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Gingivitis in Mice and the Role of Angiogenesis

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Research article

Keywords: Gingivitis, Angiogenesis, periodontal, periodontal diseases

Posted Date: September 10th, 2019

DOI: https://doi.org/10.21203/rs.2.14246/v1

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Abstract

Background Gingivitis is the first step in an abnormal oral condition, which degrades connective tissue and eventually converts to periodontal diseases leading to alveolar bone and tooth loss. Thrombospondin-1 (TSP1) and Bcl-2 are key regulators of angiogenesis. The transition from gingivitis into periodontitis has four stages and disease progression depends very heavily on changes to the surrounding vasculature structures. We hypothesized that vascular dysfunction plays an important role in the progression of gingivitis. Here we have measured Gingival Crevicular Fluid (GCF) levels in TSP1 and Bcl-2-deficient mice, in which vascular structures are affected, while also looking at the roles that age and gender play in this process.

Methods Thirty mice (15 males and 15 females) were divided into three groups of ten (n=10): group A (WT), group B (TSP1 -/-), and group C (Bcl-2 -/-) were subjected to collection of Gingival Crevicular Fluid (GCF). Saliva was collected from different groups of mice after subcutaneous injection of pilocarpine. For the WT group, GCF was monitored for 3, 4, 5 and 6 weeks and the contribution of age was also considered.

RESULTS: Samples from Bcl-2 -/- mice showed significantly lower levels of GCF and salivary secretions, while TSP1-/- mice were larger and secreted more saliva. Moreover, the TSP1-/- mice GCF level was significantly higher than that of both the WT and Bcl-2 -/- mice. C

ONCLUSIONS: The absence of Bcl-2 and TSP1 significantly affects the amount of GCF flow in mice. This current study confirms the important role that alteration of pro- and anti-angiogenic factors can play in the progression of gingivitis and in the amount of salivary secretions. Thus, this type of modulation of angiogenic proteins provides a great opportunity and a novel platform for developing enhanced oral care products of the future. TSP1 showed significantly more saliva secretion, and a larger volume of periodontal pocket. This means that higher level saliva secretion can affect periodontal pocket volume and eventually oral hygiene.

Introduction

Gingivitis is a relatively mild periodontal disease, and is the most common cause of irritated and swollen gums [1]. If left untreated it can develop into a more severe form of gum disease, known as periodontitis, which affects around 50–90% of the adult population worldwide. Gingivitis represents the beginning of gum disease and affects only the soft tissues around the teeth. During the early stages of development, gingivitis is reversible and non-destructive. It also responds well to treatment through enhanced oral hygiene practices. The two primary forms of gingivitis include: plaque induced gingivitis and systemic factor induced gingivitis [2]. Plaque induced gingivitis is caused by the buildup of bacterial plaques and the particular bacterial composition of these biofilms play an important role in disease progression. Additionally, systemic factors including certain medications or malnutrition are also known to propagate the negative effects of gingivitis. Gingivitis becomes visible as reddish inflammatory lesions within the

gingiva and this inflammation is a result of interactions between the immune system and external factors such as bacterial toxins [3]. Removing dental plaque is sometimes difficult due improper homecare which can be exacerbated by crowding and malocclusion [4]. Thus, finding alternative ways to mitigate the progression of gingivitis is highly desirable.

The initiation and progression of the gingival diseases are closely related to the development of new capillaries and increased permeability of the capillary lining [5]. Notably, a common hallmark of different types of gingivitis is an increase in capillary sprouting and increased permeability of blood vessels [6]. These changes are mediated by growth factors, primarily vascular endothelial growth factor (VEGF), which is likely released by mast cells and neutrophils inducing permeability and angiogenesis in the local area. Clinically, this presents as fluffy, irritated gums, with oozing and seepage at the later stages. Biofilm can be removed easily by maintaining good oral hygiene particularly in patients with healthy, non-inflamed tissues [7]. Thus, inhibiting angiogenesis in inflamed gingiva could provide a chance to subside enlarged gingiva and create more access during brushing, which could by itself remove the bacteria and toxins associated with inflammation [8]. In this way, it prevents the destructive effects on the supporting periodontal ligament and alveolar bone, and consequently prevents tooth loss. Nearly 50% of all adults aged 30 or older—about 65 million people—have signs of gum disease. A study titled Prevalence of Periodontitis in Adults in the United States: 2009 and 2010 estimates that 47.2 percent, or 64.7 million American adults, have mild, moderate or severe periodontitis, the more advanced form of periodontal disease [9]. In adults 65 and older, prevalence rates increase to 70.1 percent. It is important to understand that if left untreated, the progression of gingivitis to periodontitis generally occurs in four stages as originally described by Page and Schroeder (1976) and updated by several others over the years [10].

Stage 1

This is the initial stage of gingivitis and development occurs after 2 to 4 days of plaque formation [11]. In clinical terms, this stage is referred to as the healthy stage and patients do not show any clinical symptoms. However, upon microscopic analysis, this stage shows inflammatory responses, which are indicated by increased vascular activity and migration of inflammatory cells such as monocytes and neutrophils. Inflammation also occurs in the connective tissues toward bacterial products specifically bacterial polysaccharides that are present in the gingival sulcus. The migration of neutrophils is facilitated by intercellular adhesion molecule–1 (ICAM–1) while E-selectin facilitates their migration to the connective tissues. Additionally, during this stage, there are trivial changes that occur in the blood flow to the area such as an increase in vascular permeability resulting in lower levels of inflammation [12]. VEGF, also known as vascular permeability factor or vasculotropin, is an important factor for neovascularization. Angiogenesis leads to an increase in vessel profiles along the periodontal pocket wall. It has been observed that VEGF induces permeability of the fluids and proteins at estimated 50,000 times more than histamine suggesting that angiogenesis increases gingivitis. VEGF increases the actions of micro-vascular permeability and stimulates the proliferation of endothelial cells [13].

