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Marine Molluscan Shell for Dermal Regeneration: A Review

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Abstract

The dermal regeneration is the process of normal wound healing. The wound healing in mammals begins with inflammatory phase which is followed by a series of phase ultimately leading to the total restoration of the skin. The wound healing process is conserved and takes time. The process of this wound healing can be enhanced by using natural compounds. Marine molluscan shell is also seen to possess this property of enhancing the process of wound healing. Marine Molluscan shells are a highly controlled bio mineralized material which is made up of two layers of calcium carbonate, comprising an outer layer of calcite and an inner layer of aragonite. Nacre the inner lustrous layer of the molluscan shell known for its mechanical properties has layers of organic and inorganic components which make it an excellent biomaterial. The marine molluscan shell and nacre comprises an organic matrix which is made up of proteins, lipids and polysaccharides. These organic matrices are of therapeutic importance and hence the shell has been used for many biomedical applications. The application of the shell, shell matrix and nacre on osteogenesis has been widely studied. Limited studies have done for the application of skin regeneration by molluscan shells. However, the potential skin therapeutic application of nacre has not been fully exploited. The current review is about the application of the shell in dermatological application.

Keywords: Marine molluscs; Shell; Nacre; Wound healing

Abbreviations: TGase: Transglutaminase; HDF: Human Dermal Skin Fibroblasts; HSF: Human Scar Fibroblasts; WSN: Water-Soluble Components of The Nacre; ASM: Acid Soluble Matrix; AIM: Acid In-Soluble Matrix; ECM: Extra Cellular Matrix; UV: Ultraviolet; iNOS: Inducible Nitric Oxide Synthase

Introduction

Marine molluscans are diversified group of animals. They are an important group of marine animals and constitute about 23% of the entire living organism in the hydrosphere [1]. The marine molluscs are economically important as most of them are used for edible purposes and also for other applications such as crafts making, to dye cotton, yarn and clothes, etc. Hence, they have become a natural resource of economic importance [2]. The major classifications of the molluscs are Polyplacophora, Bivalvia, Gastropod and Cephalopoda of these Bivalvia and Gastropod exhibit a unique characteristic of bio-mineralization. Since the animals belonging to this class are soft bodied animals; they have adopted this characteristic of developing an external hard calcified structure. These structures protect the animals from predators and also support their growth. These bio-mineralized layer exhibit a wide range of morphologies depending upon the animal.

The molluscan shell is a biologically controlled mineralization. The shell fabrication is strictly under the

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control of cascades of genes. The entire process of shell construction is modulated by an extracellular organic matrix, a part of which is occluded in the shell during calcification [3]. Nacre, commonly known as the mother-of-pearl, is the inner lustrous layer of the molluscan protective shell. Its micron-sized compact brick wall whose texture is more than 1000 times tougher than the chemically precipitated counterpart, aragonite [4]. The nacre shows a layered structure. It has a stacked structure composed of 5% organic and 95% inorganic layers. These components pave way for the formation of the brick and mortar structure which is an important characteristic of a molluscan shell [5].These shells are of great economic importance and has a huge demand [6].

The skin is the largest human organ. It is the outer protective wrapping for the body and also act as the first line of defence in the human body. It acts as an interface between the body and the outside environment. When a skin is damaged, a natural regeneration is instigated [7]. Dermal regeneration is the biological process of skin wound healing. However, the process of wound healing may be delayed when more layers of the skin are prone to damage. To enhance the healing process, wound dressings are applied to the damaged area. Such treatments are insufficient in serious burn victims, chronic wounds, and other wounds that encompass large areas of the body [8].

From times immemorial, molluscan shells or its by products were used for maintenance of human skin. Marine pearls have been suggested for the treatment of the skin wounds. The pearl extract was seen to promote the migration of fibroblasts that could help wound healing [9]. The Water-Soluble Matrices of the pearl was also identified to enhance the fibroblast proliferation, collagen accumulation and also inhibited MMP-2 activity. The pearl fractions also significantly promoted TIMP-1 production that helps in wound healing [10]. However, the pearls are very costlier and its application for human skin will be even costlier than the conventional methods.

