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Investigation of the effects of local glutathione and chitosan administration on incisional oral mucosal wound healing in rabbits

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ABSTRACT

The aim of the present study was to investigate the effects of local glutathione (GSH) and chitosan applications on the oxidant events and histological changes that occur, during healing processes in rabbits with incisional intraoral mucosal wounds. For this purpose, discs containing glutathione and chitosan (1:1) were prepared and their physicochemical characteristics were evaluated. New Zealand white rabbits were used in in vivo studies. A standard incision was applied to the oral mucosa of rabbits. The rabbits were divided into four groups, being: an untreated incisional group ($n=6$), a group treated with discs containing GSH + chitosan ($n=6$), a group treated with discs containing solely chitosan ($n=5$) and a group treated with discs containing solely GSH ($n=5$). The levels of malondialdehyde (MDA), glutathione and nitric oxide (NOx) in the oral wound tissues were measured on the fifth day after the injury. Histological changes in the wound tissues were also investigated. The tissue MDA levels in the group treated with the disc containing GSH + chitosan were found to be lower than those in the other groups. There were no statistically significant differences in terms of tissue GSH and NOx levels between the group treated with the disc comprising GSH + chitosan and the control group that had untreated incision wounds. According to the histological findings, wound healing in the group treated with the disc containing solely chitosan was found to be better than in the other groups. The results of the experiments showed that the local application to the intraoral incision wounds of chitosan + GSH, and chitosan alone, can be effective in the wound healing processes of soft tissues and dental implants.

1. Introduction

Wound healing is a process including phases of homeostasis, inflammation, proliferation and remodeling as well as many biochemical and cellular mechanisms [1,2]. Reactive oxygen species (ROS) are associated with all stages of the healing process. ROS are produced by the inflammatory cells and play an integral role during this process. Neutrophils and macrophages constitute most of the ROS sources. Increasing free oxygen radicals causes tissue necrosis and disruption of cellular integrity, and the severity of the oxidative damage depends on the balance of the antioxidant–oxidant system [2–4]. Antioxidant administration is beneficial for healing. Nitric oxide (NO) and superoxide (O^{2-}) form the peroxynitrite ($ONOO^-$) radical and lead to lipid peroxidation, and malondialdehyde (MDA) is a relatively unchanged final product of lipid peroxidation. Growth factors such as the epidermal growth factor (EGF), transforming growth factor beta (TGF-beta), and fibroblast growth factor (FGF) initiate the growth, differentiation, and metabolism of cells, and modulate the process of wound healing. During the wound healing phases, activated neutrophils and macrophages produce large amounts of superoxides, as well as its derivatives, via the phagocytic isoform of nicotinamide adenine dinucleotide phosphate oxidases. These strong oxidants contribute to oxidative damage in cells [2,4–6].

Glutathione (GSH) is a tripeptide composed of three different amino acids: glutamate, cysteine and glycine; it has numerous important functions within cells [7]. Glutathione plays roles in catalysis, metabolism, signal transduction, gene expression and apoptosis. It is the principal intracellular non-protein thiol and plays a major role in the maintenance of the intracellular redox states. Glutathione protects tissues by neutralizing free radicals [8,9].

In cases of damage in the oral cavity, decreases in GSH levels are observed while increases are observed in the oxidized glutathione

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(GSSG) levels. With the decrease in GSH, the antioxidant mechanism is considerably deactivated and the formation of ROS, which inhibits and slows down the wound healing, gains speed [3,4].

The use of glutathione for topical wounds has been known for many years. Glutathione treatment yields successful results in the healing of wounds, particularly in those seen in diabetes patients [10–12]. Deveci et al. [11] indicated that topical GSH treatment can reduce oxidative stress and can be effective in ischemic wound healing. They prepared a GSH solution in a phosphate buffer and its gel with carboxymethyl cellulose, for topical administration. These GSH formulations were found to be effective in the healing of diabetic wounds.

Chitosan is a mucoadhesive cationic polysaccharide with high molecular weight and it is produced by the deacetylation of chitin which is naturally found in the shells of Crustaceans such as crabs and shrimps [13]. Chitosan plays an important role in wound healing. It accelerates the healing process, reduces treatment frequency and pain and it provides comfort and ease of application by protecting the wound surface. In the wound healing process, chitosan is of critical importance in the phases of complement activation, polymorphonuclear cell (PMN) and macrophage activations, fibroblast activation, cytokine production, giant cell migration and stimulation of type IV collagen synthesis [14,15]. It has a protective impact on microorganisms and stimulates the formation of tissue which has been granulated by angiogenesis. It has been revealed that chitosan performs complement activation in an alternative manner, realizing the production of C5a production. Chitosan also ensures the production of interleukin-8 (IL-8) through the stimulation of fibroblasts. As chitosan increases, the functions of macrophages, phagocytosis, interleukin-1 production, TGF-ß1 and platelet-derived growth factors are activated. In the early phase of wound healing, chitosan has also been reported to be effective in the increased infiltration of PMN cells which is followed by the collagen production of fibroblasts. Besides, the high bioadhesive feature displayed by chitosan in mucosal tissues provides a great advantage [14,16]. One of the positive effects of chitosan on the wound healing process is its antibacterial and antifungal property [16,17].

