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Review Article

In vitro Methods Used for Simulation of Skin Functions: Application in Skin Care Products

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ABSTRACT

The skin is the largest organ in mammals and serves as a protective barrier at the interface between the human body and the surrounding environment. It guards the underlying organs and protects the body against pathogens and microorganisms. Accordingly, it is directly exposed to potentially harmful microbial, thermal, mechanical and chemical influences. Simulating skin function aims for different biomaterials evaluation and for explorations of fundamental biological processes. In this mini review, we summarize *in vitro* methods that simulate skin hydration, aging and photoaging process, wound healing and menopause, high lightening novel efforts in the design of *in vitro* studies for the development of skin care products.

Keywords: Environment; Microorganisms; Skin care; Glycosaminoglycans; Structural proteins; Lipids; Skin microbiota

INTRODUCTION

The skin is the largest organ in mammals and serves as a protective barrier at the interface between the human body and the surrounding environment [1]. Two types of human skin cells, the keratinocytes and fibroblasts, that grown as monocultures are mostly used in skin research. In addition, human 3D (three dimensional)-skin models have also been developed and used for *in vitro* toxicity testing, biomaterials evaluation, and investigation of fundamental biological processes [2]. The most abundant cell type of the dermis is the fibroblasts. These cells ensure skin firmness and elasticity and regulates collagen production. In cosmetic industry research, fibroblasts are used to screen *in vitro* natural bio-compounds with potential beneficial properties to increase skin heath.

Cell culture is a process where isolated animal cells are maintained and grown outside of their bodies under controlled conditions. In skin research they are used as alternative to animal experimentation to investigate the effects of drugs and toxins in cell metabolism and survival [3]. Isolated cells can also be manipulated by transfection to investigate the role of genes in the physiology or malignancy. In the past 25 years, great efforts have been made to create substitutes that mimic human skin [4]. These skin substitutes were made possible by employing advanced tissue engineering (TE) approaches and have been used for clinical applications, promoting the healing of acute and chronic wounds, or utilized as complex human-based organ-like test systems for basic or pharmaceutical research [5]. In skin TE, various biological and synthetic materials are combined with *in vitro*-cultured cells to generate functional tissues [6]. A critical issue is the *ex vivo* expansion that is required to obtain enough numbers of the needed cells, while preserving the cells' normal phenotype and functionality. Only then these cells can be used for either the generation of skin substitutes that are suitable for transplantation or as *in vitro* test systems [3,4]. Especially the latter is of growing interest for the field of skin TE.

LITERATURE REVIEW

In this mini review, we summarize *in vitro* methods that simulate skin hydration, aging and photoaging process, wound healing and menopause, high lightening novel efforts in the design of *in vitro* studies for the development of skin care products.

Skin hydration

Skin hydration is crucial factor for many aspects of skin function such as skin aging, skin elasticity. To this, water is a very important element of every organism's health; therefore, its homeostasis is really necessary. The ability of human skin to maintain water is highly correlated with the outer skin layer-the Stratum Corneum

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(SC) - which acts as a barrier to water loss. This happens by the co-operation of water, small osmolytes, polyanionic glycosaminoglycans, structural proteins, lipids, channels, receptors and skin microbiota. Abnormalities of SC are connected with skin diseases such as atopic dermatitis eczema, psoriasis, senile xerosis, and hereditary ichthyosis [5,6]. Hydrobalance is the balance between the generation of water and the reduction of Trans Epidermal Water Loss (TWLS) and is regulated by lipids and Natural Moisturizing Factors (NMF). It is important to note that TWLS is different from perspiration [7]. The barrier and sensorial role of SC is attributed to the corneocytes which are cells without nucleus and consist of keratin fibers, amino acids and NMFs. Interestingly, UV can damage SC by destroying the skin's natural moisturizing process. It is important to note that NMFs are found only in SC cells and ensure a wet environment as in SC many hydration-dependent enzymatic reactions take place. Therefore, if water SC content is reduced, many normal enzymatic processes cannot take place leading to visible appearance of dryness, roughness, scaling, and flaking [7].