Stage 2

In the second stage of gingivitis, the early lesion develops 5–7 days after accumulation of plaque. The inflammation within the gingiva is evident, and there is significant vasodilation at this level, which can lead to an erythematous appearance. Of note, more inflammatory leukocytes, specifically lymphocytes, are recruited to the local inflammatory sites. An increase in GCF causes more transmigration of neutrophils, which can produce the growth factor VEGF, and stimulate angiogenesis and the formation of new blood vessels. As a result of edema in the gingival tissues, the gingiva may appear moderately swollen, and accordingly, the gingival sulcus can become deeper. This makes the mechanical removal of plaque significantly harder to achieve. The mast cells or neutrophils are stimulated by toxins, which release vasoactive mediators that cause vasodilation leading to increased blood flow. The tissues permeability leads to escape of exudates, which are secreted out of the sulcus as the GCF. The leucocytes and neutrophils escape by adhering to the capillary vessels [14]. After about 5–7 days of plaque accumulation, the early lesions develop. During this stage, inflammation is readily visible and significant vasodilation occurs leading to the appearance of erythematous tissues. This stimulates the defense system, which results in the recruitment of lymphocytes to the inflamed sites and disease propagation occurs. As the GCF increases in volume, the neutrophils transmigrate and produce vascular VEGF, which is capable of initiating angiogenesis and stimulating the new blood vessel formation. This leads to moderate swelling of the gingiva. At this stage, mechanical removal of the plaque is harder as the location of the bacterial challenge is established more deeply into the gingival sulcus [11].Finally, collagen can be observed to degrade in parts of the lateral and apical junctional epithelium, and these cells can proliferate into collagen depleted areas of the connective tissue to ensure barrier integrity is established.

Stage 3

In the third stage, Established lesion, based on the quantity and composition of plaque, as well as the host immune response which is under the influence of a number of other risk factors dictates whether these lesions either progress or stay stable. This constant battle between bacterial challenge in plaque accumulation and the subsequent host response to these pathogens is the foundation for periodontal disease pathogenesis. Depending on the level and composition of the plaque, gingivitis can either remain stable at this stage or develop further [15]. The established lesion stage is dominated by plasma cells. Collagen destruction can also continue due to the dysregulated release of neutrophilic lysosomal contents, including proteolytic enzymes. These cells also produce MMP–8 and MMP–9 in high concentrations, contributing to the sustained inflammatory response [16]. Notably, the pocket epithelium may be ulcerated and as a result is less able to resist the passage of a periodontal probe, and bleeding on probing is a common feature of chronic gingivitis. Although this state is manifested by high inflammation, it is still possible to reverse the disease if there is a way of controlling the plaque vessels [14]. The inflammatory changes associated with this stage are still completely reversible if effective plaque control is reinstituted. It is at this stage that gingivitis becomes chronic with the inflammation

ranging from moderate to severe inflammation. Inflammation and reddening of the gums continues due to continuous leaking of the blood plasma. The viscosity of the blood makes it difficult to move. At this stage, the red blood cells are broken down into pigments and escape to the connective tissues. This increases the level of inflammation due to the nature of the erythematous gingiva. It should be remembered that this stage is characterized by the intense chronic inflammation predominated by plasma cells. The proliferation of rete ridges arising from junctional epithelium into the connective tissue is pronounced and there is further destruction of the collagen tissues. Chronically inflamed gingiva appears edematous when clinically assessed [17].It appears boggy and is usually deep red or bluish red in color. It may appear blue due to the sluggish flow of blood, which may cause anoxemia or deficiency of oxygen in the blood.

Stage 4

In the last stage, angiogenesis expands to nourish advanced lesions. The extensive tissue damage is primarily caused by the inflammatory responses, and yet the initiating factor, the bacterial biofilm, is not eliminated. Thus, destruction of collagen fibers in the periodontal ligament continues and bone resorption progresses. Of note, the junctional epithelium migrates apically to maintain an intact barrier, and as a result, the pocket can deepen fractionally. In clinical terms, this can make it even more difficult to remove the bacteria, and to disrupt the biofilm through oral hygiene techniques, and thus perpetuating the cycle of inflammation and associated pathology. Figure1. shows a summary of progression of gingivitis and potential mechanisms and actions on surrounding tissues. The final stage of development of gingivitis is referred to as the advanced lesion stage [14]. The extensive tissue damage is primarily caused by the inflammatory response. The initiating factor which contributes to the disease is the bacterial biofilm which remains in the gingiva. If it is not removed, it will lead to the destruction of collagen fibers in the periodontal ligament and subsequently bone resorption ensues. It has been observed that removal of bacteria in active periodontally compromised lesions is significantly more difficult than in lesions limited to the gingiva. However, disturbing the bacteria by removing the biofilm using oral hygiene techniques can reverse the situation and cure the inflammation [11].Many studies indicate that the progression of oral disease depends on the architecture of vasculature. The architecture of vasculature plays an important role in bringing nutrients, oxygen, and immune cells to the site. In addition, gingivitis normally is reversible and by removal or elimination of the causing factors it will return to normal condition and disappear. Gingivitis once it has converted to periodontal disease is not reversible. Therefore, the control of this inflammatory process is imperative.

Higher rate of gingivitis in patients with systemic inflammatory disease are the result of imbalance between pro-angiogenic and anti-angiogenic factors, where pro-angiogenic factors are being over activated because of inflammation. [18]At this stage, physiological angiogenesis converts to pathological angiogenesis. The stimulation of forming new capillaries eventually turn to the chronic stage pathogenesis of multiple chronic diseases, such as psoriasis, rheumatoid arthritis, osteoarthritis, metabolic syndrome, [19] (equivalent to stage 3 and 4 of gingivitis). Higher level of gingivitis can be

found in different immune-inflammatory diseases, which all share common features of overgrowing new capillaries, and expressing inflammatory factors that support this hypothesis. Given the above information and studies about gingival diseases. Gingivitis can be categorized as “immune-driven angiogenesis”, which is highly related to angiogenesis. Higher volume of vasculature is noted in gingivitis compared to healthy gingival.

Gingivitis is initiated by alteration in vascular architecture and proceeds with higher flow rate in inflamed gingiva. Highest laser reading flow rate is reported in chronic gingivitis [20] and subsequently in acute gingivitis. Persistent gingivitis results in sprouting capillaries specifically capillary loops arching toward the epithelium [21]. But it can also lead to expanding periodontal pockets that protect the bacteria in that site. Therefore, we selected mice deficient in TSP1 as a model for enhanced pro-angiogenic activity, and Bcl–2 deficient mice as a model for diminished angiogenic activity.

Here, we investigated the role of angiogenic regulatory genes including TSP1and Bcl–2 on periodontal pocket and secretion of saliva. Periodontal Pocket has a close correlation with the volume of gingival crevicular fluid. [22]. GCF is derived from the circulation after having permeate through the diseased soft tissue of the periodontal pocket and thus the volume is an indicator of disease process.

