Cumhuriyet Dental Journal, 25(4): 302-308, 2022
DOI: https://doi.org/10.7126/cumudj. 1100449

Cumhuriyet Dental Journal

| cdj.cumhuriyet.edu.tr | Founded: 1998 | Available online, ISSN: 1302-5805 e-ISSN: 2146-2852 | Publisher: Sivas Cumhuriyet Üniversitesi

Salivary Leptin Levels in Children with Early Childhood Caries – An Interventional Study

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Research Article

History

Received: 08/04/2022
Accepted: 14/12/2022

ABSTRACT

Objectives: *To evaluate the role of salivary leptin, an adipocytokine as a non-invasive inflammatory biomarker in healthy children and in children affected by Early Childhood Caries (ECC).*

Materials and Methods *A total of 60 children between the age of (3-5) years were selected for the study. The study subjects were assigned into three groups as control (Group 1), mild to moderate ECC (Group 2) and severe ECC (Group 3). Preoperative Saliva samples were collected from all subjects and repeated after 2 months following rehabilitative intervention. Levels of salivary leptin was determined using Enzyme-Linked Immunosorbent Assay (ELISA).*

Results: *Levels of salivary leptin were significantly associated with severity of ECC. The intragroup comparison of pre and post treatment levels of salivary leptin showed significant reductions in both mild to moderate ECC and severe ECC groups following caries control. Inter group evaluation between mild to moderate ECC and severe ECC, post treatment showed statistically significant decline of leptin levels in comparison to baseline values.*

Conclusions: *Salivary leptin has a potential to be recognized as a reliable future prognostic and diagnostic inflammatory marker in children with ECC.*

Keywords: *Salivary Leptin, Biomarker, Saliva, ELISA, Early Childhood Caries.*

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How to Cite: Mokashi PR, Bhandary S. (2022) Salivary Leptin Levels in Children with Early Childhood Caries – An Interventional Study, Cumhuriyet Dental Journal, 25(4): 302-308.

Introduction

The American Association of Pediatric Dentistry (AAPD) has described Early Childhood Caries (ECC) as presence of one or more cavitated, non cavitated lesions, any restored tooth surface or tooth/teeth missing due to caries in children belonging to an age of or younger than seventy one months.[1] The detection of dental caries has been restricted to visual and radiographic aids.

Dental caries is an inflammatory response of dental pulp which can be put forth as the aggregation of cells which are inflammatory in nature thereby provoking the secretion of defence cells in the host such as Interleukin (IL-1, IL-6, IL-8) group of cells and the Tumour Necrosis Factor cells (TNF).[3]

Leptin is a polypeptide measuring 16 Kilodaltons (KDa) and is a non-glycosylated hormone.[4] Leptin performs a wide range of biologic functions which include homeostasis of lymphoid organs, osteogenesis, immune responses, and haematopoiesis and antilipemic effects.[5] Leptin is involved in the inflammatory responses such as induction of acute phase protein synthesis and stimulation of macrophages and /or T cells.[6] Leptin along with its receptors are structurally and functionally similar to the groups belonging to Interleukin family (IL-1, IL-6, IL-8).[7] Leptin is seen in chronic periapical lesions due to infection of dental pulp usually as a sequel to dental caries, and recently gingiva was also considered as a cellular source of leptin which is found in the gingival crevicular fluid of both, healthy and inflamed gingiva.[10-11] Recently, it has been documented that leptin has direct consequences on the pulpal stem cells, thereby being a chief regulator of the cells of mesenchymal somatic origin.[11] Pulpitis is signalized by the aggregation of cells such as dendritic cells, neutrophils, macrophages and the lymphocytes.[6] Molecules like leptin which are at an interface between metabolism and immunity reflect the abnormal immune responses. Development of latest therapeutic approaches for anti-leptin therapy are taking pace thus the blockade of leptin with antagonists could inhibit the activity of bioavailable leptin and reduce its pro-inflammatory effects.[8-9]

