-4%	-6%

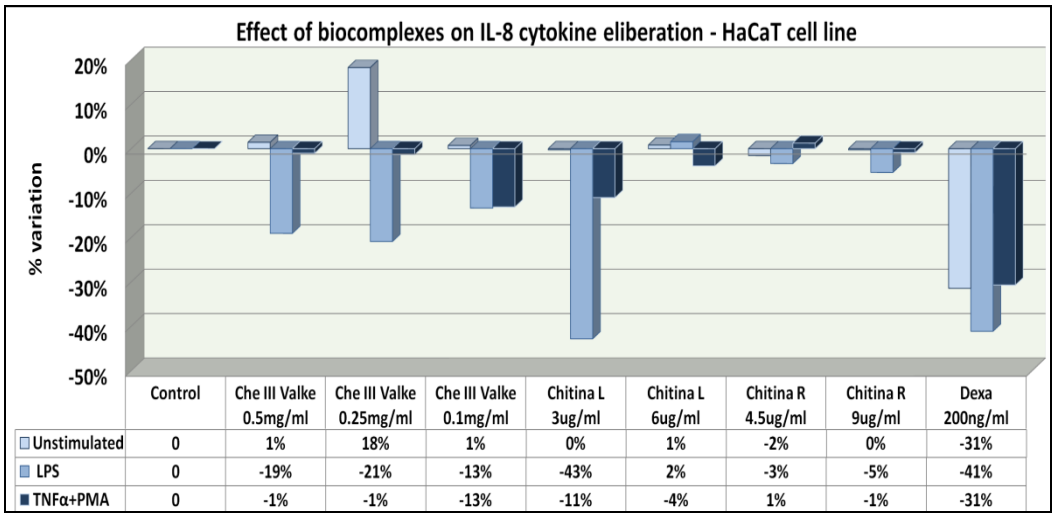
Fig. 3. Keratinocytes' response in terms of intracellular O_2^- to pro-inflammatory stimulation

Inflammatory status evaluation: IL6 and IL8 cytokines

As we previously mention, in order to demonstrate the anti-inflammatory effect of active principles, „in vitro" experimental models were applied to modulate the expression of proinflammatory cytokines in conditions of mimicking inflammation by non-specific prooxidant and pro-inflammatory stimuli (TNFα + PMA stimulation) and bacterial inflammation (LPS stimulation). Keratinocytes were cultivated 24h, followed by treatment with test substances for 48h, from which 24 h stimulation. After this experimental time, extracellular media were collected and store at -70^{o}C for further investigations. The extracellular cytokines detection were done throug Capture – Beads - Analysis technique, as we previously describe, and flow cytometry analyzed. Results from succesive experiments are presented in the figures below, expressed as % of variation compared with the control cells.



	Control	Che III Valke 0.5mg/ml	Che III Valke 0.25mg/ml	Che III Valke 0.1mg/ml	Chitina L 3ug/ml	Chitina L 6ug/ml	Chitina R 4.5ug/ml	Chitina R 9ug/ml	Dexa 200ng/ml
Unstimulated	0	-4%	4%	-24%	-11%	18%	37%	-10%	-4%
LPS	0	-5%	-4%	-40%	-19%	2%	6%	4%	-19%
TNFα+PMA	0	-11%	-15%	-14%	-12%	-10%	-2%	7%	-39%



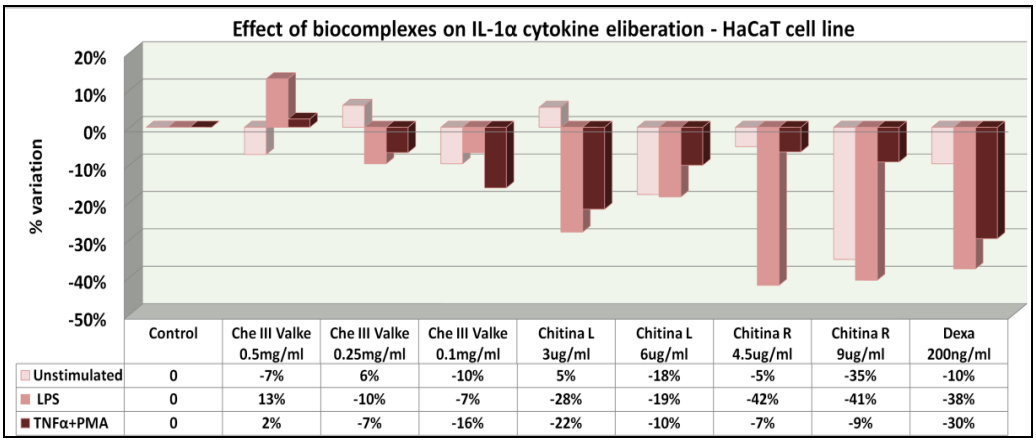
	Control	Che III Valke 0.5mg/ml	Che III Valke 0.25mg/ml	Che III Valke 0.1mg/ml	Chitina L 3ug/ml	Chitina L 6ug/ml	Chitina R 4.5ug/ml	Chitina R 9ug/ml	Dexa 200ng/ml
Unstimulated	0	1%	18%	1%	0%	1%	-2%	0%	-31%
LPS	0	-19%	-21%	-13%	-43%	2%	-3%	-5%	-41%
TNFα+PMA	0	-1%	-1%	-13%	-11%	-4%	1%	-1%	-31%

