Original Article
Diphenhydramine exert protective action against experimental periodontitis in rats via attenuation of oxidative stress and inflammation

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Received June 9, 2019; Accepted September 11, 2019; Epub November 15, 2019; Published November 30, 2019

Abstract: The present study enumerates the effect of Diphenhydramine (DPH) against periodontitis and its possible mechanism of action. The experimental periodontitis (EP) was induced in Male Wistar rats via administration of endotoxins obtained from the E.Coli. The effect of DPH was investigated on the various parameters and biomarkers of EP. The antioxidant status was found to be greatly enhanced after DPH administration as compared to EP group. The expression of lysosomal enzymes, such as, cathepsin B, cathepsin D, β-glucuronidase and acid phosphatase were found to be significantly reduced together with decrease in acute phase protein, C-reactive proteins and fibrinogen in DPH treated group. The architecture of gingival tissues of rats was improved in the DPH treated group as evidenced via histopathological analysis. In western blot analysis, the expression of NLRP3, TNF-α and iNOS was decreased in a dose-dependent manner with increase in caspase-1 activity in DPH treated group. In conclusion, our results showed that DPH exerts protective effect against periodontitis via modulating oxidative stress and inflammation.

Keywords: Diphenhydramine, periodontitis, oxidative stress, inflammation

Introduction

The oral diseases are considered as a major burden on developing nation with marginal setting, where these diseases are not taken up seriously [1]. The increase in awareness for oral hygiene and care has significant contribution to reducing the burden of oral disease. Despite this, across the globe, persons belonging to the disadvantaged and socially marginalized class are still affected by the oral illness [2]. Dental caries and periodontal diseases (tooth loss) are the two major ailment of oral cavity, and the later is seen affecting large population of adults because of improper hygiene and presence of systemic infections [3]. Periodontitis (PD) is an inflammatory condition characterized by the presence of inflammation and destruction of periodontal tissues which result in tooth decay. Various studies documented that the most frequent cause of periodontitis is bacterial infection. The bacterial infection after the plaque in teeth causes production of numerous toxins, enzymes and its metabolites which are responsible for the generation of inflammatory response. This inflammation damages connective tissues and alveolar bone necessary for holding the teeth in its place via releasing proinflammatory cytokines such as, prostaglandin (PG), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-12 (IL-12), and tumor necrosis factor-α (TNF-α) [4-6]. The bacterial infection in acute periodontitis recruits polymorphonuclear leukocytes (PMN), which act as the primary mediators of the host response. The activated PMN produce a large amount of reactive oxygen species (ROS) which induces lipid peroxidation and DNA damage to aggravate periodontal tissue erosion. This suggested that periodontal inflammation might be associated with systemic oxidative stress. Thus, novel therapeutics under development against PD is concentrated towards limiting the damage caused by oxidative stress and inflammation [7].
Diphenhydramine (DPH) is pharmacologically classified as first-generation H1 receptor antagonist to treat allergies, common cold and itching [8, 9]. Studies suggested that diphenhydramine exhibit anticholinergic [10], sedative [11], antivertigo [12] antiemetic [13], antidyskinetic [12], and local anesthetic activity [14, 15]. It has been found that, antihistaminics are able to control the inflammation. However, till now, no study has reported the effect of antihistaminic agent against PD. Thus, encouraged by the above, we intended to investigate the effect of DPH on the experimentally induced periodontitis in rats.

Materials and methods

Diphenhydramine was obtained from Sigma Chemical Co., USA. For the animal experimentation, male Wistar rats weighing approximately 250 g were procured from central animal house of the institute and housed in polypropylene cages under strict veterinary supervision and maintained in control rooms with 12 h light/dark cycle. Animals were fed with standard laboratory diet and water ad libitum.

Preparation of endotoxin

The endotoxin required for animal experimentation was produced from E.coli organism. Initially, E.coli was cultured on nutrient agar and incubated overnight to obtain optimum colony count and then washed with phosphate buffered saline (PBS). The liquid collected after washing was thoroughly mixed with phenol in separating funnel. After keeping for some time, the separated water phase was collected and again extracted with phenol. The whole separation process was repeated thrice. The resulting supernatant was precipitated with ethanol and sodium acetate. The precipitate containing endotoxin was easily isolated by centrifugation at 10,000 g for 10 min and dried in air.

