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Gingipain Injection Affects Intestinal Oxidant-Antioxidant Status and Alkaline Phosphatase in Overfed Zebrafish

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ABSTRACT

Objective: *Porphyromonas gingivalis* (*P. gingivalis*), a major periodontopathogen, is associated with overfeeding disorders, including metabolic syndrome. Gingipains are one of the most powerful endotoxins of *P. gingivalis*. Our aim was to reveal the effects of gingipain injections on the intestinal oxidant-antioxidant status and alkaline phosphatase (ALP) activity in overfed zebrafish.

Materials and Methods: Four groups of healthy adult zebrafish were placed in random tanks as C: Control (n=15); GP: Gingipain (n=15); OF: Overfeeding (n=15); and OF+GP: Overfeeding+Gingipain (n=15) groups. At the end of the experiment, levels of intestinal lipid peroxidation (LPO) and ALP, glutathione S-transferase (GST), and catalase (CAT) activities were evaluated.

Results: Intestinal LPO was significantly lower in the GP and OF groups compared to C. Gingipain injection in OF (OF+GP) significantly elevated LPO when compared to C, GP, and OF groups. ALP activities decreased significantly in the GP, OF, and OF+GP compared to C. GST activities increased significantly in the GP when compared to C. Decreased GST activities were observed in the OF and OF+GP. This decrease was less in OF+GP. CAT activities significantly decreased in all groups when compared to C.

Conclusion: Our findings demonstrate that gingipain injection alters the ALP activity and intestinal oxidant-antioxidant status in overfed zebrafish.

Keywords: Gingipain, zebrafish, oxidative stress

INTRODUCTION

Porphyromonas gingivalis (P. gingivalis), a gram-negative anaerobic bacteria, is involved in the etiology of periodontitis. Periodontitis is generally characterized by bacteria-induced inflammation, which damages the tissues around teeth resulting in teeth loss. This strong keystone periodontopathogen, highly related to severe periodontitis, is an effective colonizer of the oral epithelium. *P. gingivalis* can infiltrate periodontal tissues and get over the host's immune response. Using a panel of virulence factors, it destabilizes innate immunological and inflammatory responses (1). Capsule, lipopolysaccharide (LPS), fimbriae, and cysteine proteases are some listed virulence factors of *P. gingivalis* (2).

P. gingivalis' extracellular cysteine proteinases, known as gingipains, are major endotoxins among these virulence

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factors. The gingipains are composed of Lys-gingipain (Kgp) and Arg-gingipain (Rgp) (RgpA and RgpB). Gingipain R is specific for arginine, while gingipain K is specific for lysine residues. Gingipains cleave amino acid chains right after lysine or arginine residues at the C-terminal region. In the physiology of the bacteria, gingipains' proteolytic activity is critical for adhering to host surfaces, obtaining nutrition through protein degradation, and promoting further colonization. While present in circulation, gingipains are also associated with fibrin(ogen)olytic activity and can interact with and degrade plasma proteins (3). Gingipains are responsible for 85% of *P. gingivalis'* extracellular proteolytic activities and play a major role in periodontitis. They activate host matrix metalloproteinases, inactivate immune defense by degrading immunological components, and cleave immune cell receptors, causing immune dysregulation and inflammation (2).

P. gingivalis and its endotoxins, including gingipains are detected in circulation and organ lesions (4, 5). The host defense molecules produced against these endotoxins together with bacteria itself play a significant role in the relationship between systemic diseases and periodontitis (6). The current trend topic for periodontal research is the connection between periodontitis and metabolic syndromes, including obesity and diabetes mellitus (7, 8). Recent research has also suggested that *P. gingivalis* cause an imbalance in the microbiota of intestinal tissues and exacerbate metabolic problems (9).