Methods

All animal care and procedures were in agreement with the Principles of Laboratory Animal Care and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health. The source of all mice was Jackson Lab (Sacramento, CA USA). The housing and condition of mice was the same as our previous investigation [23] performed at the University of Wisconsin- Madison, WI.

Breeding colony and preparation of animals:

Thirty mice (15 males and 15 females) including three groups (WT, TSP1 -/- and Bcl–2 -/-) were selected, five males and five females per group were arranged in three groups. We maintained a breeding colony of WT, TSP1-/-, and Bcl–2 +/- mice, and the progenies with the desired genotype were used.

GCF Collection:

GCF collection was performed according to methods presented by Matsuda et al. [24]. Briefly, mice were anesthetized and placed in a holder for easier handling, and then were fresh weighed absorbent paper points (Spident, Meta Biomed Co, Incheon, Korea) around the second molar at the left and right sides. After 10 min, the paper points from both sides were collected and then weighed using an analytical balance to a sensitivity of ±0.05 µg. In order to avoid bleeding, the bleeding cases were excluded from

study. procedure are visulaized on the isolated mandible on figure 3, but we should mention that this step was done in alive mice.

Saliva Collection

Saliva collection was performed according to methods presented by *Saghiri et al.* [23]. Briefly, mice were anesthetized and placed in a holder for easier handling, and then were given pilocarpine 80 mg/kg in 0.1 mL subcutaneously. The saliva was collected in two 5-min intervals using a simple suction device. The animal's nose remained unobstructed for unhindered respiration. According to *Saghiri et al.* [23], tubing was used to collect the saliva from the oral cavity, while the nose was allowed to remain free for unhindered respiration. Saliva collection was performed every 15–30 sec for 10 min and accumulated on ice. The investigator was masked to the mice genotype for saliva collections. This section evaluation of samples was performed on postnatal day 21 only. All animals were euthanized with CO_2 according to Laboratory Animal Care and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health by the end of this investigation.

Statistical Analysis:

Data of GCF and saliva collection were analyzed by two-way ANOVA, Levene's, and Kruskal Wallis tests.

Results

Current investigation used A two-way ANOVA test to determine the effect of two nominal predictor variables on a continuous outcome variable. That is, it compares the mean differences between groups that have been split on two independent variables (called factors). in addition, we wished to determine the effect of gender (male and female) and the mouse genotype (WT, TSP1 -/-, and Bcl–2 -/-) on the volume of GFC and saliva collected. Thus, the study compared the mean differences in the volume of GFC and saliva (dependent variable) collected by gender and the genotype (two independent variables).

To determine the status of oral hygiene and periodontal inflammation, we determined the volume of GCF production in these mice. Across all the various types of mice, including: WT, TSP1 -/-, and Bcl–2 -/-, the volume of GCF collected was higher for female mice compared to that of male mice. Mean volume of GCF collected was very low for Bcl–2 -/-mice among both male and female mice, however, TSP1 -/- had the highest average GCF volume regardless of gender (Fig.4).

Comparison of Variance using Levene's test

Results indicated that the P-value (0.381) is greater than 0.05, which indicates that the variance of the volume of GCF is equal across the groups. The results indicated that gender has an effect on the volume of GCF collected, thus, there is a significant difference in the mean volume of GCF collected between the

male and female mice (*P = 0.005).* Similarly, the test also showed a significant effect of mice genotype on the volume of GCF collected. Thus, there was a significant difference in the mean volume of GCF collected from the three types of mice, whether wild type (WT), TSP1 -/-, or Bcl–2 -/- (*P = 0.000).* However, there was no significant effect of interaction between the gender and the mice genotype on the mean volume of GCF collected (*P = 0.382).* Thus, the mean volume of GCF collected from any mice genotype was not influenced by the gender. Pairwise Comparison of mean volume of GCF by gender indicated thaton the average female mice had a higher GCF volume (*mean = 212, C.I = [199.909, 224.358])* compared with that of male mice (*mean = 186.000, C.I = [173.775, 198.225]),* and this difference was statistically significant (*P = 0.005)* as shown in the pairwise comparison table. In addition, Pairwise Comparison of mean volume of GCF by mice genotype revealed that the volume of GCF was higher TSP1 -/- mice (*mean = 315.10, C.I = [300.128, 330.072]),* followed by the wild type mice (*mean = 198.500, C.I = [183.528, 213.472]).* Whereas Bcl–2 -/- mice recorded the lowest mean GCF volume. These differences are statistically significant (*P = 0.005).*

Saliva Data Results

The statistical results that collected from Saliva are shown in Figure 3, and indicated the mean volume of saliva collected is higher for Wild (WT) and TSP1 -/- male mice compared to the same genotype of female mice. However, female Bcl–2 -/- mice had a higher volume of saliva relative to that of Bcl–2 -/- male mice.

Comparison of Variance using Levene’s test

This tests the null hypothesis that the error variance of the volume of saliva is equal across the groups. Results indicated that the p-value (0.002) is less than 0.05, which indicates that the variance of the volume of saliva is unequal across the groups.

Testing the effects of Gender and Type of mice on the volume of collected saliva

Statistical Results indicated that the gender had an effect on the volume of saliva, thus, there is a significant difference in the mean volume of saliva collected between the male and female mice (*P = 0.008).* Similarly, the test also showed a significant effect of the mice genotype on the volume of saliva collected. Thus, there was a significant difference in the mean volume of saliva collected among the three types of mice, whether wild type (WT), TSP1 -/-, or Bcl–2 -/- (*P = 0.000).* However, there is no significant effect of interaction between gender and mice genotype on the mean volume of saliva collected (*P = 0.113).* Thus, the mean volume of saliva collected for any of the three mice genotypes was not influenced by gender. Pairwise Comparison of mean volume of saliva by gender confirmed thaton the average male

mice had a higher volume of saliva (*mean = 1.052, C.I = [0.980, 1.124])* compared to that of female mice (*mean = 0.909, C.I = [0.837, 0.981]),* and this difference is statistically significant (*P = 0.005).*

Pairwise Comparison by mice genotype.

These results indicated that the saliva volume was higher for TSP1 -/- mice (*mean = 1.732, C.I = [1.644, 1.820]),* followed by the wild type (*mean = 0.988, C.I = [0.900, 1.076]),* whereas Bcl–2 -/-mice recorded the lowest mean volume of saliva. These differences are pairwise statistically significant (*P = 0.005).*

GCF volume collected from WT type mice at different time points (3, 4, 5 and 6 weeks)

The result form the role of age on GCF volume is shown in Fig. 6. This indicated that volume of GFC collected from the wild type mice at different time points decreased with an increase in time for female mice. It increased a bit in week four and then decreases afterwards (from week 5 onwards). Generally, we observe a downwards trend in the GFC volume collected from WT mice across both sexes.