Fig. 4. Keratinocytes'proinflammatory cytokines' expression modulated by chitin and keratin

Cheratin Valke is active mostly on IL6 regulatory pathway in inflammation caused by bacterial infection (polysaccharide stimulation). Chitin acts mainly on IL8 inhibition in LPS stimulated keratinocytes, only those isolated from entomological sources. Weaker response have noticed for TNFα + PMA stimulation. Interleukin (IL)-6 plays a central role in acute inflammation and also, together with IL8 induce chemotaxis of leukocytes into a wound. As well as, IL-6 signaling is responsible for the switch to the reconstruction phase of the healing process. Integrating these results, keratin fragments and chitin took part in the regulation of wound healing as a critical step for non-healing wounds: inappropriate proinflammatory signaling can result in wounds that take much longer to heal and are at risk of infection.

Irritative status evaluation: IL-1α cytokine

IL1α cytokine have a close relationship to wound remodeling as well as to skin irritation. It is considered that inflammatory cytokines may become one of the markers for wound age estimation, but further studies will confirm this ipothesis. Our investigations concern this cytokine modulation in respect with keratine and chitin 48h treatment of the keratinocytes' monolayer. Results of the experimental models of inflammation previously described are presented in the folowing picture.



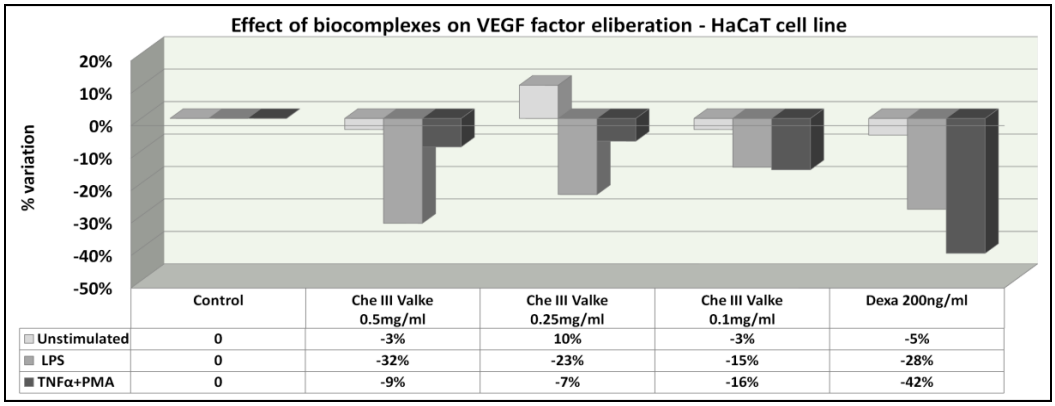
	Control	Che III Valke 0.5mg/ml	Che III Valke 0.25mg/ml	Che III Valke 0.1mg/ml	Chitina L 3ug/ml	Chitina L 6ug/ml	Chitina R 4.5ug/ml	Chitina R 9ug/ml	Dexa 200ng/ml
Unstimulated	0	-7%	6%	-10%	5%	-18%	-5%	-35%	-10%
LPS	0	13%	-10%	-7%	-28%	-19%	-42%	-41%	-38%
TNFα+PMA	0	2%	-7%	-16%	-22%	-10%	-7%	-9%	-30%

Fig. 5. Keratinocytes'pro – irritating factor IL1α, modulated by chitin and keratin