Though the shells of nacre are of economic importance, the shells some edible molluscan shells are considered as wastes. However, these shells are also made up of the similar matrix proteins. The matrix proteins present in marine molluscan shells and the nacre have made it a valuable biomedical product. It has led to the usage of the shells in the zoo therapy. Since ancient times marine molluscan shell has been used in the treatment of human diseases. Nacre has also shown promising mechanical properties [11] and also showed excellent biological properties such as the biocompatibility and biodegradability. This has led to the use of the nacre directly in the field of the bone tissue engineering. It is known to induce the proliferation of the osteoblasts [12-16]. The nacre contains one or more signal molecules capable of activating osteogenic bone marrow cells. The osteogenic effects of the nacre particles were studied on a sheep model. The cavities induced in the bones of the sheep were filled with the nacre and formation of the new layers was observed. There was significant activation of the bone formation and increased mineralization [17]. Osteoclast precursor cells were grown on a Nacre substrate and shown to differentiate into active osteoblast [18]. The Water-soluble Matrix of the Pinctada maxima on the human osteoblast was found to increase the Bcl-2 protein associated with the cell cycle and hence it reveals the mechanism of the osteoblast proliferation [19].

Apart from the usage of nacre for bone regeneration studies, due to its biocompatibility and biodegradability, nacre is also known to have impact on the human skin. Hence, it has found its application in cosmetics industries. The current review deals with the study of impact of marine molluscan shell on the skin cells and to correlate various works to study the mechanism underlying in the usage of molluscan shell in human dermatological applications.

Studies on the Impact of Molluscan Nacre on Skin Regeneration

The nacre extracted from the inner shell layer of the giant oyster, *Pinctada maxima*was powdered and mixed with autologous blood and a mouldable paste suitable for implantation was prepared. The prepared nacre paste was implanted onto IOPS WISTAR rats. The implants were inserted on the ventral surface of rats at the junction of the thorax and abdomen. After implantation the histological studies were performed. The sutures on the implanted site were observed to be healed very fast and the incisions were healing within first day. The implant got dissolved and no evidence of necrosis was observed. The nacre was not encountered by any macrophages and collagenase was detected around all the residual nacre fragments. Angiogenesis was observed and the capillaries were seen to be organized in concentric networks at some distance from the implant but they were seen directed towards the implant. The fibroblasts were seen to be recruited in larger number which was very active. The fibroblasts were seen to be attracted towards the nacre. Collagen type I was seen as a dense network and Collagen type III was also observed. These two secretions can be closely related to the fibroblasts. Decorin, an important ECM protein was also seen at the site of the implants [20].

The lipids extracted from the nacre of *Pinctada margartifera* was on the artificially delipidated skin

explants. The profile of the lipids extracted from the nacre showed 0.5% of Cholesterol sulphate, 2.07% Hydroxylated ceramides, 1.03% Non-hydroxylated ceramides, 5.97% Cholesterol, 6.24% Fatty acids, 4.46% Triglycerides, 0.7% Cholesterol acetate and 79.03% Squalene like lipids. This lipid was tested on the delipidated skin explants. The expression of flagging in the skin explants was investigated according to monoclonal antibody labelling monitored with fluorescent microscopy. The immunofluorescence indicated that the nacre lipids induced an overexpression of flagging on the dehydrated skin, higher than the level expressed in the control explants. The expression of the Transglutaminase (TGase) was also conducted. It was concluded that the nacre lipids can supplement the deficient stratum corneum lipids. However, the molecule responsible for the activation of filaggrin and the repression of TGase was not identified. It was concluded that nacre and nacre extracts of the genus *Pinctada* have biological activities either on the bone or skin lineage [21].

The *in vitro* toxicity assessment of inner calcified layer of the shell of *Pinctada margartifera* was conducted on three different dermal cell lines representing various layers of the human skin. The different cells were HaCaT cells, a human derived immortalised keratinocytes cell line, primary human dermal skin fibroblasts (HDF) and primary human scar fibroblasts (HSF). The significance of testing on the normal and the scar cell lines can be correlated with the application of the nacre in the cosmetic applications. A live dead assay was conducted on these cell lines to test the cytotoxicity. The primary human scar fibroblasts (HSF) showed cytotoxicity at a concentration of 2.5mg/ml of nacre after 24 hours and 72 hours of incubation, whereas toxicity was observed at a concentration of 0.5 mg/ml in HaCaT cells after 24 hours. However, the cytotoxicity in HaCaT cells was not observed after 72 hours of incubation. The primary human dermal fibroblasts did not show any cytotoxicity at any concentration. Morphological changes were observed in the HDF and HSF at a higher concentration of 2.5 mg/ml. These morphological changes were postulated to be linked with the Reactive Oxygen Species levels in the cells. The Oxidative stress was tested using the cell permeable fluorogenic probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). The result revealed that there were no significant changes in the ROS levels in the cells. The study concluded that the alteration in the cell morphology was mainly due to the regulation of cell motility through geometrical constraint in the presence of nacre [22].