Early childhood caries (ECC) is a multifactorial disease and has been an enigma to researchers since past decades. There has been a correlation between ECC and dietary factors.[1-3] Thus, leptin bridges the gap between nutritional status and immune competence, serving as a primary regulator of the two.[6] However, the role of leptin in ECC remains unclear and this study could be an effort towards validating this molecule as an easily accessible and prognostic marker in clinical settings in future; hence the inevitability to perform this study. Hence the study was conducted with the objective to assess and quantify salivary leptin levels in healthy children and in children with Mild to

Moderate and Severe ECC, (S-ECC) pre and post treatment of carious lesions.

Materıals And Methods

The study was conducted in the Department of Paediatric and Preventive Dentistry in collaboration with Department of Biochemistry at the host University. The permission of the Institutional Ethical Committee was obtained prior to the study. Informed consent was taken from the parents of the children participating in the study.

Sample size distribution

60 children within the age group of 3-5 years visiting the Department of Pediatric and Preventive Dentistry at the parent institute were selected for the study. The children were divided into 3 groups of 20 each:

Group 1 – Caries free children as controls.

Group 2 – Children with mild to moderate early childhood caries (ECC) (1 or more cavitated, filled smooth surfaces in primary maxillary anterior teeth or dmfs score >4)

Group 3 – Children with severe early childhood caries (S-ECC) (1 or more cavitated, missing (due to caries), or filled smooth surfaces in primary maxillary anterior teeth or a decayed, missing, or filled score of ≥4 (age 3), ≥5 (age 4), or ≥6 (age 5) surfaces constitutes S-ECC.)[12]

The inclusion criteria comprised of: (1) Children between the age group of 3-5 years who were willing to participate in the study. (2) Children affected by mild to moderate ECC without pulpal involvement. (3) Children affected by severe ECC with not more than 3-4 pulpally involved carious lesions. (4) Healthy children with no dental caries belonging to the same age group and who are willing to participate in the study. The exclusion criteria comprised of: (1) Children who are medically compromised and with special healthcare needs. (2) Uncooperative children (3) Children who were on antibiotics, non-steroidal anti-inflammatory drugs in past 3 months which may curb the inflammatory expression cells. (4) Patients presenting with generalized gingival inflammation.

A thorough dental examination was carried out on all children using WHO Oral Health Assessment Form for children 2013 and the oral findings were recorded for each patient and a profile was maintained for ease of follow up till completion of the study. Baseline salivary samples were collected from all the three groups.

Saliva sample collection

Saliva samples were collected from the children in Group 1, Group 2 and Group 3. All samples of unstimulated saliva were collected within a 10 minutes period between 9 am and 11 am to minimize any possible effect of diurnal variation.[3] Saliva samples were collected by Draining method or the passive drooling method, in which the subjects was asked to sit up straight with the head bent down and mouth open to spit the saliva passively from the lower lip to drool approximately 1–2 millilitres (ml) of unstimulated whole resting saliva into the non- graduated sterile plastic test tube for 5 minutes.[13] Samples were then transported to Central Research Laboratory and centrifuged at 4000 revolutions per minute (rpm) for 15 minutes at 4ºC.The supernatants were stored at -70ºC. [13]

Rehabilitative intervention

The children from group 2 and group 3 were treated according to the requirement for caries control, which included advice on oral health education, diet counselling, oral prophylaxis, restorative procedures, topical fluoride application and pulp therapy. The patients in group 2 and group 3 were recalled after a period of 2 months and post caries control saliva sample was collected. The collected saliva for all the groups was analysed for salivary leptin levels by using salivary ELISA kit.

ELISA for determination of salivary levels of leptin

High-sensitivity commercially available Human Leptin Enzyme-Linked Immuno Sorbent Assay (ELISA) kit was used for determining the levels of leptin in saliva samples of the subjects.[14]

All 60 saliva samples (with the volumes 2 ml) collected by using Salivates in 10 minutes were used for this study after rinsing their mouths thoroughly with water.