Figure 6 shows that time has a significant effect on the volume of GFC for the wild type mice *(P = 0.000).* That is, the time point at which the experiment was conducted has an effect on the amount of GFC collected from the wild type mice. However, gender differences were not statistically significant with *P = 0.137.* The results displayed in Figure 4 indicated that the time has significant effect on the volume of GFC from the wild type mice (*P = 0.000).* That is, the time point at which the experiment was conducted has an effect on the amount of GFC collected from the wild type mice. However, gender was not statistically significant with *P = 0.137.*

Discussion

The current study investigated the impact of angio-regulatory genes, namely TSP1 and Bcl–2 expression on GCF volume and periodontal pocket volume as indicators of gingivitis. We also measured the levels of saliva secretion in these mice. Mice share structural, functional and genetic traits with humans [25]. Moreover, powerful molecular and genetic tools developed in the past two decades make mice an ideal animal model to study complex traits. Previous studies indicated that secretion of saliva has a close correlation with periodontal disease including gingivitis by enhance the mechanisms of plaque mineralization [26, 27]. In addition, the development and maturation the periodontal problems could be influenced by angio-regulatory genes such as Bcl–2,TSP–1 expression. However, the apparently diminished potential angiogenic activity In Bcl–2-/- mice could prevent the formation of GCF volume.

GCF is an inflammatory exudate that is normally considered an indicator of periodontal disease. Our results showed that the GCF for WT mice increased during 3–6 weeks of age. Gingivitis is not simply caused by the presence of plaque but is also dependent on the host response including inflammation and

changes to the vascular density of the periodontal apparatus. Thus, the results of the current study revealed that the alteration vasculature density in Bcl–2-/- and TSP–1-/- mice, may adversely affect GCF secretion. Future studies will focus on the impact of exogenous factors designed to target these types of angio-regulatory genes and the exciting role they will play in mitigating the inflammatory process in gingivitis.

Angiogenesis can be regulated by many factors including trace elements [28–31] and it is critical for regeneration and healthy tissue particularly in oral and maxillofacial area [32]. By targeting specific pathways like angiogenesis that are known to play a crucial role in the development of periodontal disease, we may be able to intervene in this process at an earlier stage and dramatically alter the course of the disease. As periodontal health is the goal of our research, we should focus our efforts as close to that baseline goal as possible. Rather than attempting to simply combat advanced disease once it reaches an irreversible stage, prevention as we described previously is of paramount importance. Due to the global scale and prevalence of gingivitis and periodontal disease, the potential epidemiologic impacts of this work are extraordinary. By studying disorders like gingivitis that affect nearly the entire global population, every breakthrough can have a profound impact. It is for that reason that we focused our work on early intervention of such a prevalent and complicated disease. Additionally, the assumption that the systemic health of the periodontal patient does not play a key role in disease pathogenesis has been thoroughly debunked in the literature and the American Academy of Periodontology's position on the correlation between gum disease and other systemic diseases including heart disease and diabetes is very clear.

This work was supported by an unrestricted award from Research to Prevent Blindness to the Department of Ophthalmology and Visual Sciences, Retina Research Foundation, P30 EY016665, P30 CA014520, EPA 83573701, EY022883, and EY026078. NS is a recipient of RPB Stein Innovation Award.

Conclusion

The control of gingival inflammation through regulation of angiogenesis may have positive impacts not only for oral health but systemic health as well.

In this particular study, a mouse model was selected to evaluate periodontal disease progression in humans. The use of rodent models for this exact purpose has been extensively documented in the literature and in particular, the genetic manipulation of mouse models have shown great promise in evaluating the subsequent host response and their role in the disease process[25].

We believe this area should be investigated further because too many of our current treatments for combating periodontal disease is focused on the bacterial challenge alone. However, as we described previously, periodontal destruction is due not only to the presence of bacterial plaque but due to the destructive mechanisms inherent within our own host response. By focusing on the host response to a bacterial challenge, we may approach treatments from a new perspective. The overuse of antibiotics in medicine and dentistry is known to have enormous societal impacts including bacterial resistance and

reduced effectiveness of some of our life saving medications. By identifying new targets in the treatment of some of our most prevalent diseases such as gingivitis, we may yet again circumvent another massive obstacle and fight epidemics on a global scale.

Abbreviations

GCF: Gingival crevicular fluid

Pg: Porphyromonas gingivalis

BOP: Bleeding on probing

CAL: Clinical attachment level

Declarations

Consent for publication

On behalf of all the authors, corresponding author of the current manuscript hereby declare that the above mentioned manuscript which is submitted for publication is NOT under consideration elsewhere.

Availability of data and material

Upon of request

Competing interests

The views expressed in this paper are those of the authors and do not necessarily reflect the views or policies of the affiliated organizations. The authors hereby announced that they have active cooperation in this scientific study and preparation of present manuscript. Authors confirm that they have no financial involvement with any commercial company or organization with direct financial interest regarding the materials used in this study.

Authors' contributions

Designed the experiment: MAS, N. S. Conducted the experiment: MAS, AA Analyzed/interpreted data: MAS, AA, AF, MMM, SM, NS, Wrote the article: MAS, NS; Proofed/revised article: MAS, AF, NS. It is announced that all authors read and approved the manuscript.

Acknowledgements

This publication is dedicated to the memory of Dr. H. Afsar Lajevardi [33], a legendry Pediatrician (1953–2015) who passed away during this project. We will never forget Dr. H Afsar Lajevardi's kindness and support.

References

1.Pihlstrom BL, Michalowicz BS, Johnson NW: Periodontal diseases. *The lancet* 2005, 366(9499):1809–1820.

2.Tatakis DN, Trombelli L: Modulation of clinical expression of plaque-induced gingivitis: I. Background review and rationale. *J Clin Periodontol* 2004, 31(4):229–238.

3.Graves DT, Fine D, Teng YTA, Van Dyke TE, Hajishengallis G: The use of rodent models to investigate host–bacteria interactions related to periodontal diseases. *J Clin Periodontol* 2008, 35(2):89–105.

4.Mariotti A: Dental plaque-induced gingival diseases. *Ann Periodontol* 1999, 4(1):7–17.

5.Yu C, Abbott PV: An overview of the dental pulp: its functions and responses to injury. *Aust Dent J* 2007, 52:S4-S6.