Although, there is not much work done *in vitro* for the simulation of skin hydration process there are some *in vitro* models proposed for use such us 2D cell cultures based on normal human epidermal keratinocytes (NHEK) and 3D reconstructed human epidermis (RHE). By using these *in vitro* models we can evaluate hydration by measuring the stimulation of the epidermal differentiation (filaggrin, involucrin, transglutaminase, cytokeratins), the synthesis of sebaceous and epidermal lipids (ceramides, cerebrosides and phospholipids), the expression of extracellular matrix components (glycosaminoglycans, hyaluronic acid, proteoglycans, ECM receptors and proteases) and the expression of markers of epidermal cohesion and intercellular cell junctions (claudin, occludin, desmogleins, integrin V, collagen IV, collagen VII, connexins and aquaporines) [7,8]. Specifically, focusing on *in vitro* 3D Reconstructed Human Epidermis (RHE) which is composed by normal human dermal fibroblasts (NHDF) and NHEK cell types, allows a short-term clinical dimension of skin hydration since this model considered to be similar to human skin as far as differentiation markers, morphology and functional characteristics are concerned. RHE is metabolically and mitotically active while it exists in many stages of maturity [9]. On the other hand, focusing on 2D cell cultures based on NHEK we can have a molecular insight of skin hydration process targeting the transcripts of genes that encode proteins important in this process.

Aging and photoaging

Aging is a normal and time dependent biological process which is related to changes on skin appearance and functionality and it is caused by both intrinsic and extrinsic factors. Typical extrinsic factors are UV exposure, IR exposure, smoking and hormones status [10,11]. In terms of clinical aspects, aged skin seems to be pale, thin, dry and has wrinkles, irregular hair growth and insufficient sweat. It also has sagging, increased laxity, insufficient perspiration, thinning of nail plates and loss of fat tissue leading to the formation of hollowed cheeks and eye sockets [10-12]. In terms of molecular biology, aging reduces self-renewing capability of the epidermis and changes vital thermoregulation function of eccrine sweat glands. Specifically, collagen which is the most representative protein in the extracellular matrix and due to age, undergoes gradual fragmentation leading to loss of skin mechanical properties and dermal cell functions. The decrease of collagen in extracellular matrix (ECM) leads to reduced skin strength, wrinkle formation and to the formation of a microenvironment which helps tumor initiation and progression. It is important to note that the decrease in collagen happens due to the overexpression of matrix metallopeptidases (MMPs) that happens naturally as a result of aging. Moreover, aging affects pigmentation, immunity, vasculature, innervations, wound healing, adipose and fat homeostasis. Furthermore, under normal aging process the basal keratinocyte proliferation is reduced leading to thinning of dermis and epidermis junctions (DEJ). The flattening of DEJ has many consequences such as reduction of the exchange surface between epidermis and dermis, reduction of nutrient flux, reduction of keratinocyte proliferation, reduction of epidermal resistance to shearing forces and reduction of surface lipid production, increasing incidence of xerosis, pruritus and skin irritation in elderlies.

On the other hand, photo-aging is that type of aging that happens due to UV exposure and is a cumulative process whose degeneration rate is related to the frequency, duration and intensity of solar exposure. This rate is correlated with the skin pigmentation status. Photo-aged skin has deep wrinkles, reduced elasticity, dryness, laxity, rough textured appearance, broken-appearing blood vessels, increased epidermal nerve fibers, cancerous lesions, precancerous lesions, accumulation of amorphous elastin-containing material in the upper dermis, increased sensory nerves and pigmentation disorders. Furthermore, the SC turnover rate and transit time is reduced due to photo-aging. The appearance of photo-aged skin is different from the naturally aged skin and is caused by the damage of the connective tissue of the dermis [11-16]. As far as pigmentation is concerned, an interesting study showed that cumulative dermal fibroblasts modifications are highly correlated with the hyperpigmentation status seen in photo-aging [16]. A possible mechanism of aging proposes that reactive oxygen species (ROS) and Manganese Superoxide Dismutase (MnSOD) destroy skin components such as lipids and proteins. To add to this, UV light generates hydroxyl radicals (OH) which induce the expression of MMPs leading to extracellular matrix component degradation such as collagen [10,11,13,17]. ROS are normally produced, by the electron transport chain of the aerobic metabolism in the mitochondria and therefore cells have developed mechanisms that convert ROS into less toxic metabolites in order to prevent their accumulation. Skin, seems to have the biggest concentration of ROS compared to other organs [18,19]. Apart from collagen and skin connective tissue degradation , UV has many consequences for the cell such as alteration of immune response, DNA damage, RNA damage, protein damage, lipid damage and mitochondrial DNA (mtDNA) damage [11,12]. An interesting study proposed that UV can also have an effect on transforming growth factor beta (TGFb)/Smad pathway which is highly linked to collagen I production. Moreover they propose that carbazole (FICZ), which is a key chromophore produced by human keratinocytes in response to UVB and UVA, is involved in photo-aging and therefore it could be used as a potential target to prevent this process [17].