Each supernatant was divided into three aliquots and stored at−70 C until analysis. All samples were stored without adding any preservatives (only overnight wait).[14] Each well was filled with a wash buffer of 400 microliters (µL) and then the solution was pulled out using a squirt bottle, manifold dispenser, or auto washer. The plate was then blocked by adding 300 µL of Block Buffer to each well. This was then incubated at room temperature for 1-2 hours. Leptin conjugate reagent was added in the standard and the testing wells and covered with a plate sealer followed by incubation at room temperature for 1 hour. Optical Density of every well was assessed promptly using a microplate reader set to 450 nanometers (nm). The salivary leptin levels were then calibrated in ng/ml and tabulated. [14]

Statistical Analysis

Data obtained was compiled on a MS Office Excel Sheet (v 2019, Microsoft Redmond Campus, Redmond, Washington, United States). Data was subjected to statistical analysis using Statistical package for social sciences (SPSS v 26.0, IBM). Inter group comparison (>2 groups) was done using one way ANOVA followed by pair wise comparison using post hoc power analysis. Intra group comparison was done using paired t test (upto 2 observations) Comparison of frequencies of categories of variables with groups was done using chi square test. For all the statistical tests, $p<0.05$ was considered to be statistically significant, keeping α error at 5% and β error at 20%, thus giving a power to the study as 80%. * = statistically significant difference ($p<0.05$)

Results

The children who reported to the Department of Pediatric and Preventive Dentistry, within the age group of

3 to 5 years, were screened for the presence of dental caries. 20 children with no clinical signs of dental caries and who had good oral hygiene were recruited as the controls, by convenience sampling. In the mild to moderate ECC group (Group 2) and Severe ECC (S-ECC) group (Group 3), a total of 20 children each were recruited as study group before beginning the caries management protocol. Out of the total 60 children, 42 were females and 18 males. Upon completion of the study, a total of 3 patients in Group 2 and 1 patient in Group 3 who failed to appear and follow through with the study protocol were excluded in the mild to moderate ECC and S-ECC study groups. (Table 1)

The mean value of the salivary leptin levels estimated in the control group (Group1) of children was 7.63 nanogram per milliliter (ng/ml) (±0.529 ng/ml) Similarly, mean values for the pretreatment salivary leptin levels estimated in the mild to moderate ECC (Group2) and S-ECC (Group 3) study groups were 8.89 ng/ml (±0.613n/ml) and 11.81ng/ml (±0.531ng/ml), respectively. (Table 2) The post treatment (caries control) salivary leptin levels estimated in the mild to moderate ECC and S-ECC study groups were 8.06 ng/ml (±0.55ng/ml) and 7.51 ng/ml (±0.72ng/ml), respectively after a span of 2 months. (Table 3)

In an intra group comparison of Group 2, the mean of leptin levels before intervention and after intervention was 8.80 and 8.06 respectively. Using paired t test, it was derived that there was a statically significant difference seen for the values between the time intervals (p> 0.01) with higher values at T0. (Figure 1)

In an intra group comparison for Group 3, it was noted that the mean of leptin levels before intervention and after intervention was 11.78 and 7.51 respectively. Using paired t test, it was derived that there was a statically significant difference seen for the values between the time intervals (p> 0.01) with higher values at T0. (Figure 2)

In an intergroup comparison using one way ANOVA between Group 1, Group 2 and Group 3 before treatment (T0) was found to be highly statistically significant with a p value of 0.0 (Figure 2)

In an intergroup comparison using one way ANOVA between the two groups after 2 months post caries control (T2) was found to be highly statistically significant with a p value of 0.015. (Figure 3)

Discussion

The present study is the first to demonstrate the expression of salivary leptin in children with ECC. The leptin levels are typically elevated during infection and inflammation. Moreover, after exposure to inflammatory stimuli, the levels of circulating leptin and leptin expression in the adipose tissue increases. Thus, it appears that proinflammatory mediators such as TNF- α and IL-1, which upregulate leptin expression contributing in turn to the creation of a loop of acute phase reactants that influence each other in promoting the development of chronic inflammation.