6.Arias HR, Richards VE, Ng D, Ghafoori ME, Le V, Mousa SA: Role of non-neuronal nicotinic acetylcholine receptors in angiogenesis. *The international journal of biochemistry & cell biology* 2009, 41(7):1441–1451.

7.Page RC: The pathobiology of periodontal diseases may affect systemic diseases: inversion of a paradigm. *Ann Periodontol* 1998, 3(1):108–120.

8.Madianos P, Bobetsis Y, Kinane D: Generation of inflammatory stimuli: how bacteria set up inflammatory responses in the gingiva. *J Clin Periodontol* 2005, 32:57–71.

9.Eke PI, Dye B, Wei L, Thornton-Evans G, Genco R: Prevalence of periodontitis in adults in the United States: 2009 and 2010. *J Dent Res* 2012, 91(10):914–920.

10.Page RC, Schroeder HE: Pathogenesis of inflammatory periodontal disease. A summary of current work. *Laboratory investigation; a journal of technical methods and pathology* 1976, 34(3):235–249.

11.Listgarten MA, Mayo HE, Tremblay R: Development of dental plaque on epoxy resin crowns in man: a light and electron microscopic study. *J Periodontol* 1975, 46(1):10–26.

12.Lang NP, Schätzle MA, Löe H: Gingivitis as a risk factor in periodontal disease. *J Clin Periodontol* 2009, 36:3–8.

13.Padma R, Sreedhara A, Indeevar P, Sarkar I, Kumar CS: Vascular endothelial growth factor levels in gingival crevicular fluid before and after periodontal therapy. *Journal of clinical and diagnostic research:*

JCDR 2014, 8(11):ZC75.

14.Bartold PM, Van Dyke TE: Periodontitis: a host-mediated disruption of microbial homeostasis. Unlearning learned concepts. *Periodontol 2000* 2013, 62(1):203–217.

15.Marsh PD: Microbial ecology of dental plaque and its significance in health and disease. *Adv Dent Res* 1994, 8(2):263–271.

16.Makela M, Salo T, Uitto V-J, Larjava H: Matrix metalloproteinases (MMP–2 and MMP–9) of the oral cavity: cellular origin and relationship to periodontal status. *J Dent Res* 1994, 73(8):1397–1406.

17.Scott D, Singer D: Suppression of overt gingival inflammation in tobacco smokers–clinical and mechanistic considerations. *Int J Dent Hyg* 2004, 2(3):104–110.

18.Flemmig TF, Shanahan F, Miyasaki KT: Prevalence and severity of periodontal disease in patients with inflammatory bowel disease. *J Clin Periodontol* 1991, 18(9):690–697.

19.Lira-Junior R, Figueredo CM: Periodontal and inflammatory bowel diseases: Is there evidence of complex pathogenic interactions? *World J Gastroenterol* 2016, 22(35):7963.

20.Kindlova M: The blood supply of the marginal periodontium in Macacus rhesus. *Arch Oral Biol* 1965, 10(6):869-IN868.

21.Egelberg J: The blood vessels of the dento-gingival junction. *J Periodontal Res* 1966, 1(3):163–179.

22.Teles R, Sakellari D, Teles F, Konstantinidis A, Kent R, Socransky S, Haffajee A: Relationships among gingival crevicular fluid biomarkers, clinical parameters of periodontal disease, and the subgingival microbiota. *J Periodontol* 2010, 81(1):89–98.

23.Saghiri MA, Asatourian A, Gurel Z, Sorenson CM, Sheibani N: Bcl–2 expression is essential for development and normal physiological properties of tooth hard tissue and saliva production. *Exp Cell Res* 2017, 358(2):94–100.

24.Matsuda S, Movila A, Suzuki M, Kajiya M, Wisitrasameewong W, Kayal R, Hirshfeld J, Al-Dharrab A, Savitri IJ, Mira A: A novel method of sampling gingival crevicular fluid from a mouse model of periodontitis. *J Immunol Methods* 2016, 438:21–25.

25.Saghiri MA, Orangi J, Asatourian A, Sheibani N: Validity and Variability of Animal Models Used in Dentistry. *Advances In Human Biology* 2015, 5(2):1–16.

26.Macgregor I: Smoking, saliva and salivation. *J Dent* 1988, 16(1):14–17.

27.Acharya A, Kharadi M, Dhavale R, Deshmukh VL, Sontakke A: High salivary calcium level associated with periodontal disease in Indian subjects—a pilot study. *Oral health & preventive dentistry* 2011, 9(2).

28.Aaltonen A, Tenovuo J, Lehtonen O-P: Increased dental caries activity in pre-school children with low baseline levels of serum IgG antibodies against the bacterial species Streptococcus mutans. *Arch Oral Biol* 1987, 32(1):55–60.

29.Saghiri MA, Asatourian A, Orangi J, Sorenson CM, Sheibani N: Functional role of inorganic trace elements in angiogenesis—Part II: Cr, Si, Zn, Cu, and S. *Crit Rev Oncol Hematol* 2015, 96(1):143–155.

30.Saghiri MA, Asatourian A, Orangi J, Sorenson CM, Sheibani N: Functional role of inorganic trace elements in angiogenesis—Part I: N, Fe, Se, P, Au, and Ca. *Crit Rev Oncol Hematol* 2015, 96(1):129–142.

31.Saghiri MA, Orangi J, Asatourian A, Sorenson CM, Sheibani N: Functional role of inorganic trace elements in angiogenesis part III:(Ti, Li, Ce, As, Hg, Va, Nb and Pb). *Crit Rev Oncol Hematol* 2016, 98:290–301.

32.Saghiri MA, Asatourian A, Sheibani N: Angiogenesis in regenerative dentistry. *Oral surgery, oral medicine, oral pathology and oral radiology* 2015, 119(1):122.

33.Saghiri MA, Saghiri AM: In Memoriam: Dr. Hajar Afsar Lajevardi MD, MSc, MS (1955–2015). *Iranian Journal of Pediatrics* 2017, 27(1):1.

Figure Legends

Figure1. shows a summary of progression of gingivitis and potential mechanisms and actions on surrounding tissues. Bcl–2 knockout mice generally have lower weight that other type of mice.

Figure 2. Steps for GCF Collection.

Fig 3, The mean and trend of GCF collection from Wild type (WT), TSP1 -/-, and Bcl–2 -/-, the average (mean) volume of GCF (µg).

Figure 4. The Mean and SD for Saliva collected from Wild type (WT), TSP1 -/-, and Bcl–2 -/- (mL).