Two promising *in vitro* models are used so far, to simulate the aging process of the skin. The first based on 3D RHE model supported by a comparative study between normal and irradiated RHE model [14]. Interestingly, irradiated RHE showed some special characteristics compared to normal RHE such as decreased keratinocyte viability,

increased permeability of caffeine, testosterone and nanocarriers, while release of interlleukin-1 (IL-1) and interlleukin-8 (IL-8) and increased number of senescence-associated -galactosidase positive keratinocytes indicated stress mediated cellular senescence. Moreover, irradiated RHE had thinner stratum corneum possibly due to flattened keratinocytes and/or exfoliated corneocytes. The second model is based on a pigmented skin model comprising a melanocyte-containing epidermis cultured on a living fibroblast embedded-dermal equivalent [15]. This skin model gave important information for the involvement of dermal fibroblasts in skin pigmentation, from the microscopic to the macroscopic level [15]. Last but not least some helpful biological markers of aging as proposed and used by Damiani et al are β-galactosidase activity, p16 expression and proliferation rate. Specifically, normal human dermal fibroblasts were serially cultured under 21% and 5% of CO2 and it was shown that in the condition of 21% compared with 5% there was an increase in the markers of aging and senescence while there was upregulation of MMPs, downregulation of COL1A1 and increased DNA damage [16]. Last but not least, it has been reported that UV irradiation activates *in vitro* at least three pathways including the mitogen-activated kinase pathway (MAPK), the stress-activated kinase pathway (SAPK) and the p38 pathway [10].

Wound healing- *in vitro* scratch assay

Wound healing is a normal, age dependent and evolutionary conserved process which aims to maintain skin integrity after an injury [20,21]. However, the outcome of the wound healing process is not the same between different organisms. It consists of three partially overlapping phases: Hemostasis and inflammation, proliferation and tissue remodeling [21,22]. It would be important to note that the wound healing process in human is very complicated as it needs coordination between many cell types, especially keratinocytes and fibroblasts [22]. After injury the accumulation of platelets lead to the formation of a blood clot whose function is to prevent blood loss and make the initiation of molecular cascades which lead to the formation of an early ECM that will act as a scaffold of cellular attachment and proliferation [22].The blood clot consists of platelets, cross-linked fibrin fibers, plasma fibronectin, vitronectin, and thrombospondin [23]. The damaged epithelium and the blood clot release chemotactic factors that recruits inflammatory cells from the surrounding tissues to the damaged cite through blood circulation. The first to arrive are the neutrophils whose function is to kill microorganisms in the early wound. Then in the site of damage we have monocytes which differentiate into macrophages and are responsible for clearing apoptotic neutrophils and orchestrating tissue-specific functions during the different stages of wound healing. Then we have Mast Cells (MC) which co-ordinate many aspects of the wound healing process. All these cells apart from growth factors and cytokines also produce toxic mediators such as ROS and proteases which are harmful for the surrounding area. Interestingly some studies propose that neutrophils and macrophages are rather inhibitory than enhancing to the wound healing process [22,23]. This phase takes about 72 hr in order to be completed [20]. As the first phase subsides, the endothelial activation and the degradation of endothelial basement membrane are followed by angiogenesis which happens in response to Fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF). This process is very important as the new blood vessels are responsible for the supply of nutrients, oxygen and metabolite exchange. Simultaneously, fibroblasts migrate into the wound in response to TGF-1, Platelet-derived growth factor (PDGF) and fibroblasts growth factor (FGF), where they proliferate and produce new ECM, including proteoglycans, hyaluronic acid, collagen, and elastin. To add to this, some fibroblasts differentiate into myofibroblasts and are responsible for wound contraction and the deposition of additional matrix. The new tissue formed is called granulation tissue and allows re-epithelization. During re-epithelization, keratinocytes and dermal appendages migrate to the bed of wound in order to cover it with new epidermis. Interestingly, the migration of keratinocytes is related with an Epithelial-Mesenchymal Transition (EMT) like process giving epithelial cells a migratory ability. This phase's duration varies from some days to weeks [20,22,23]. During the tissue remodeling phase of wound healing, keratinocytes stop their migration and revert their EMT like phenotype.