One of the most interesting aspects that medical and dental scientific community research is the possible connection between oral inflammatory processes due to infection and systemic health.[15] Saliva serves as a medium to connect these two. The use of saliva as a diagnostic tool is limited because of lack of validation arising from brief knowledge on the understanding of biomolecules and their importance to disease etiology, combined with the insufficient high-sensitivity detection systems.[14]

ECC always commences with an initial stage; starting with white or brown spots, appearing along the gingival line. In the moderate stage, cavitation starts from the enamel reaching upto dentin and in the severe stage, the caries process progresses further into the pulpal tissue.[1-3] Therefore, our study groups were categorized as mild to moderate ECC (without pulpal involvement), and severe ECC (SECC) (with minimum of 4–6 pulpally involved teeth). We hoped that this grouping could provide an insight not only the variation of expression of leptin levels in relation to severity of carious lesions, but also the prognosis following caries control protocol.

Leptin is expressed in the serum of healthy individuals in the range of 5-18 nanograms per millilitre (ng/ml) and in the saliva in the range 1-10 nanograms per millilitre (ng/ml).[16] The release of local cytokines promotes expression of molecules implicated in mineralization.[10] The pleiotropic behaviour of leptin is reinforced by the widespread distribution of leptin receptor (LEPR).[17] LEPR shows structural similarity to the class I cytokine receptor family. According to the multifunctional role of leptin, this fully-active isoform of LEPR is expressed not only in the hypothalamus, where it takes part in energy homeostasis, but also is present on peripheral tissues as well as on hematopoietic cells and on all types of immune cells involved in both innate and adaptive immunity.[18-19]

Considering the caries control protocol, the time required for stabilization of oral biofilm following restorative procedures and for setting in of new routines of oral hygiene practices, a post treatment follow-up time period of 2 months was adopted. This is in accordance with observations made by Marsh PD, Van Der Hoeven JS *et al*., and Winnier JJ *et al.*[20]

In the present study, a dropout of 3 children from Group 2 and a dropout of 1 child from Group 3 was observed. Data was tabulated with the estimated salivary leptin levels in those patients who complied and completed the caries control protocol set out for the study. This observation of dropouts from our study is in line with previous literature where it has been documented that parental avoidance behavior and lack of understanding of the seriousness of the implications of dental caries on the general health and wellbeing of the child. Thus, it becomes important to establish caries preventive and control protocols to engage parents in community and self-directed awareness programs. These play an important role during the early years of a child's life, even before eruption of the first primary tooth. [21-22]

In healthy children, the range of salivary leptin levels was 7.11 to 8.32 ng/ml with a mean of 7.63 ng/ml. The normal salivary leptin levels ranges between 1–10 ng/mL, directly reflecting the amount of energy stored in the

adipose tissue, thus, obese individuals typically produce higher leptin than leaner individuals. Leptin levels can also be influenced by the taste perception of individuals. This wide range of salivary leptin can be due to its instability as a result of increased LEPRs in the oral cavity.[23]

Whereas, in children with mild to moderate ECC, the range of baseline mean salivary leptin levels was 7.8 to 9.76 ng/ml with a mean of 8.9 ng/ml. This could be because, in mild-moderate ECC there is a developing nidus of bacteria and this increasing bacterial load may contribute to the rise of the pro inflammatory cytokines. Hence, an increase from the normal range can be witnessed.[3,16]