Generation of experimental periodontitis (EP)

The EP was induced by administering E.coli endotoxin as isolated above. The rats received intragingival injection with either 10 μl of saline or 1 mg endotoxin dissolved in 1 ml of saline for every other six days to induce periodontitis. After induction rats were divided into various treatment groups. The Group 1 corresponds to control, while animals belonging to Group 2 were further divided into five sub-groups. The first three sub-groups correspond to DPH treatment (2.5, 5 and 10 mg/kg of DPH administered once per day over the 6-day protocol via oral intubation), while the rest two include standard (Naproxen, 20 mg/kg) and EP control group. Each group contained 6 animals each.

The animals were sacrificed by cervical decapitation under anaesthesia after the experimental period, and venous blood was collected. The histopathological evaluation was performed on maxillary halves of the bone and teeth of the right maxillary halves of the animals.

Estimation of biochemical parameters

The effect of DPH was determined on \( \text{H}_2\text{O}_2 \), superoxide anion, myeloperoxidase (MPO) activity and lipid peroxides (LPD). The activity of cathepsin B, cathepsin D, β-glucuronidase and acid phosphatase was also determined as per the standard protocols in various treated groups. The activity of C-reactive protein (CRP) in serum was also estimated using the standard protocol. Assay of fibrinogen was performed using a standard assay kit (Fibriquik ®Organon Teknika, USA) according to the manufacturer’s protocol. The level of ascorbic acid, α-tocopherol, ceruloplasmin and reduced glutathione (GSH), superoxide dismutase and catalase (CAT), glutathione peroxidase (GPx) and glutathione-transferase (GST) were also estimated in serum. Moreover, protein concentration in serum was determined using crystalline bovine serum albumin as a reference standard.

Histopathological analysis

The tissues from the maxillary halves were dissected out, fixed in 10% buffered formalin solution for 24 h, processed and embedded in paraffin. Thin sections (3 μm) were cut and stained with heamatoxylin and eosin and were analysed with light microscopy.

Western blot analysis

The gingival tissues were mixed in a buffer that comprise of 10 mM Tris (pH 7.5), 1.5 mM MgCl₂, 10 mM KCl, and 0.1% Triton X-100. The tissues were homogenized and the nuclei were isolated after centrifugation at 7500 rpm. The resulting supernatant was collected and stored at -80°C.
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for the analysis. The nuclear protein was extracted as supernatant at 4°C by resuspending the nuclei pellet in the buffer, followed by 1 h incubation and microcentrifugation for 15 min. The concentration of protein was estimated by BCA assay. Thereafter, the total protein extract (50 µg) was separated on a 15% SDS-PAGE and probed with primary antibodies (1:1,000 in 1% BSA/TBS-T) overnight at 4°C. The membranes were then washed twice for 15 min each in TBS-T and incubated with HRP-conjugated goat anti-mouse or rabbit antibodies (1:10,000 in 1% BSA/TBS-T).

Statistical analysis

All data are presented as mean ± SD of three independent experiments. Data were statistically analyzed by one-way analysis of variance followed by the Newman-Keuls post hoc test using statistical software GraphPad Prism 5.0 (California, USA). The P value <0.05 was considered as statistically significant.

Results

Effect of DPH on the oxidative stress

The effect of DPH was investigated on the oxidative stress markers such as superoxide, hydroxyl radicals, lipid hydroperoxides, and reactive non-radical compounds including singlet oxygen, hydrogen peroxide and results have been shown in Figure 1. It has been found that, in EP-untreated group, the level of these oxidative stress markers found to be enhanced as compared to control. While in DPH treated group, these enhanced levels were found to be restored near to normal as compared to EP group. Thus, it has been suggested that, DPH

Figure 1. Effect of DPH on ROS and lipid peroxides in experimentally induced EP. *P<0.01 vs. control, **P<0.05, "P<0.01 vs. EP group.
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A

Ascorbic Acid (mg/dl)

Control  EP  2.5 mg/kg  5 mg/kg  10 mg/kg  Std.