Moreover, myofibroblasts become apoptotic, angiogenesis stops, a cellular scar is formed, collagen type III is gradually dominated by collagen type I and the disorganized collagen fibers are rearranged and aligned. This phase can last from months to years while its defects are linked to excessive wound healing or chronic wound [20,22]. Notably, it has been shown that the wound healing process is promoted in moist environments proposing that water and hydration are positive effectors of the healing response. Specifically, moist environment indicatively accelerates wound healing, reduces scar formation, promotes epithelization rate, reduces pain perception and reduces wound infection rates [24]. Another important part of successful wound healing is the existence of undifferentiated cells in the basal layer of the skin epithelium. New studies have shown that there are two dermal lineages giving rise to the upper and lower dermis respectively. Furthermore, regeneration phase can be affected by inflammation in the wound area. Collectively, some of the most important wound healing regulators are EGF family, prostaglandin E2 (PGE2), TGFb1/Smad, TGFb1/TGFb3 ratio, MMPs, KGF, recombinant human bFGF, recombinant human epidermal growth factor (FGF) and PDGF [20,23,25]. Interestingly, there are many proposals of *in vitro* methods that aim to simulate wound healing process of human skin. Firstly, an *in vitro* model which consisted of keratinocytes and fibroblasts in a 3D viscose rayon scaffold. Then a 3D multilayered reconstructed human epidermal cellular construct while many others still use single cell monolayers as they are simple and cost-effective. A recent innovation was the construction of a de-cellularized epidermis that was re-seeded with keratinocytes and fibroblasts. In the same study in order to study wound healing process they used a week viable 3D skin wound healing model which consists of a collagen type I construct with fibrin-filled defects [20,23]. Specifically, they seeded keratinocytes in the upper side of the collagen construct and then they formed a wound which they filled with fibrin. Then fibroblasts were added in two ways into this construct. In the first case fibroblasts were added directly in the collagen construct while in the second case they were added via fibrin beads, in two concentrations, in order to control the spatial arrangement of fibroblast according to keratinocytes. The results showed that the bigger keratinocyte migration was seen when keratinocytes and fibroblasts were in a close proximity [24]. Also, the bigger keratinocyte migration was seen in the constructs with

small cell growth proposing that fibroblasts promote keratinocyte migration over proliferation in the wound healing process. Finally the two known regulators- TGF-b and b-FGF- were also increased when keratinocytes where in a close proximity with fibroblasts [25].

Menopause

Menopause is a normal biological process that happens in women as a result of the reduction of ovarian hormones such as estrogen and the increase of androgens and it is defined by twelve months of amenorrhea after the final menstrual period. Menopause can cause severe skin and hair problems in women such as alopecia, skindryness, loss of skin elasticity and skin atrophy leading to increased anxiety, reduced self-esteem, and low quality of life [26,27]. Although, many therapies have been proposed, aiming to reduce the menopause symptoms, none of them succeeded to support treatment in menopausal disorders [18]. An extremely interesting and useful *in vitro* method for simulation of menopause in normal human dermal fibroblasts (NHDF) cells, which consisted of four individual steps, was proposed by Remoue et al [25]. According this study, at first step, the NHDF cells were seeded and let to reach confluence while in the second step NHDF cells were cultured in order to simulate collagen synthesis and deposition under non-menopausal conditions. In the third step, NHDF cells were cultured with medium which lacked growth factors and serum under non-menopausal conditions. Finally, in the fourth step, which was the test phase, NHDF cells were cultured under menopausal conditions and were compared with NHDF cells cultured under non menopausal conditions. The menopausal conditions achieved for NHDF cells under specific hormonal treatment [26]. In this study was shown that under menopausal conditions cell proliferation, extracellular collagen deposition, collagen III/collagen I ratio were reduced while the release of MMP, and especially MMP1 and MMP3, is increased. Surprisingly, it worth to be mentioned, during the first hours of incubation with menopausal, hormone concentrations there was a reduction in procollagen I and procollagen III expression while the next hours there was an increase suggesting that there is a mechanism that regulates collagen synthesis under these conditions. It was proposed that the decreased collagen deposition despite the increased procollagen expression was correlated with increased MMP release, post-translational modifications and folding processes of collagen [26]. As it was previously mentioned, menopause causes adverse effects on skin which are probably related to collagen loss. However, it is not clear if these effects are more related to age or to menopause [27,28].

Interestingly, another study proposed that collagen loss is more related to age than to postmenopausal years [26]. Specifically, in menopause there has been described a reduction in post-translational modifications of collagen and a reduction in the amount of hydroxyproline and glycosylated hydrolysine in collagen type I [29]. Also, it has been proposed that during menopause, levels of collagen IV are reduced in DEJ and that this reduction is age related while there is involvement of TGF-1 signaling [27]. It was previously stated that TGF-1 signaling is related to the synthesis of many extracellular matrix macromolecules and to collagen biosynthesis [30]. In 1990, it was found that skin cells also have estrogen receptors which are reduced during menopause and are responsible for the symptoms of menopause. The expression of these receptors is inversely correlated with the levels of ferritin under menopausal conditions [27,31]. Moreover, it was shown that under menopausal conditions there was less effective healing possibility due to the decreased levels of TGF-b while there were differences in metabolism.

CONCLUSION

Finally yet importantly, the low levels of estrogen led to the reduction of the polymerization of glycosaminoglycans, the elastin fragmentation and granular degeneration and the reduction of the mitotic activity of epidermal basal layer.

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