In children with severe ECC, the range of baseline mean leptin levels at T0 was 11.08 to 12.57 nanograms (ng/ml) with a mean of 11.81ng/ml. This rise in levels is in accordance with various studies conducted by Menon MM *et al.*[4] 2020 and Vrinda Sharma *et al.*[3], 2012 on the role of inflammatory cytokines in S-ECC. S-ECC is associated with an increase in pulpal involvement thereby leading to a rise in the circulating inflammatory markers.[14] An in vitro study conducted by Martin Gonzalez et al i*n* 2013 to investigate the presence of leptin in healthy and inflamed human dental pulp tissue also concluded the elevated expression of leptin in the inflamed pulp tissue samples than in healthy tissues. This can be due to the role of leptin as an autocrine as well as paracrine pathway and therefore it may play a role in pulpal/periapical inflammatory and immune responses similar to that of the white adipose tissues.[16]

Thus, post caries control in mild to moderate ECC group, after a span of 2 months, the range of salivary leptin levels was observed to be 7.24 to 8.53 ng/ml with a mean of 8.069 ng/ml. This is in accordance with a study conducted by Menon et al in 2020 where they evaluated salivary IL-6 levels pre and post intervention and found a reduction in the IL-6 levels post intervention after 2 months.[4] This was as a result of reduction of bacterial colonies and inflammatory load post intervention of pulpitis.

In severe ECC group, the range of salivary leptin levels was observed to be 6.35 to 8.33 ng/ml with a mean of 7.51 ng/ml. As this group was associated with an increased pulpal inflammation, this decline in the levels of salivary leptin post caries control can be supported by the fact that leptin has direct consequences on dental pulp stem cells, and it has been reported that.[24] Leptin is associated with an increased expression of CC-chemokine ligand 20 (CCL20).[25] CCL20 expression is persuaded by stimulus with bacteria related to caries progression, invading deeply into the dentinal tubules, as well as by proinflammatory cytokines in the inflamed pulpal lesions.[26]

In a comparative evaluation between the baseline levels of healthy children and children with mild to moderate ECC, a statistically significant increase of levels was seen. While the baseline values also showed a statistically significant upsurge between mild to moderate ECC and children with severe ECC. Leptin regulates the immune response, both innate and adaptive responses, not only in normal but also in pathological conditions.[17] Consistent with this, the leptin levels are increased upon infectious and inflammatory stimuli such as lipopolysaccharides, turpentine, and cytokines.[16,27]

Similarly in a comparative assessment between children with mild to moderate ECC and children with severe ECC, a fall in the salivary leptin levels was seen post treatment as compared to the healthy children. A statistically significant fall in the levels was also witnessed between the mild to moderate ECC and severe ECC group, post caries control, thus stating that as the pulpal inflammation is controlled, there is a fall in the salivary leptin levels.

In comparison before and after intervention in children with mild to moderate ECC, the mean difference of salivary leptin levels was 0.73 ng/ml whereas in the children with severe ECC the mean difference was 4.26 ng/ml. Thus, a statistically significant difference in the levels of leptin was appreciated at the different time intervals with higher value at the baseline. As the amount of pulpal inflammation was higher and chronic in the children with severe ECC, a drastic rise and fall of the normal values were in accordance with the support of literature.[16,24]

The post treatment rehabilitative procedures helped in reducing the inflammatory and the bacterial load in the oral cavity. Cytokines are produced by the activation of monocytic macrophagic cells and act as mediators of infection, inflammation and immunological processes in defence to the bacterial irritation.[25] Pulpal symptoms are generally explained by increase in intra-pulpal pressure due to edema. The levels of salivary leptin could be correlated to the extent of inflammation and edema in the pulp, in addition to its role as a mediator of host response following tissue injury and infection. As salivary leptin is correlated to the immune defence mechanisms a period of 2 months was chosen for the inflammatory reactions to subside.