The effect of the water-soluble components of the nacre (WSN) of *Pteriamartensii* on the wound healing also showed higher biocompatibility when compared to that of the powdered nacre. The application of the WSN lead to less discomfort when compare to that of the powdered nacre. The effect of this WSN was studied on female porcine model (*Sus scrofadomesticus*). The animal was anesthetized and the fur of the animal was shaved. The second degree burn wound was induced in the shaved area using metal bars. The WSN was sprayed directly on to the wound at a time interval of two days. The WSN was sprayed at a concentration of 0.4% W/V. The skin was examined by various methods. The observation revealed that the recruitment of the fibroblast was higher in the areas where the WSN was applied. These fibroblasts play an essential role in the wound healing. The WSN treated models also showed quick healing when compared to that of the control models. The WSN stimulated fast granulation and tissue formation and also thick collagen tissue formation.

The wound healing in the model was assumed to have occurred via different routes. Either the WSN induced the extracellular matrix which will directly stimulate adhesion, proliferation, and migration of the cells that helps in the wound healing or by recognition of large matrix proteins or unknown stimuli by their cognate partners present on the cytoplasmic membrane of cells that induce the activation of signalling molecules that are involved in the de novo synthesis of proteins, including collagen, that will participate in wound healing. In the study it was found that the collagen synthesis was higher. In order to confirm the role of WSN, its activity was also studied *invitro* by using the scratch cell assay. The Murine fibroblast NIH3T3 cell line was used in the analysis to assess the cell proliferative and the cell migration inducing capacity of the WSN. The WSN was seen to induce the cell proliferation and had no role in the cell migration. The accelerated wound healing potential was associated with the proliferation of the fibroblasts. However, the gene responsible for the collagen synthesis was seen to be highly expressed in the cells. It was also observed that the calcium also can induce the collagen gene expression. It was concluded that the WSN played a synergistic role in the wound healing process. The identification of the soluble factors present in the WSN is critical for the development of any WSN based therapeutics [23].

The potential biological activities of shell matrix components extracted from the shell of the marine scallop *Pecten maximus*was studied on human fibroblasts in primary culture. The Water Soluble Matrices (WSM), Acid Soluble Matrix (ASM) and Acid In-soluble Matrix (AIM) were extracted from the nacreous shell powder of *P. maximus*. The extracted matrix was suspended in the PBS at a concentration of 4mg/ml and was filtered in

0.22µmmesh prior to use. The ASM of the scallop nacre did not alter the Fibroblasts metabolic activity and also did not induce cell proliferation. The AIM and WSM of the shell decreased the metabolic activity of the fibroblasts and also did not stimulate the proliferation of the cells. However, the extracts modulated the Extra Cellular Matrix (ECM) protein gene expressions. The ASM was observed to increase the mRNA levels of COL1A1, COL1A2, COL3A1 and MMP1 at a steady state level. The ASM also was seen to improve the level of expression of the mRNA corresponding to p65 and TIMP1. The AIM decreased the levels of COL1A1 and COL3A1 expressions and increased the expression of MMP-1. No significant changes were observed in the expression of the TIMP1 and p65 in the cells treated with AIM. The WSM of the shell also did not significantly improve the levels of the COL3A1, TIMP1 and p65. ASM was found to enhance the synthesis of Collagen type I after exposing the cells for 48 hours. ASM also was found to stimulate the synthesis of the sulphated glycosaminoglycan's synthesis at a concentration of 500µg/ml and 1000 µg/ml. AIM enhanced the activity of the MMP1.

The enhancement in the expression of the type I collagen gene by ASM was mainly due to the transcription control involving c-Krox, probably CBF, Sp1 and Sp3, CBF and the three zinc-fingers being transactivators of COL1A. WSM also was also reported to have impact over these transcription factors. The wound healing potential of the ASM and WSM was also studied through the *invitro* wound closure assay. The ASM and WSM had no role over the wound closure. These extracts did not promote the movement of the fibroblasts. The effect of these ASM and WSM over the ECM involved in cell movement were studied. The fibronectin receptors β1 integrins were observed to be distributed all over the cell surface and also in punctuated structure with respect to fibrillar adhesion [24].