B

α-Tocopherol (mg/dl)

Control  EP  2.5 mg/kg  5 mg/kg  10 mg/kg  Std.

C

Ceruloplasmin (mg/dl)

Control  EP  2.5 mg/kg  5 mg/kg  10 mg/kg  Std.

D

GSH (U/mg)

Control  EP  2.5 mg/kg  5 mg/kg  10 mg/kg  Std.

E

CAT (U/mg)

Control  EP  2.5 mg/kg  5 mg/kg  10 mg/kg  Std.

F

SOD (U/mg)

Control  EP  2.5 mg/kg  5 mg/kg  10 mg/kg  Std.

G

GPx (U/mg)

Control  EP  2.5 mg/kg  5 mg/kg  10 mg/kg  Std.

H

GPT (U/mg)

Control  EP  2.5 mg/kg  5 mg/kg  10 mg/kg  Std.
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**Figure 2.** Effect of DPH on non-enzymatic antioxidants and enzymatic antioxidants in serum in endotoxin induced experimental periodontitis. *P<0.01 vs. control, †P<0.05, ‡P<0.01 vs. EP group.

**Figure 3.** Effect of DPH on lysosomal enzymes in endotoxin induced experimental periodontitis. *P<0.01 vs. control, †P<0.05, ‡P<0.01 vs. EP group.

might exert protective effect against EP via mitigating the enhanced oxidative stress after induction of EP.

The effect of DPH was determined on the non-enzymatic anti-oxidant system, such as ascorbic acid, ceruloplasmin, GSH, α-tocopherol and antioxidant enzymes such as catalase, GPx, SOD and GST. The results are presented in Figure 2. The DPH showed significant modulation of the activity of the above studied antioxidant system, which were found consistent with effect of DPH on curbing the generation of free radical.

**Effect of DPH on the lysosomal enzymes**

The effect of DPH was investigated on lysosomal enzymes, such as, cathepsin B, cathepsin D, β-glucuronidase and acid phosphatase. The results are presented in Figure 3. Results showed that, the level of these enzymes were found to be elevated in EP group as compared to control. Whereas, upon administering DPH, their level were drastically reduced in a dose-dependent manner as compared to EP.

The concentration of acute phase protein, for instance, C-reactive proteins and fibrinogen was found to be elevated in the EP treated group, while DPH caused significant reduction of these enhanced enzymes as shown in Figure 4.

**Assessment of effect of DPH on histopathology of gingival tissues**

In the histopathological examination, the EP treated group showed increased cellular permeability as evidenced by increase in the con-
Figure 4. Effect of DPH on the acute phase proteins in endotoxin induced experimental periodontitis. *P<0.01 vs. control, #P<0.05, **P<0.01 vs. EP group.

Figure 5. Histopathological examination of DPH on the gingival tissues. (A) Control group (B) EP, (C); DPH (2.5 mg/kg), (D) DPH (5 mg/kg); (E) DPH (10 mg/kg); (F) Standard.

These observations are more prominent as compared to control group, where normal manner of gingival tissues was observed. The histopathological lesion as evidenced in EP group was restored to near normal and cellular permeability was reduced in a concentration dependent manner in DPH treated group.
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Effect of DPH on the NLRP3 inflammasome pathway and other mediators

As shown in Figure 6, the effect of DPH was investigated on the mediators of NLRP3 pathway. It has been found that, the level of NLRP3 and other proteins, such as, TNF-α, iNOS was found to be increased, with decrease in caspase-1 in EP rats. The DPH administration causes reduction in the level of NLRP3, TNF-α and iNOS, with increase in caspase-1 level, in a dose-dependent manner.