The metabolic syndrome, formerly referred to as insulin resistance, is a group of risk factors including elevated triglycerides and fasting glucose, reduced high-density lipoprotein, high blood pressure and abdominal obesity that increase the risk of type 2 diabetes mellitus (T2D), cardiovascular diseases and even death. In metabolic syndrome, inflammation in adipose tissues and imbalance in intestinal flora are thought to be possible reasons in this abnormal systemic status. As a consequence of continuous exposure to high-fat diets, the composition of gut microbiota is altered and the intestinal mucosa barrier is damaged, allowing enterotoxins to enter systemic circulation. Rapid adipocyte hypertrophy caused tissue hypoxia and induced elevated adipokines, including tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), stimulating macrophages to release pro-inflammatory substances resulting in increased systemic inflammation and insulin resistance (9).

Intestinal alkaline phosphatase (ALP) is a major player in maintaining intestinal homeostasis and health. Obesity is one of the chronic inflammatory conditions that have been linked to altered ALP expression. Intestinal ALP is essential in the activation of bacterial Lipopolysaccharides (LPS) through dephosphorylation of its lipid A moiety, resulting in a non-toxic monophosphoryl section. This dephosphorylated monophosphoryl lipid A is not capable of forming a complex with the host's toll like receptor 4 (TLR4) (10).

Oxidative stress, the imbalance between oxidants and anti-oxidants, as a result of increased free radicals is involved in the molecular mechanisms underlying many diseases. In order to countervail these harmful molecules, the anti-oxidant enzymes including catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST) and glutathione peroxidase in addition to antioxidant molecule, glutathione (GSH) are produced.

The increased oxidative stress is considered to be an essential factor in the relation between periodontitis and over-eating induced-metabolic syndrome. Intestinal homeostasis and health are also involved in the molecular mechanisms related with obesity and metabolic syndrome. Increased serum Reactive oxygen species (ROS) are present in these conditions, and a pro-inflammatory state is expected to have a reciprocal influence on the molecular mechanisms (7). Patients with periodontitis may experience greater levels of oxidative stress induced-inflammation due to the deranged endogenous antioxidant defense system driven by the overproduction of lipid peroxidation products at inflammatory sites (11).

Due to their various benefits, including their small size, brief life cycle, availability in huge numbers, and low maintenance requirements, zebrafish have become a prominent vertebrate model organism for biomedical research. Zebrafish are used in numerous areas, including genetics, biomedicine, neuroscience, toxicology, pharmacology, as well as the modeling of human disease (12). Overfeeding-caused metabolic syndrome diseases such as obesity and diabetes have been well documented in zebrafish models as well (13-15). *P. gingivalis* and gingipains related research have been recently conducted in zebrafish, but in these studies and other animal models, the specific effects of gingipains have not yet been explored. Despite *P. gingivalis'* many other virulence factors such as LPS, capsule, and fimbria, which all create a cumulative effect in bacterias' pathogenicity, it is important to underline the specific effects of gingipain itself in order to reveal this powerful endotoxins' toxicity (16-18). Here in this study, our aim was to assess the impact of gingipain injections on the intestinal oxidant-antioxidant status and ALP activity in overfed zebrafish to explore the relationship between periodontopathogens' virulence factors and metabolic diseases and to underline the role of gingipain in these complicated pathological mechanisms. We also assessed intestinal ALP because of its regulatory role in intestinal homeostasis in the case of overfeeding situations, which is an inflammatory condition related with altered ALP expression.

MATERIALS AND METHODS

Animals and Treatment

The animal experiments conducted in this study were performed according to the European Communities Council Directive of 24 November 1986 (86/609/EEC). The entire procedures used were authorized by the Marmara University Animal Care and Use Committee (38.2021mar). According to the ARRIVE (Animal Research: Reporting of *in Vivo* Experiments)