Studies on the Impact of Molluscan Shells on Skin Regeneration

Apart from molluscan nacre, shells of molluscs have also been reported to have skin regeneration properties. The effects of matrix macromolecular components extracted from the shells of the marine bivalves *Mytilus edulis* and Crassostrea gigas was studied on human dermal fibroblasts. The ASM, AIM and WSM were prepared from the shells of these bivalves and suspended in PBS at a concentration of 4mg/ml. The ASM of *M.edulis* was observed to induce changes in the metabolic pathways at a concentration of 250µg/ml and metabolic activity was observed to be increased by 233% at 1000µg/ml when compared to the control. The ASM of the *C. gigas* seem to have no effect on the fibroblast metabolic activity up to a concentration of 50 µg/ml but at higher concentrations, they decreased the cell metabolic activity. The WSM of *M.edulis* also was observed to increase the metabolic activity only at a concentration of 1000 µg/ml however the WSM of *C. gigas* did not have any impact on the fibroblast activity. The experiments on the impact of these extracts on the proliferation of fibroblasts. With regard to *M.edulis* 250 µg/ml of ASM and 1000 µg/ml of WSM are capable of inducing proliferation whereas only the 1000 µg/ml of ASM of *C.gigas* was observed to induce cell proliferation. The ASM and WSM of the *C.gigas* and *M.edulis* decreased the levels of the mRNA of COL1A1 and also the protein synthesis. The reduction in the Collagen gene and protein expression can be correlated with the increased expression of the MMP1. The MMP-1 mRNA levels were significantly increased up to 12 folds for ASM of *M. edulis* and up to 31 folds for ASM of *C. gigas*. The expression of TIMP 1 was reduced by extract of *C. gigas* and the extract of *M. edulis* had no impact over the TIMP 1 expression. The decrease in the expression of the collagen can also be correlated to the expression of the p65 after incubation of 48 hours with the extract of *M. edulis* but there was no increase in the expression further. *C. gigas* extract had no impact over the expression of p65 [25].

The Water-soluble fraction and Water-insoluble fraction of the shells of *Patinopectenyessoensis* was subjected to various assessments. The Water-soluble fraction was found to have at least three kinds of proteins with molecular masses of 90 kDa, 20 kDa and 17 kDa. Each protein was then separated as fractions and the fractions 16-18 that inhibited elastase and trypsin activities also contained free radical scavenging substances. The fraction 27 had α-chymotrypsin activation capacity. However, the protein responsible for the skin regeneration has not been identified [26]. The photoprotective activity of the scallop shell water extract of *Patinopectenyessoensis* using the cultured rat skin keratinocyte cells was studied. The extract protected keratinocytes against Ultraviolet (UV) B induced cell damage (sun burns). It was concluded that the scallop shell water extract may protect skin keratinocyte cells against UV-B-induced damage, either by antioxidant activity or by promoting the growth of skin keratinocyte cells. So, this extract was reported to be used as a material for skin protection [27]. The effect of shell extract of the *Patinopectenyessoensisinvivo* on Male wistar rats was also investigated.

The erythema and eschar were reported to be observed after application of scallop shell extract when compared to application of the vehicle control. The histological studies of the animal tissue also showed the recovery in epidermal layer. The result suggested that scallop shell

extract activates rat keratinocyte cells and promotes the turnover of skin stratum corneum [28]. This shell extract also increased the the mRNA expression levels of type I collagen, MMP-1 and TIMP-1, suggesting that the scallop shell extract may activate collagen metabolism in skin fibroblast cells. About 1.3 fold increase in the collagen content was observed. *In vivo* studies also revealed that the topical application of the scallop shell extract to rat dorsal skin increased the collagen content in the skin tissue. These results suggest that the scallop shell extract may be an effective agent in the treatment of photo aged and aging skin, which undergo collagen loss [29]. The Scallop shell extract also inhibits squalene monohydroperoxide-induced skin erythema and wrinkle formation in rat [30].