The leptin levels have decreased post caries control protocols in both the groups. Thus, indicating that undergoing caries control treatment helps to reduce their inflammatory status. An increased nidus of inflammation in the oral cavity may lead to hindrance in the immunologic, metabolic, physical and emotional development of the child.[2-3] Thus, it is important to diagnose and treat the pulpal inflammation but also is important to counsel the parents. ECC is a pathologic condition of multifactorial nature. Hence, along with the bacterial load, various dietary and oral hygiene maintenance practices also need to be incorporated to get a desirable reduction of the inflammation of the oral cavity.[3,13] Moreover, the diagnostic and prognostic significance for ECC has not been explored till date. Thus, from the present study we can conclude that salivary leptin holds a true potential to be a future prognostic and diagnostic inflammatory marker. Also, a drastic fall in the levels of leptin can be appreciated post rehabilitative intervention of ECC, stating its role in the immune and inflammatory reactions.

Conclusions

Leptin bridges the gap between nutritional status and immune competence, serving as a primary regulator of

the two. Salivary leptin holds a true potential to be a future prognostic and diagnostic inflammatory marker. Also, a drastic fall in the levels of leptin can be appreciated post rehabilitative intervention of ECC, stating its role in the immune and inflammatory reactions and in determining the status of pulp in such patients.

This study could be an effort towards validating it as a non-invasive accessible and prognostic biomarker in clinical settings in future.

Limitations and Recommendations

On the basis of the observations in our study, we would like to propose the following recommendations for future research:

1. Small sample size and shorter duration of follow up, hence requires a more exhaustive study with a larger sample size and longer follow ups in establishing the role of leptin in assessing the pulpal status of patients with ECC.
2. Further studies may be recommended for estimation of salivary leptin to establish a reference range in children. This will validate the results of our pioneer study.
3. The role and mechanism of salivary adiponectin hormone family in oral defence processes needs to be further understood in healthy and pathological conditions.
4. More longitudinal studies can be done with salivary leptin levels as a good biomarker for comparing the efficacy of various caries control treatment protocols.

Conflict of Interest

No Conflicts of Interest

Source Of Funding

Nil

Table 1: Summary of Sample recruitment and dropouts in the study.

Group	Sample size estimated (participants required)	Participants recruited (pre-treatment saliva collected) ml	Participants completed (post- treatment saliva collected) ml
Control (No ECC)	20	20	-
Mild to Moderate ECC (ECC)	20	20	17 (-3)
Severe ECC (S-ECC)	20	20	19 (-1)

Table 2: Numerical Summary for Pre-treatment salivary leptin levels in children from Group 1, Group 2 and Group 3 (ng/ml)

	Group	N	Mean (ng/ml)	Standard deviation
1.	Control (No ECC)	20	7.63	± .529
2.	Mild to Moderate ECC	20	8.89	± .613
3.	Severe ECC (S-ECC)	20	11.81	± .531

Table 3: Numerical Summary for Post-treatment salivary leptin levels (ng/ml):

	Group	N	Mean (ng/ml)	Standard Deviation
1.	Control (No ECC)	-	-	-
2.	Mild to Moderate ECC	17	8.06	± 0.55
3.	Severe ECC (S-ECC)	19	7.51	± 0.72