Figure 5. The Mean and SD for GCF volume (µg) collected from WT type mice in different time points (3, 4, 5, and 6 weeks).

Figures

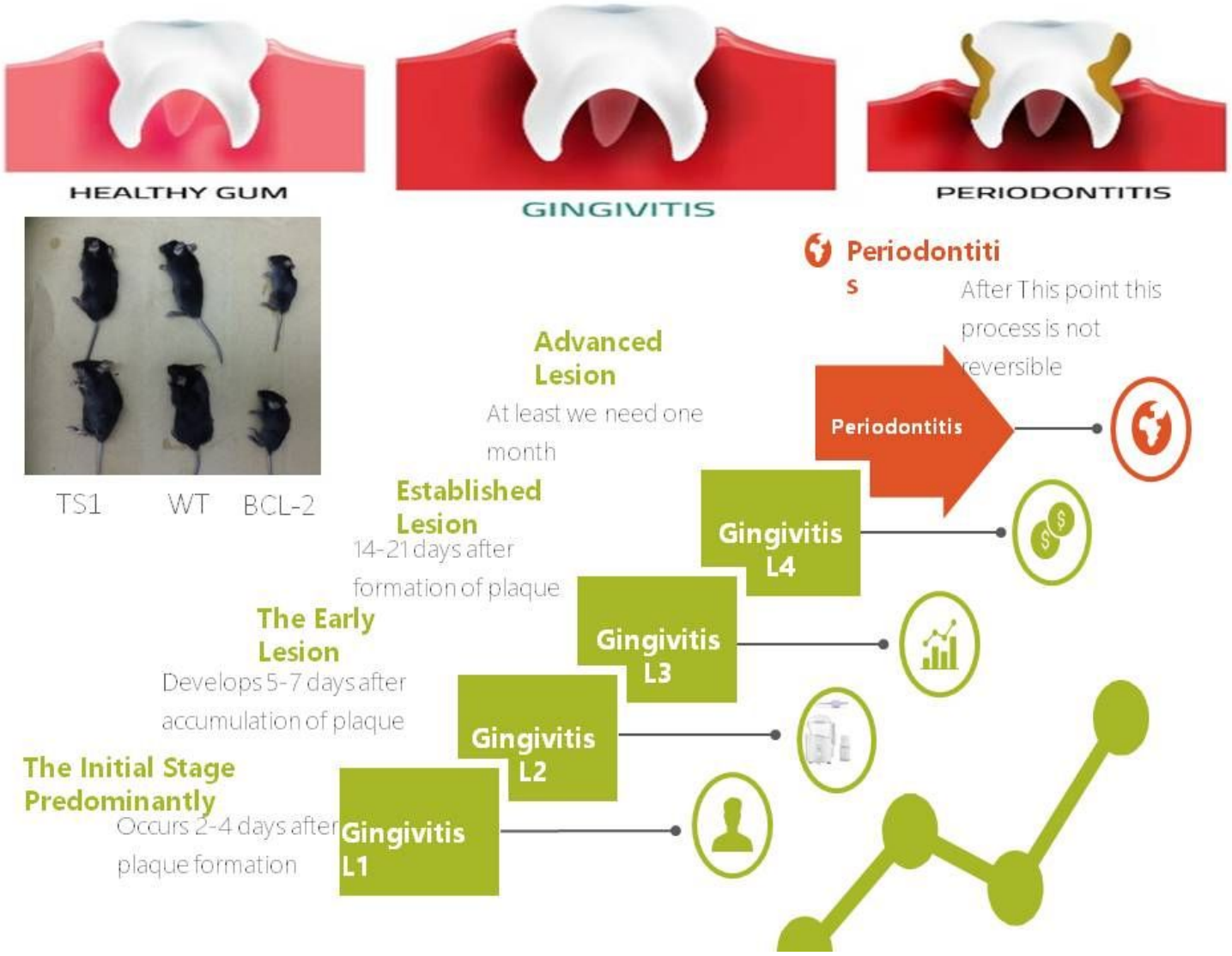

Figure 1

shows a summary of progression of gingivitis and potential mechanisms and actions on surrounding tissues. Bcl-2 knockout mice generally have lower weight that other type of mice.

