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ARTICLE

BONE TISSUE ENGINEERING USING BIOMATERIAL SCAFFOLD WITH ANTIOXIDANT PROPERTY

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Abstract

Oxidative stress plays an important role in the biological compatibility of many biomaterials due to inflammation. To improve the biocompatibility of engineered material, biodegradable polymers that have antioxidant properties built into their backbone structure have high relative antioxidant content. Antioxidant properties in the material are therefore a potential avenue to reduce oxidative stress-related pathologies and may provide prolonged, continuous attenuation of oxidative stress while the polymer or its degradation products are present. The antioxidant activity of the scaffold was proved using cell line. Scaffold reduced reactive oxygen species generation in cells and protected cells from oxidative stress-induced cell death. This antioxidant property can be useful for tissue engineering application where oxidative stress is a concern.

Keywords: Scaffold, antioxidant, tissue engineering, oxidative stress, inflammation

Introduction

Antioxidants are compounds capable to either inhibit or delay the oxidation processes which occur under the influence of atmospheric oxygen or reactive oxygen species. In the defense mechanism of the organism, antioxidants are involved against the pathologies associated with the attack of free radicals [1].Reactive oxygen species (ROS) play crucial roles in various physiological processes such as cell signaling and host innate immunity. However, when ROS are overproduced, they may damage biomolecules in vivo and cause diseases such as cardiovascular or neurodegenerative diseases. Oxidative stress is usually involved in various inflammatory tissues representing an important target for the development of numerous therapeutic strategies [2]. Therefore various probes for the in vitro or the in vivo detection of ROS have been developed for the diagnosis of the oxidative stress relevant diseases. Due to their potential applications in biomedical field oxidation, responsive polymers also have attracted great interest.

There are two types of oxidative species in the human body: reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS include the superoxide anion and hydrogen peroxide which are primarily spawned by oxygen reduction, as well as their derived reactive species including the hydroxyl radical, hypochlorous acid, peroxyl radicals, and singlet oxygen. Most commonly RNS includes nitric oxide and its derivatives, such as peroxynitrite and nitrogen dioxide [3]. At appropriate concentrations, ROS or RNS have biologically important functions in a wide range of physiological processes such as cell signaling, apoptosis or proliferation. For example, hydrogen peroxide is directly or indirectly involved in signaling pathways in many cells, mainly via redox reactions. Nitric oxide also is known as the endothelium - derived relaxing factor, can relax vascular smooth muscle. The key oxidants for host innate immunity against microbial infection are hydroxyl radical and peroxynitrite [4]. However, ROS and RNS are double - edged swords, if overproduced; these oxidative species may damage cell biomolecules such as lipids, proteins, and DNA and leads to cell death. By endogenously or by exogenous stimuli such as UV ray, gamma radiation and xenobiotic may be induced overproduction of ROS, resulting in oxidative stress, a biological feature that is closely implicated with various pathological disorders [5]. In addition, oxidative stress is usually associated with inflammatory tissues [6].

In the past few decades, the biocompatibility of implantable materials has evolved from simply meaning inertness, via the lack of a deleterious response [7]. The biocompatibility of implantable materials was defined by Williams' is that "the ability of a biomaterial to perform its desired function without eliciting any undesired effect, but generating a beneficial cellular or tissue response" [8].An important module of a biomaterial's response that can have an influence on the performance of medical devices yet is often overlooked in oxidative stress in biomaterials science. When a biomaterial induces an inflammatory response due

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to biological responses, leukocytes release various cytokines and chemokines and generate reactive oxygen species (ROS) (e.g., superoxide, hydroxyl radicals, and hydrogen peroxide) [9]. Pro-oxidant molecules and compounds react with synthetic biomaterials and damage DNA, proteins and lipids, potentially impairing the normal function of cells. Both in vitro and in vivo, the detection of ROS is currently being used to characterize the inflammatory host tissue response to biomaterials [10]. The generation or presence of oxidative stress is particularly important to biodegradable polymers as the local accumulation of polymer degradation products generate ROS [11, 12]. In fact, excess generation of ROS is a significant cause of toxicity for many biodegradable materials [13, 14, 15, 16].