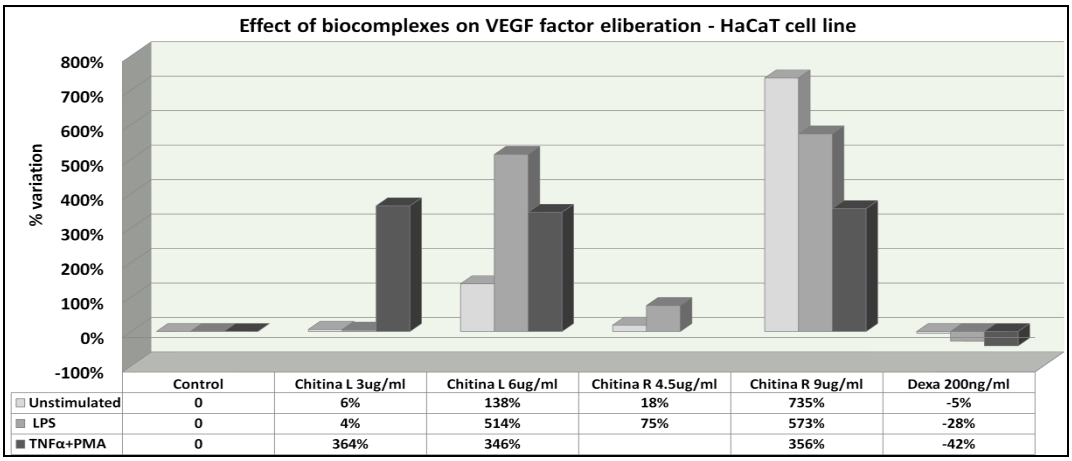
Chitin is more active than cheratin inhibiting the IL1α release, both varieties tested: from entomological and marine sources, similar with Dexamethasone, the antiinflamatory drug. This effect is mainained in both types of stimulation, systemic and bacteria - like. The relevance of these proved action consist in the potential aplications of the active principles on sensitive / burn / injured skin.

Pro-angiogenic signaling: VEGF factor

VEGF (vascular endothelial growth factor) is a multifactorial agents that stimulates wound healing through cellular mechanisms, including collagen deposition, angiogenesis and epithelization. It induces vascular permeability and the influx of inflammatory cells into the site of injury. Its significance in the wound closure and epidermal repair, as well as its interrelated actions with the others soluble factors presented in this paper selected it in our experimental design of chitin and keratin evaluation at epidermal level. Results (fig.6) expressed as % compared with the control were calculated from concentration (pg/ml) found in the culture media treated with the active compounds.



	Control	Che III Valke 0.5mg/ml	Che III Valke 0.25mg/ml	Che III Valke 0.1mg/ml	Dexa 200ng/ml
Unstimulated	0	-3%	10%	-3%	-5%
LPS	0	-32%	-23%	-15%	-28%
TNFα+PMA	0	-9%	-7%	-16%	-42%



	Control	Chitina L 3ug/ml	Chitina L 6ug/ml	Chitina R 4.5ug/ml	Chitina R 9ug/ml	Dexa 200ng/ml
Unstimulated	0	6%	138%	18%	735%	-5%
LPS	0	4%	514%	75%	573%	-28%
TNFα+PMA	0	364%	346%		356%	-42%

Fig 6. Pro-angiogenic factor VEGF modulated by chitin and keratin

Differentiated results were obtained, keratin Valke inhibits the release of VEGF, but chitine increased it dramatically in the extracellular environment. These effects reccomend chitine as a potent pro-angiogenic mediator through VEGF pathway, increasing also the vascular permeability. In contrast, keratine inhibits this factor's release, reducing the angiogenesis signaling and preventing the hypervascularization. Every compound has to be correctly applied in proper formulations based on their differentiated effects.

Conclusions

The experimental design covered the main inflammatory pathways governed by extracellular modulation of different signaling cascades (TNFα, PMA, LPS) at epidermal level, in order to define the efficacy profile of chitin and keratin fractions. The intracellular oxidative stress was induced by the chosen stimuli, chitin L having moderate activity in counteracting these effects (Inhibits H_2O_2 and O_2^- generated by pro-inflammatory stimulation).

In bacterial infections simulated in vitro by stimulation with LPS polysaccharide, the anti-inflammatory effects induced by keratin Valke on IL6, an acute phase cytokine, are noticeable. Also, the anti-inflammatory effect is supported by the inhibition of IL8, the chemokine responsible for the recruitment of neutrophils to the inflammatory site, a phenomenon achieved by most of the tested compounds.

In chronic wounds, with systemic inflammation installed, process simulated by TNF alpha and PMA stimuli, but also in the case of over-infected lesions (LPS stimulation), the pro-angiogenic factor VEGF is activated especially by chitin, suggesting a sustained regeneration potential of the vasculature, at the deep tissue level.

A special mention has to be done to the anti-irritating effect induced by chitin and keratin Valke on IL1α, a valuable result in long term healing process, especially for difficult wounds, resistant at usual treatment.

Results directed the further development of chitin and keratin based – treatments, strengthen their role in epidermal inflammation remission with lack of irritation and starting the angiogenesis for the new tissue reconstruction.

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