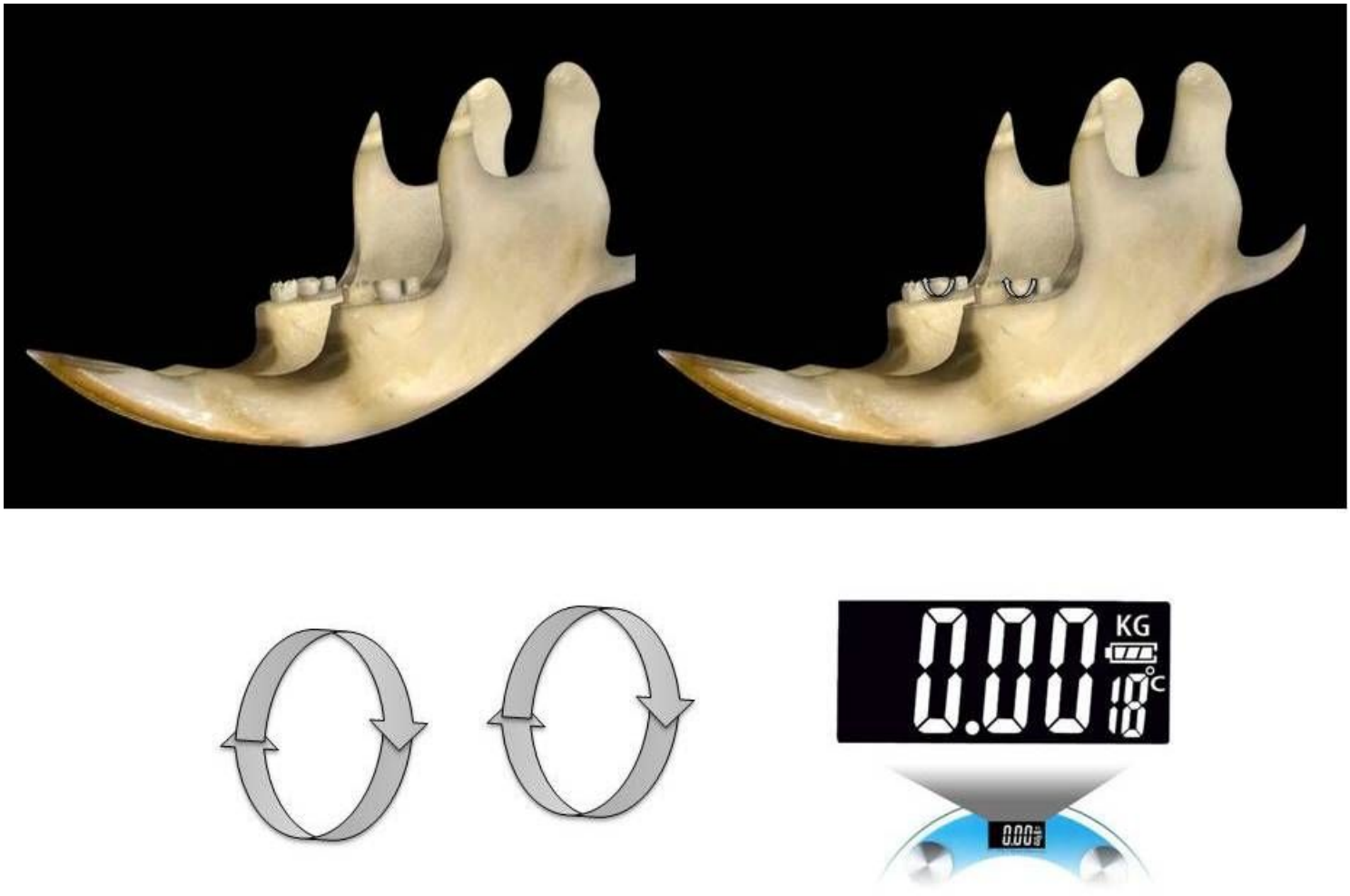

Figure 2

Steps for GCF Collection.

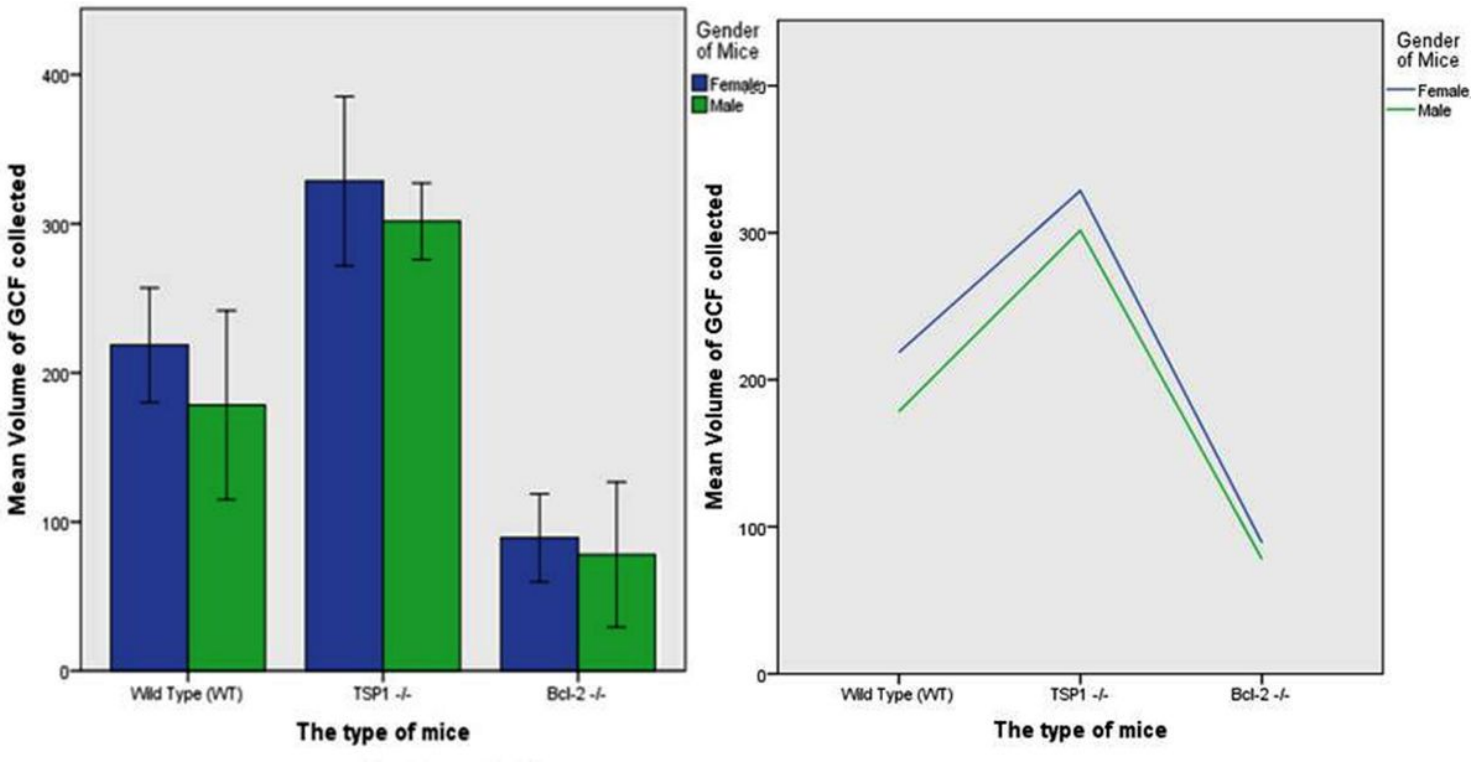

Figure 3

The mean and trend of GCF collection from Wild type (WT), TSP1 -/-, and Bcl-2 -/- , the average (mean) volume of GCF (μg).