Discussion

Periodontitis has been classified as inflammatory disease mainly caused by bacterial infection and has been associated with numerous ailments, such as heart diseases, diabetes, cancer and pregnancy complication [16, 17]. In periodontitis affected individuals, the bacterial-antigen host response promotes local recruitment of neutrophils at the gingival site and releases of proteolytic enzymes which results in systemic inflammatory response. Moreover, the critical importance of oxidative stress in periodontitis could not be overlooked [18-21]. It is well documented that oxidative stress caused by the production of excessive amounts of ROS plays a key role in pathogenesis of periodontitis conditions [22-25]. Under influence of inflammation, ROS concentration has been greatly increased due to altered antioxidant mechanism causing cellular damage (proteins, lipids, and DNA) if not neutralized by anti-oxidant substances. Various studies showed that, enhanced level of ROS is the characteristic hallmark of depleted anti-oxidant levels in gingival crevicular fluid and are held accountable for the chronic local activation of inflammation of periodontal tissues and its destruction [26-28]. Various animal models suggested that higher levels of lipid peroxidation, hydrogen peroxides, and oxidative DNA damage are linked with experimental periodontitis [28-31]. Prompted by the above, initially, the effect of DPH was evaluated against the oxidative stress in the experimentally induced periodontitis. It has been revealed that, DPH causes restoration of antioxidant defence system via modulation of superoxide, hydroxyl radicals, lipid hydroperoxides, and reactive non-radical compounds including singlet oxygen, and hydrogen peroxide. The effect of DPH was also investigated to confirm its activity against the non-enzymatic anti-oxidant system, such as ascorbic acid, ceruloplasmin, GSH, α-tocopherol and antioxidant enzymes such as catalase, GPx, SOD and GST. According to previous results, it has been found that DPH causes positive modulation of these markers and restored their level near to normal. Thus, it could be suggested that, DPH causes significant improvement of antioxidant defence mechanism via enhancing free radical scavenging activity. The oxidative stress in periodontal disease recruits neutrophil which causes gingival tissue destruction level of lysosomal enzymes in gingival tissues of the patients [32-34]. In the present study, DPH causes downregulation of cathepsin B, cathepsin D, β-glucuronidase and acid phosphatase as compared with the disease group suggesting its beneficial effect against periodontitis. Acute phase proteins are considered as an important marker for the inflammation, and can be called as acute phase reaction [35-38]. The activity of these proteins were greatly enhanced (>25%) in response of cytokines during inflammation (IL-1, IL-6, TNF-α). In DPH treated group, the level of C-reactive proteins and fibrinogen protein markers was significantly reduced. This result was found in agreement with previous study where DPH showed significant anti-inflammatory effect by reducing inflammation in myocardial injury after organophosphate poisoning in rats [39]. The beneficial effect of DPH was further confirmed against periodontitis via observing the histology of gingival tissue of the treated and EP-group. It was found that, DPH causes improvement of architecture of the gingival tissues as compared to EP group. This result further confirmed the protective effect of DPH against periodontitis. It has been confirmed that DPH showed antioxidant activity in microglial cells and might be helpful in mitigating neurodegenerative diseases originated due to oxidative stress [40]. Inflammosomes are multi-protein signalling complexes that trigger the activation of inflammatory caspases and the maturation of interleukin-1β [41-43]. The NLRP3 inflammasome is found aberrantly activated in many inflammatory conditions, thus behaving as a promising target for anti-inflammatory therapies [44]. In the present study, the effect of DPH was investigated on NLRP3 inflammasome by western blot analysis. It has been found that DPH causes significant decline in the level of NLRP3, TNF-α and iNOS, with increase in caspase-1 level, in a dose-dependent manner.
Figure 6. Effect of DPH on the inflammatory mediators including the NLPR3 inflammasome pathway. Values represent the mean ± SD and are representative of three independent experiments. **P<0.01 vs control, ***P<0.01 vs. EP.
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Conclusion

In the present study, diphenhydramine showed beneficial effect against periodontitis via decreasing oxidative stress and associated inflammation. However, more studies are needed to investigate the clinical significance of DPH against periodontitis.

Acknowledgements

The studies received financial support from Guangdong Science and Technology Department Key Laboratory Open Fund (KF201712-0102). The experiments were approved by the Animal Care and Use Committee of the Hospital of Stomatology, Sun Yat-sen University, Guangzhou, China (AEC/HS/13). The study protocols were approved and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Sun Yat-sen University.

Disclosure of conflict of interest

None.

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