The shells of the *Haliotisdiversicolor* have been used as a traditional Chinese medicine. The shell gas been used for the treatment of poorly managed ulcers or traumatic wounds of skin. The extract of the shell was extracted and powdered. The powdered extract was then added to the culture media and used on the cell lines. For experimenting on the animal, the powered extract was added to the mineral oil. The invitro analysis on RAW 264.7 cells showed that the shell extract decreased the inducible nitric oxide synthase (iNOS) expression thereby alleviating the inflammation and enhanced the functions of macrophages. In rat model the effect of the extract on the burn injury was analysed. The results revealed that the extract decreased neutrophil infiltration, promoted wound healing by increasing the collagen I content and also promoted the expression of transforming growth factor-beta 1 (TGF-β1) protein which in turn converted the collagen during the tissue remodelling phase. Thus, the gastropod shell also was proved to have skin regeneration property [31].

In Indian Siddha medicine the shells of marine mollusc *Crypraeamoneta* has been used. The effect of the processed shell powder of *C.moneta* on rat model showed that the wound healing effect of the shell powder was very effective. The shell powder was made into an ointment and was applied on the animal. The scar was reported to be observed in the treated animal on the eight day which was not seen on the untreated animal [32].

Discussion

In mammalian species the wound healing process is divided into three phases. The inflammation, tissue formation, matrix formation and remodelling. The transition from one phase to another depends on the maturation and differentiation of the main cell populations. Among them fibroblasts play a major role [33-35].

The first event occurring after injury is the formation of a blood clot and due to the action of fibrin fibres, the clot is stabilized and is “invaded” by several infiltrating cells, such as neutrophils, macrophages, mastocytes, platelets, and, possibly, by bacteria and toxins, which are counteracted by host-generated H_2O_2. Neutrophils will be massively infiltrating the wound during the first 24 hours postinjury are attracted by numerous inflammatory cytokines produced by activated platelets, endothelial cells, as well as by degradation products from pathogens. Macrophages massively infiltrating the wound two days postinjury produce intense phagocytic activity [36]. The second phase that lasts about two weeks is characterized by neo-angiogenesis and granulation. Next in the reepithelialisation process, keratinocytes from the wound edges migrate over the wound bed. This migration of the keratinocytes is facilitated by the production of specific proteases, such as collagenase by the epidermal cells to degrade the extracellular matrix. Now the activated fibroblasts migrate to the wound bed and form the granulation tissue. Both growth factors and reactive oxygen species (ROS) produced by the granulation tissue will favour proliferation and differentiation of epithelial cells, restoring epithelial barrier integrity. The last stage of the wound healing process consists in a gradual involution of the granulation tissue and dermal regeneration. This step is associated with apoptosis of my fibroblasts, endothelial cells, and macrophages. The remaining tissue is therefore composed mostly of extracellular matrix proteins, essentially collagen type III that will be remodelled by metalloproteinase produced by epidermal cells, endothelial cells, fibroblasts, and the macrophages remaining in the scar and then replaced by collagen type I [37,38].

The above process shows that the skin regeneration is a highly conserved process. The application of nacre of *Pinctada maxima* to the site of injury onto an animal model has been reported to healthe wound on the first day [20]. The study reported that Collagen type I and Collagen type III was observed on the site of injury and these two secretions closely related to an activated fibroblast. Usually in a mammalian system the process occurs only after two weeks in the natural process of wound healing. It also proved that the recruitment of the fibroblast at the site of injury was higher in the areas where the Water-Soluble Matrix of Nacre (WSN) of *Pteriamartensii* was applied [23]. The WSN was observed to stimulate fast granulation and tissue formation and also thick collagen tissue formation. The above results help us in understanding the effect of the nacre on skin

regeneration. Similarly, the extract from the shells of marine molluscs increased the mRNA levels of COL1A1, COL1A2, COL3A1 and MMP1 at a steady state level [25].

This shows that shells of marine molluscs also have impact over the mammalian skin regeneration. The *invitro* and the *invivo* results shows that the expression of collagen is increased when treated with nacre and shells of marine molluscs. Collagen is essential for healthy maintenance of skin. The lipids present in the nacre are also known to moisturize the skin. Hence the nacre has been identified a potential ingredient in the skin related cosmetics. The effect of the nacre and its extract on the scar tissue has also been extensively studied. However apart from creams and formulations the new generation methods of using molluscan shells for human skin regeneration have to be studied. Many farmed molluscan shells have to be studied for its applications for biomedicine and can fetch extra earning for farmers instead of wasting the shells. This unexploited potential of molluscan shells for skin pharmaceutical applications has to be studied further for wider applications

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