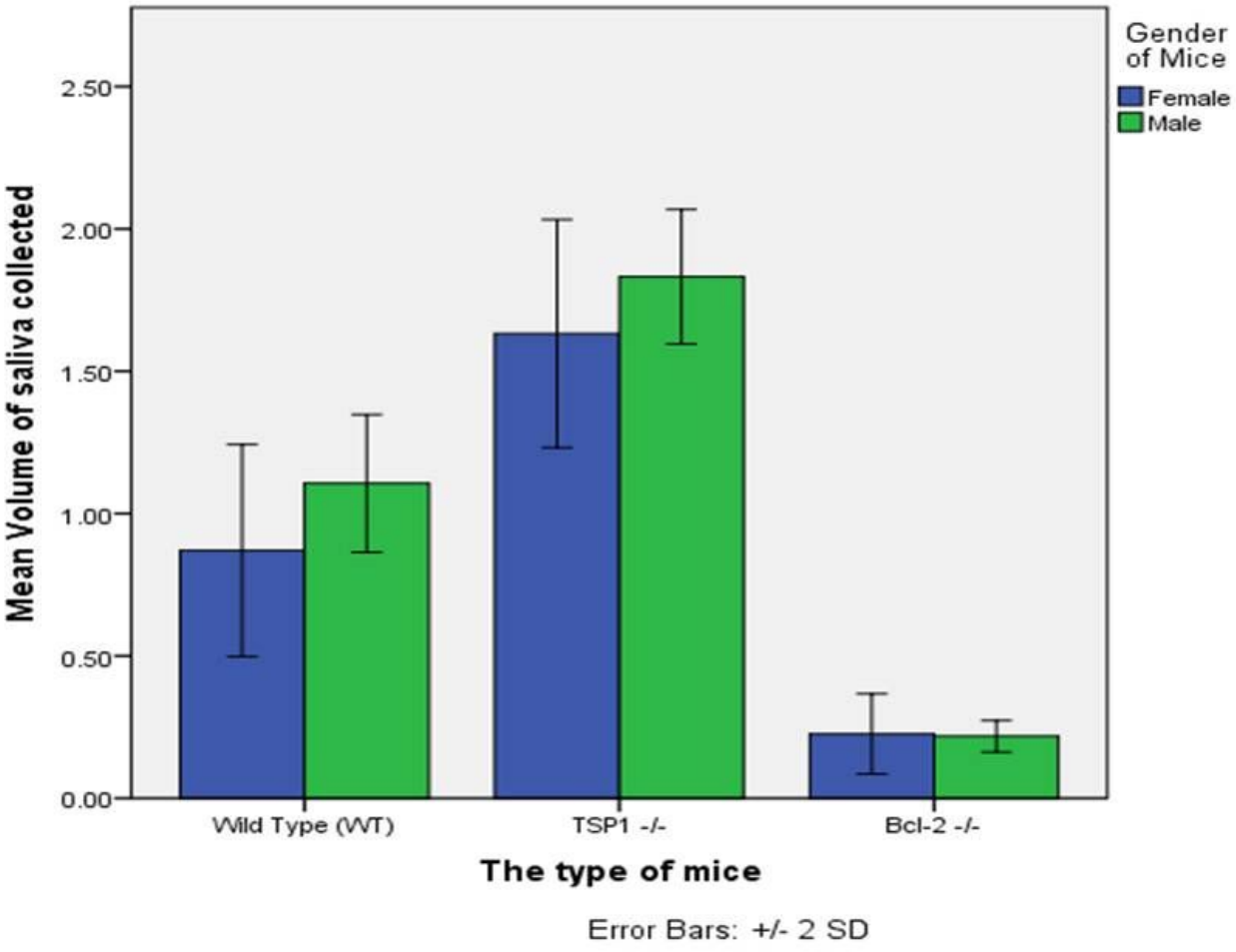

Figure 4

The Mean and SD for Saliva collected from Wild type (WT), TSP1 -/-, and Bcl-2 -/- (mL).

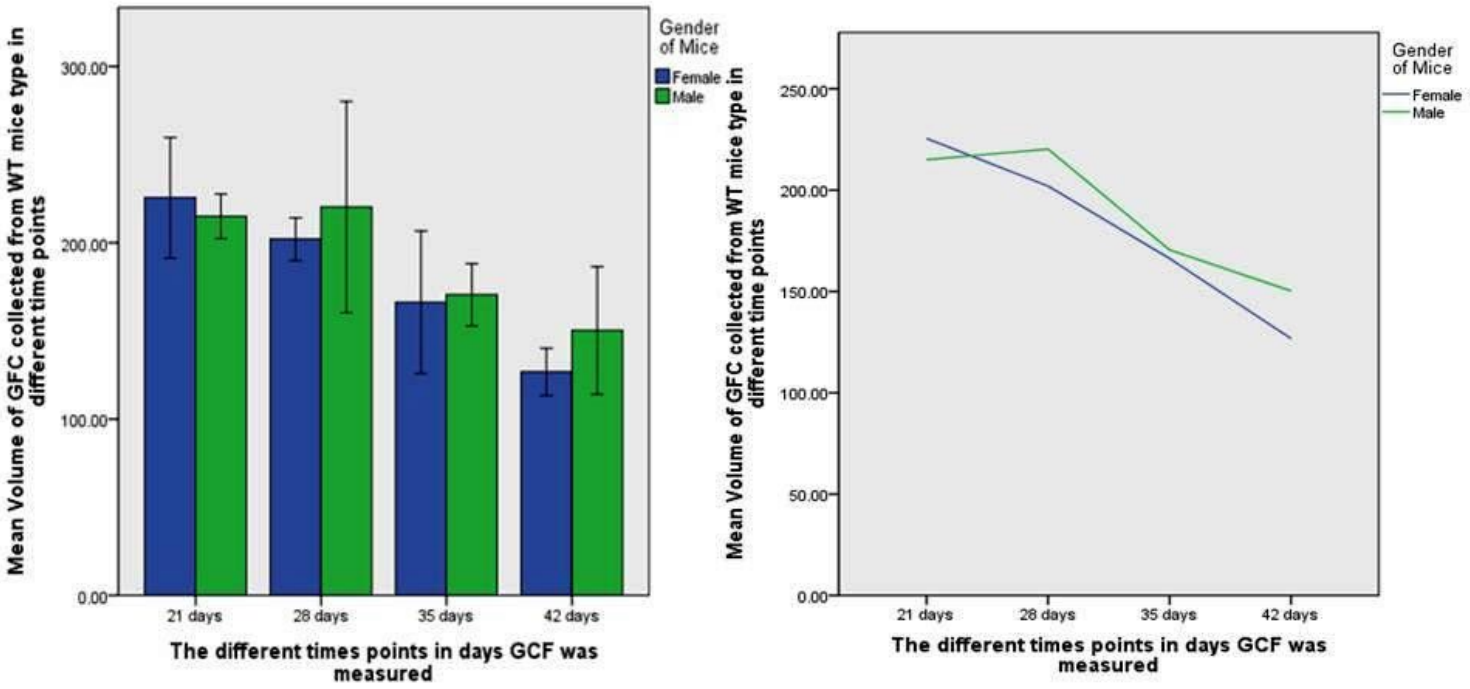

Figure 5

The Mean and SD for GCF volume (µg) collected from WT type mice in different time points (3, 4, 5, and 6 weeks).