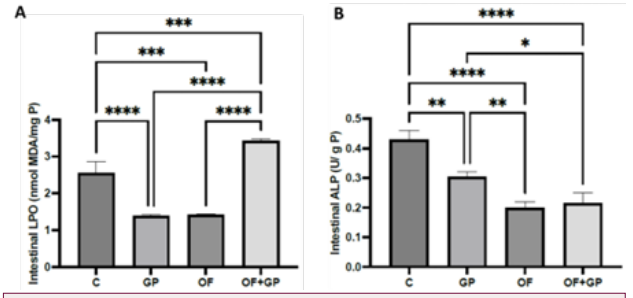

Figure 1. A: LPO levels **B:** ALP activities of the groups. Values are given as means ± SD; n= 6. **** p<0.0001; *** p<0.001, ** p<0.01; * p<0.05. LPO: Lipid peroxidation, ALP: Alkaline phosphatase, C: Control; GP: Gingipain, OF: Overfeeding, OF+GP: Overfeeding+Gingipain,

guidelines, care was taken to utilize as few animals as possible in the research. Zebrafish (AB/AB strain, wild type) were kept in an aquarium rack system (ZebTEC, Tecniplast, Italy) at a 14/10-hour light/dark cycle and 27–28 ± 1^{0}C temperature. Four groups of zebrafish (4-6 months old) were placed at random as C: Control (n=15); GP: Gingipain (n=15); OF: Overfeeding (n=15); and OF+GP: Overfeeding+Gingipain (n=15) groups. The control group was fed with 20 mg of fish food/fish/day, while the overfed zebrafish groups were fed with 120 mg/fish/day of commercial fish food (3.39 kcal/g; TetraMine; Tetra; Germany) over six daily feeding periods using an automated feeding system (Yinsheng T8800; Yinsheng; China). Overfeeding procedure was continued for 15 days. The content of tetramine consisted of a minimum of 51% crude protein, 11% crude oil, 2.3% calcium, 1.5% phosphorus and a maximum of 3% crude fiber, 6.5% moisture and 15% ash. The granule proportions of tetramine were between 0.65 mm and 0.36 mm. The tanks were cleaned and filled with fresh water daily. Gingipain (MyBioSource, Inc., USA, MBS969681, Recombinant *P. gingivalis* Gingipain R1(rgpA), partial) at 186 nmol/L was intraperitoneally injected into fish in the GP group. Gingipain dosages were chosen and applied in accordance with range finding research (19). 6 hours after injection applications the zebrafish were sedated and euthanized. Intestinal tissues of the zebrafish were taken, and for upcoming analyses, replicate samples of the tissues were made.

Biochemical Analyses

The total protein concentrations were measured using the Lowry et al. method, and the findings were provided per protein (20). Malondialdehyde (MDA) levels were measured using the Yagi method, and they were analyzed as thiobarbituric acid reactive compounds that are formed as byproducts of lipid peroxidation (LPO) (21). CAT activity was assessed by using the modified method of Aebi (22). For the conjugation of GSH, GST is required, and the activity of GST was measured using a spectrophotometer at 340 nm (23). ALP activity was measured according to Walter and Schutt method (24).

Statistical Analyses

For the statistical analyses GraphPad Prism 9.0 (GraphPad Software, San Diego, USA) was used and the data were presented as mean ± standard deviation. Dunn's multiple comparison test was run after the Kruskal Wallis test to compare the data. The Pearson correlation test was applied to evaluate the relationship between the oxidant and antioxidant parameters. Significant data were defined as those with a p-value lower than 0.05 (p<0.05).

RESULTS

When compared with the control group, overfeeding and gingipain injections yielded a significant decrease in intestinal LPO (p<0.001 and p<0.0001, respectively). However, gingipain injections increased LPO level significantly in overfed zebrafish (OF+GP) when compared to the GP, OF, and control groups, (p<0.0001, p<0.001, and p<0.001, respectively) (Figure 1A).