Senel et al. [18] developed the chitosan gel and film formulations containing chlorhexidine gluconate to ensure a long term active agent concentration in the oral cavity. At the end of their in vitro release studies, considering its antifungal activity, bioadhesive characteristics and high viscosity, the optimum formulation was reported to be a 2% chitosan gel formulation. As the active agent discharge from chitosan gel occurs with an extended release, it was concluded to be an appropriate delivery system for the treatment of local diseases in the oral cavity.

Local application of the drug formulation may have advantages in terms of high local drug concentration on tissues, decrease in possible systemic side effects and controlled drug release. The effect of local drug application, including different active ingredients such as L-NAME [19], EGF [20], TGF-beta [21] and aminoguanidin [22] on the oxidant and anti-oxidant parameters of the incision wound tissue of rabbit oral mucosa have been shown in previous studies. In the wound area, different parameters are evaluated and even if the active ingredients are different, the parameters playing in the healing process are the same.

Both chitosan and glutathione have positive effects on the healing process, therefore the objective of the present study was to investigate the effect of local glutathione and chitosan applications on the oxidant events and histological changes occurring during incisional oral mucosal wound healing process in rabbits. To our knowledge, this study is the first to seek the effect of local exegoneous GSH and chitosan administration on incisional oral wounds.

2. Materials and methods

2.1. Materials

The active ingredient L-glutathione reduced (assay ≥ 98%) was obtained from Sigma–Aldrich, USA. It was stored at 2–8 °C. Chitosan (highly viscous; deacetylation value 75–85%; molecular weight 500.000–700.000) was also obtained from Sigma–Aldrich. The viscosity of the chitosan (1% in acetic acid) was measured with a viscometer (DV-III + Rheometer TC502, Brookfield, USA) and found to be 650 ± 8 cP at 37 °C.

Other materials used for the in vitro and in vivo studies such as sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate anhydrous, disodium hydrogen phosphate dihydrate, and sodium chloride were obtained from Merck, Germany. 5,5′-Dithio-bis-(2-nitro-benzoic acid), sodium citrate tribasic dihydrate, trichloroacetic acid, thiobarbituric acid, butyl hydroxy toluene, vanadium (III) chloride, hydrochloric acid, naphthalene diamine dihydrochloride, phosphoric acid and sodium nitrite were obtained from Sigma–Aldrich, USA.

2.2. Animals

New Zealand rabbits, male (n = 22), 4 months old, and weighing 2.0 ± 0.4 kg (mean ± SD) were housed in clear plastic cages and fed with rabbit pellets and tap water.

2.3. In vitro studies

2.3.1. Physicochemical and stability studies of the active ingredient

The particle size of GSH was determined using the laser diffraction method (Laser-Diffraction Particle Sizer, Sympatec Gmbh, System-Partikel-Technik, Germany), and Fourier transform infrared analyses of the drug, chitosan and a physical mixture of drug:polymer (1:1) were performed using FT-IR (Thermo Nicolet-6700, USA).

GSH was assayed spectrophotometrically, in a procedure based on the reaction of *GSH* with 5,5′-dithiobis(2-nitrobenzoic acid) (*DTNB*) to give a yellow colour at 410 nm [23,24]. The method was validated.

Differential scanning calorimetry (DSC) (DSC Q 100 TA Instruments, England) analysis was carried out on the drug, chitosan and the physical mixture of drug + polymer (1:1) to evaluate whether there was any incompatibility between GSH and chitosan. Samples were heated from 30 to 300 °C at a heating rate of 10 °C min^{-1} under a nitrogen atmosphere.

The stability of GSH was investigated in a pH 6.6 buffer at 18 °C and 37 °C. Stability experiments at 37 °C were also performed by passing nitrogen gas through a pH 6.6 buffer. The results were evaluated according to zero and first order reaction kinetics and the half-life of GSH was calculated. The pH 6.6 buffer was chosen for these experiments, because this pH is appropriate for the oral cavity.

2.3.2. Preparation of discs

The discs containing glutathione and chitosan were prepared under aseptic conditions. All glass materials were sterilized by heat drying. For the preparation of discs, chitosan and glutathione were mixed to the ratio of 1:1 (w/w). The powder mixture was compressed using a tablet compression machine. 100 mg discs containing 50 mg GSH and 50 mg chitosan were prepared, the effective dose of glutathione for wound healing being 50 mg. Compressed discs were kept at 2–8 °C and protected from light in tightly closed containers. They were used on the same day. Physicochemical