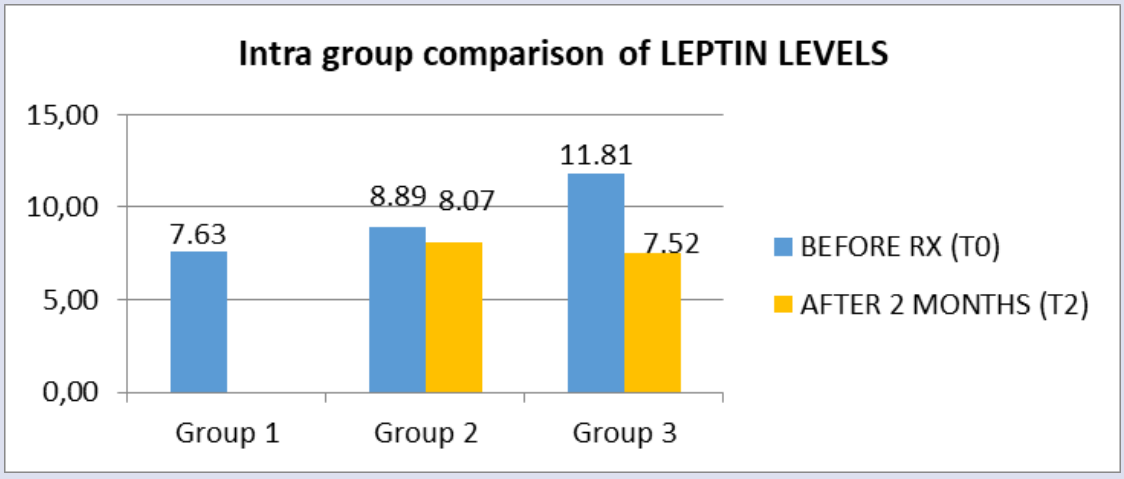

Figure 1. Intra group comparison of leptin levels between the control, pre-treatment and post treatment values of the two study groups

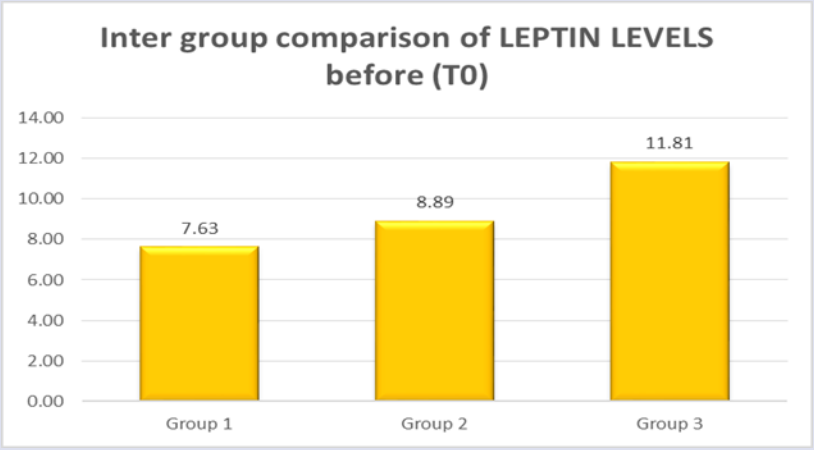

Figure 2. Intergroup comparison of leptin levels before treatment (T0)

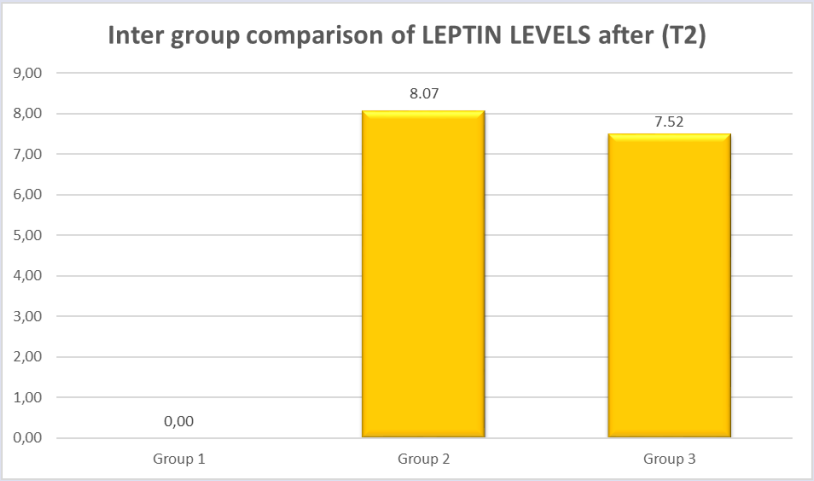

Figure 3. Inter group comparison of leptin levels after treatment (T2) Intergroup comparison between the 3 groups before treatment (T0) was found to be highly statistically significant with a p value of

0.0 Inter group comparison between the 3 groups after 2 months post caries control (T2) was found to be highly statistically significant with a p value of 0.015.

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