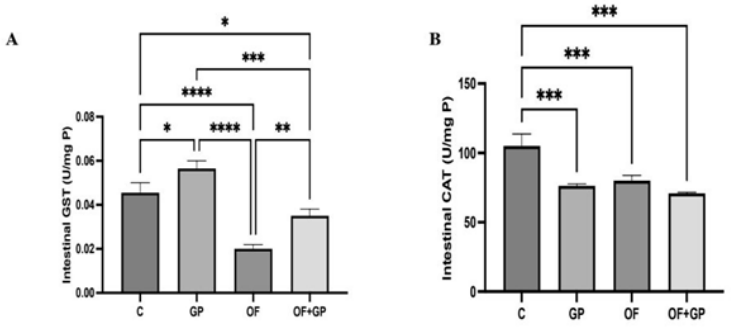

Figure 2. A: GST **B:** CAT activities of the groups. Values are given as means ± SD; n= 6. **** p<0.0001; *** p<0.001, ** p<0.01; * p<0.05. GST: Glutathione S-transferase**,** CAT: Catalase, C: Control; GP: Gingipain, OF: Overfeeding, OF+GP: Overfeeding+Gingipain

Intestinal ALP activities decreased significantly by gingipain injections and overfeeding in all three groups (GP, OF, and OF+GP) when compared to the control group ($p<0.01$, $p<0.0001$, and $p<0.0001$, respectively) (Figure 1B).

Intestinal GST activities were significantly elevated by gingipain injections when compared with the control group ($p<0.05$). However, decreased GST activities were observed in the OF and OF+GP groups ($p<0.0001$ and $p<0.05$, respectively). Gingipain injections led to increased GST activities in the overfed zebrafish (OF+GP) ($p<0.01$) (Figure 2A).

Intestinal CAT activities decreased significantly by gingipain injections and overfeeding in all three groups (GP, OF, and OF+GP) when compared with the control group ($p<0.001$). No significant change was observed among GP, OF, and OF+GP (Figure 2B).

In the GP group, LPO was positively correlated with CAT activity ($r=1$; $p<0.0001$), and negatively correlated with GST activity ($r=-1$; $p<0.0001$). In the OF group, LPO was positively correlated with GST activity ($r=1$; $p<0.0001$); whereas a negative correlation was found between LPO and CAT activity in the same group ($r=-1$; $p<0.0001$). In the OF+GP group, LPO was negatively correlated with CAT and GST activities ($r=-1$; $p<0.0001$).

DISCUSSION

P. gingivalis and one of its most significant endotoxins, gingipain, have been detected in organs and the circulatory system. The inhibition of gingipains with the specific inhibitors in *P. gingivalis* infected mice reduced neuroinflammation and brain infection (4). Oral administration of *P. gingivalis* exacerbates the severity of drug-induced colitis in mice (5). These and many other data demonstrate that *P. gingivalis*, key periodontopathogen, and its endotoxins are associated with many systemic disease pathologies. The current trend topic for periodontal research is the relation between periodontitis and metabolic syndromes, including obesity and diabetes mellitus, which are related with overfeeding (7, 8). Many studies about the relation with periodontopathic bacteria and intestinal disorders have indicated that *P. gingivalis* generate the dysbiosis of gut microbiota resulting in the aggregation of metabolic disorders, recently (9).

Dynamic relationships between the intestinal epithelium, the microbiota, and the human immune system are necessary for intestinal homeostasis. Intestinal homeostasis is maintained by a variety of regulatory mechanisms, and a malfunction in these pathways may lead to the chronic inflammatory pathological evident in inflammatory bowel disease (25).

The oral administration of *P. gingivalis* dramatically worsened colitis in the experimental colitis model. By lowering the expression of tight junction proteins *in vivo*, the ingested *P. gingivalis* damaged the colonic epithelial barrier in mice. The disruption of the epithelial barrier specific to *P. gingivalis* was shown by *in vitro* permeability experiments employing the intestinal epithelial cell line (26).