Due to an imbalance between the production of oxidants and the antioxidant defense mechanism, oxidative stress may also be a pathophysiological response resulting in a net increase in ROS [17]. Biomaterials that can counter the effects of oxidative stress and inhibit excessive ROS generation in a constant manner may be a useful tool for therapies that target these medical problems. A significant advance in the field of bone tissue engineering is the development of synthetic scaffold materials that possess both the mechanical and chemical properties for bone development [18]. In bone tissue engineering, the importance is off antioxidants are of because at the site of a bone fracture the antioxidants can suppress the number of free radicals produced and protect the surrounding tissue from further damage [19, 20]. Nowadays, the considerable efforts have been devoted to fabricating degradable conductive scaffold and these scaffolds have shown excellent the biocompatibility, physical properties and controllable chemical modification [21–26].

Materials and Methods

The L929 fibroblast cell lines used in the study were procured from National Centre for Cell Sciences (NCCS) Pune. The cells were maintained in DMEM media containing 10% FBS and incubated at humidified atmosphere containing 5% CO_2 at 37°C. When the cell culture reached confluence, the cells were trypsinized and sub cultured and these cells were used for further studies. All other chemicals and reagents used in the study were of analytical grade quality.

Neutral Red Uptake (NRU) Assay

Cells were treated with scaffold and incubated for different time intervals in duplicate at 37°C. After incubation, a solution of Neutral red, a vital dye is added to the well plates. The plates were incubated to allow neutral red uptake by the cells at standard culture conditions. After 2 hours incubation, PBS was added to the wells to decanted excess neutral red. For evenly distribute the dye, the plates were placed on a plate shaker to fully extract the neutral red in each well and absorbance was measured at 540 nm.

Glutathione Reductase

The reaction mixture contain 2.6ml PBS buffer (0.12M) with 0.1ml EDTA (1.5mM),0.1ml oxidized glutathione (65.3mM) and 0.1ml cell lysate. To the above solution 0.05ml of NADPH (9.6mM) was added and absorbance was read at 340nm.

Reduced Glutathione

The treated cells were homogenized using 4ml precipitating reagent containing 1.67g of glacial metaphosphoric acid, 0.2g of EDTA and 30g of NaCl in 100ml distilled water. The solution was allowed to stand for 5 minute and filtered.3ml of phosphate solution was added to 2ml of filtrate. 1ml of DTNB solution was added to all tubes and absorbance was taken at 412nm.

Statistical Analysis

All experiments were performed using 3 biologically independent replicates. All results were reported as mean ± standard variance.

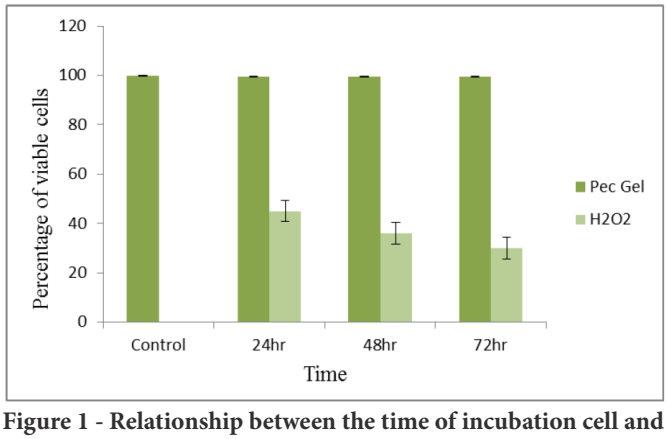

Figure 1 - Relationship between the time of incubation cell and scaffold with neutral red and the absorbance at 540 nm after the assay of neutral red. L929 cells were seeded in a culture plate and incubated for 24 hr, 48 hr and 72hr. Values are expressed as mean ± SD of triplicate.

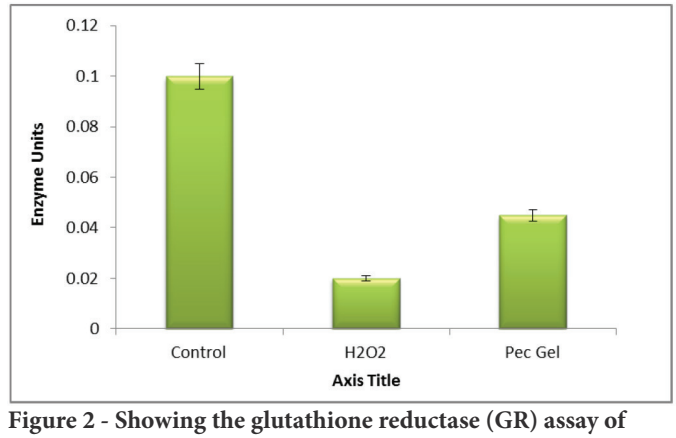

Figure 2 - Showing the glutathione reductase (GR) assay of cells treated with H_2O_2 induced oxidative stress on mouse macrophage cells L929. Values are expressed as mean ± SD of triplicate.