In this study, we wanted to assess the impacts of gingipain injection on the intestinal oxidant-antioxidant status and ALP activity in overfed zebrafish. We found that gingipain injections decreased intestinal ALP activity. Through interactions with the local microbiota, nutrition and the gut, intestinal ALP has an important function in intestinal homeostasis and health. The function of intestinal ALP in the intestine is to dephosphorylate extracellular nucleotides like uridine diphosphate as well as harmful microbial ligands such as LPS. Intestinal ALPs' capacity to control the microbial environment by establishing a complicated interaction between the microbiota, food, and the intestinal mucosal surface is also crucial in these issues. The gut microbiota and homeostasis can be affected by overfeeding because it changes intestinal ALP expression and activity (27).

Obesity is one of the chronic inflammatory conditions that have been linked to altered intestinal ALP expression (10). The results of our study showed decreased intestinal ALP activity in the overfed group. This finding indicates the diminished protective effect of intestinal ALP due to overfeeding on intestinal hemostasis.

In this study, the activities of CAT and GST were evaluated as they are the main antioxidant enzymes that might detoxify free radicals in the case of GP injection and overfeeding. CAT enzymes defend cells against free radical damage by degrading hydrogen peroxide (22), and GSTs may detoxify the end products of LPO and they can prevent the formation of lipid hydroperoxides (23).

Gingipain injections decreased intestinal CAT activity and LPO in the intestines. The reduction in CAT activity may be associated with the use of CAT to inhibit the increase in LPO associated with gingipain toxicity-induced inflammation. Similarly, the decreased LPO levels in the OF group may be associated with decreased CAT activity in the same group due to its use as an antioxidant to prevent the increase in LPO, consistent with the negative correlation between LPO and CAT observed in this group. Decreased LPO in the GP injected group may also be attributed to the substantial increase in the GST activities in the same group. In order to combat foreign compounds or oxidative stress products, GSTs catalyze the conjugation of reduced glutathione, resulting in the formation of less reactive molecules that are easily eliminated (28). Accordingly, in our study, increased GST in the gingipain-injected group may be suggested to prevent the increase in LPO levels due to gingipain injection. Decreased GST activity in the overfed group is consistent with the reported decrease in the adipose tissue GST enzyme content of high fat fed mice (29). However, gingipain injections in the overfed group caused an elevation in the GST activities to overcome oxidative stress induced by gingipain, as evidenced by increased LPO in the same group. Moreover, gingipain injections caused a significant elevation in GST activity, whereas overfeeding caused a reduction in

intestinal GST activity. Lower GST activity in the gingipain-injected overfed group, when compared with the gingipain-injected group, demonstrated that overfeeding diminished antioxidant response and even the gingipain challenge was not enough to augment this impairment.

As far as we are aware, there are only a few studies that have used gingipain *in vivo* models directly (18). It would be advantageous to increase the number of studies on the direct use of gingipain in zebrafish, given the benefits of this animal model, including their use in toxicological studies and the modelling of systemic diseases. Moreover, to our knowledge, this is the first study to report the effects of gingipain injections on the intestinal oxidant-antioxidant status and ALP activity in overfed zebrafish. Our study showed that gingipain disrupts the oxidant-antioxidant balance and ALP activity in the intestines due to overnutrition and provided important information on the effects of periodontal pathogens on intestinal health in metabolic diseases. We believe that our findings will guide future studies related to the connection of systemic diseases and periodontitis pathology.

Ethics Committee Approval: This study is approved by of Marmara University Animal Experiments Local Ethics Committee (38.2021mar).

Authors' Contributions: Conception/Design of Study- G.G., E.E.A., K.N.K.; Data Acquisition – G.G., M.B.; Data Analysis/Interpretation – G.G., M.B., İ.Ü., D.C., E.E.A., K.N.K.; Drafting Manuscript– G.G.; Critical Revision of Manuscript- E.E.A., K.N.K.; Final Approval and Accountability– G.G., M.B., İ.Ü., D.C., E.E.A., K.N.K.

Conflicts of Interests: The authors declare that they have no competing interests.

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