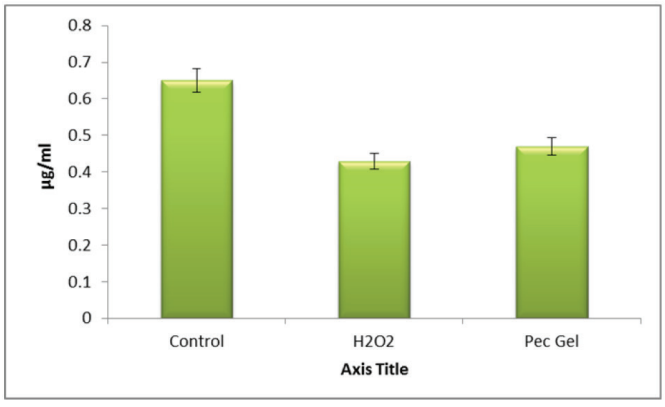

Figure 3 - Showing the reduced glutathione activity (GSH) oxidative stress induced L929 cells. Values are expressed as mean ± SD of triplicate

Results and Discussion

The modes of action of antioxidants are exerting by inhibiting the formation of reactive oxygen species, by either inhibition of enzymes or by chelating trace elements. The antioxidant assay is widely used to evaluate the free radical scavenging effect of natural antioxidants. Oxidative stress is due to an imbalance between antioxidants and reactive oxygen species results in ROS production, leading to cellular damage .

The Neutral Red (NR) Cell Viability assay delivers a quantitative estimation of the number of viable cells in a culture condition [29].This assay is based on the ability of viable cells to incorporate and bind neutral red and primarily accumulates in lysosomes. In order to estimate the cell viability, the cells were treated with H_2O_2 and PEC GEL scaffold. Results showed that more number of the cells were viable at cells treated with scaffold than the treated cells. The results were compared with the reference control, the cell alone. NRU assay (Fig.1) the results showed above 96% cell viability at the scaffold treated cells at maximum time exposure.

Cytotoxicity and cell viability assays are depend upon various cell functions such as cell membrane permeability, enzyme activity, cell adherence. Cell viability is based upon the ATP production, co-enzyme production and nucleotide uptake activity [30]. This assay was based on the ability of viable cells to take up the supravital dye neutral red. Reactive oxygen species (ROS) are a vibrant biomarker of intracellular oxidative stress, which is spawned by a variety of cellular biochemical processes [31]. Increases in intracellular ROS contribute to a number of diseases via by cell membrane damage; mitochondrial dysfunction and DNA damage [32]. Biocompatible materials are should be either free radical scavengers or do not alter the ROS level in biological systems. The results (Fig.2.) shows that glutathione reductase (GR) activity increased in PEC GEL scaffold treated cells compared to the control and lowest GR activity showed by the cells treated with H_2O_2.

Enzyme Glutathione-S-transferase (GST) plays a role in scavenging endogenous oxidants. Altered enzyme activity resulting from inherited polymorphisms, cause in increased oxidative stress [33]. The results (Fig.3.) of reduced glutathione activity (GSH) showed that macrophage cells treated with H_2O_2 showed moderate GSH activity compared to control and cells treated with PEC GEL scaffold. Reduced glutathione (GSH) is the master antioxidant present in mammalian cells to prevent ROS caused cell damage. ROS levels are necessary to induce cellular responses causing cell death [34].

There is generous proof and consent that reactive oxygen species are indispensable for redox signaling, hormone synthesis and intracellular killing of bacteria [35, 36, 37]. But if ROS is not appropriately controlled by neutralization and compartmentalization, they may damage DNA, proteins and lipids [38]. Prevention and repair of damage by ROS is mediated in an intensive action of antioxidative systems and DNA repair mechanisms. Anything beyond their capacities may cause irreversible damage followed by cell death [39, 40– 42].

Conclusion

In bone tissue engineering, one of the main goals is controlling oxidative stress to allow initial regeneration of new tissue. Antioxidant property of a biodegradable scaffold is valuable where cellular oxidative stress is an important factor in tissue engineering. Biodegradable scaffold showed high relative antioxidant properties which have built into their backbone structure. The anti-oxidant compounds in particular are often presented in the scaffold as part of preventing oxidative stress.

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