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Histopathological and Morphometrical Studies on the Effect of Ozonized Water on the Periodontium of Rats

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Abstract: Topical application of ozone has been used in management of gingivitis and periodontitis with no reliable data obtained regarding the direct effect of ozone on the periodontal tissues. The idea of the present work is to study the effect of local ozonized water on the periodontal tissues of rats. In this study, 120 male rats were used and the rats were divided into 4 main groups each 30: group I: this group did not receive any ozone application. Group II received one ozone application per week for 1, 3 and 6 months. Group III received two ozone applications per week for 1, 3 and 6 months. Group IV received three ozone applications per week for 1, 3 and 6 months. Each ten rats from each group were sacrificed after 1, 3 and 6 months. Bone cells count and fibroblasts number were done using the image analysis system in the cervical, middle and apical parts of the root. Bone cells count and fibroblasts showed a significant increase in groups II, III and IV which received one, two or three ozone applications for one or three month as compared with the control group. On the other hand using ozone three times per week for six months showed starting of the decrease in number of bone cells. a significant increase in number of fibroblast cells as compared with the controls in the groups, which received one, two, or three ozone applications for one or three months. In group which received three ozone applications for three and six months there was a starting of decrease in number of fibroblasts. The histopathological examination of the samples revealed presence of normal histological structure of gingival epithelium in the groups, which received one or two ozone applications for one month. The other groups showed some structural alterations in the histopathological samples examined. In the periodontal ligament, there was increased number of fibroblasts. As regarding the bone cells there was a noticed increased numbers of bone cells along the different groups but also for a limited time period. Ozone should be carefully ued with special attention to the dose and time period of application to produce a beneficial effect without reaching any adverse side effects. It was recommended to use ozone in local applications on the periodontal tissues once a week for a period not exceeding one month.

Key words: Ozonized water · Bone cells · Fibroblast cells · Histopathological · Gingival epithelium

INTRODUCTION

Periodontal disease, a group of inflammatory disorders, is considered to represent bacterial infection in which certain bacteria appear to play a significant role in inducing and maintaining the inflammatory process [1, 2]. Periodontitis, one category of the recent classification of periodontal diseases is the most common type of periodontal diseases that result from extension of the inflammatory process initiated in the gingiva to the supporting periodontal tissues [3, 4].Various treatment modalities have been utilized aiming at optimal plaque control as a main causative factor in the etiology of periodontal disease and also in aiming of promoting healing and repair of damaged periodontal tissues. Oral hygiene instructions with plaque control evaluation were done. Scaling and root planning together with removal of plaque-retention factors were also performed together with the use of various antiseptic gels, dentifrices and mouth rinses in order to reduce the pathogenic bacterial flora in the oral cavity to aid healing. Also different chemical plaque control agents in addition to using of anti-adhesive agents and antimicrobial agents either systemically or locally have been used with some success

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[5-7].The topical application of ozone has long been used but the literature regarding the direct effect of ozone on the oral tissues is still obscure [8].

Therefore, the objective of this study is to detect any histopathological changes that may occur due to the local application of ozonized water as subgingival irrigation on the periodontium of normal rats.

MATERIALS AND METHODS

Experimental Animals: One hundred and twenty male rats (100-150 g) obtained from the Animal House Lab, National Research Centre, Cairo, Egypt were used in this study. Rats were divided into four groups, 30 rats each: **Group I:** served as control group. **Group II:** received ozone once weekly. **Group III:** received ozone twice weekly. **Group IV:** received ozone. Ozone application was made as a local subgingival irrigation of the periodontal tissues 1, 3, or 6 months. At the end of each period, ten rats of all groups were sacrificed after 1, 3 and 6 months after ozone application The two incisors of the lower jaw and its supporting structures were excised and were preserved in 10 % saline formalin.. The used dose in rats is 36 μg/L according to Paget and Barnes [9]. Periodontal pockets were irrigated with 2.7 ml of ozonized water for 11 seconds per application.

Histopathological and Histochemical Examination: Formalin fixation followed by 10% ethylene diamine tetra acetic acid (EDTA) fixation for 6-8 weeks for decalcification. Paraffin sections (5μm) were prepared and stained with hematoxylin and eosin stain for histopathological and morphometric studies. Other sections were stained with Feulgen stain for DNA analysis.

Morphometeric Measurements: The morphometeric measurements were done using Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd, Cambridge, England). The morphometeric measurements were carried out with optical magnification of 200 X on hematoxylin and eosin stained slides. Fibroblasts and bone cells were counted in the cervical, middle and apical portion of the root incisor.

RESULTS

Bone Cells Count: Table 1 shows the bone cells count of the cervical, middle and apical parts of alveolar bone of rats received different numbers of applications of ozone (1, 2 and 3/week) for different periods (1, 3 and 6 months).

Table 1: Bone cells count in different positions of the alveolar bone after different applications of ozone

Applications	Time	Cervical	Middle	Apical
Group 1	1	43.2±6.1	44.6±5.1	44.4±4.4
	3	44.6±6.8	46.6±5.6	45.3±4.1
	6	45.7±6.5	47.9±5.8	46.7±4.9
Group 2	1	52.7±5.7	54.5±4.3	55.2±4.9
	3	63.1±6.4	63.5±3.3	64.8±5.8
	6	64.3±4.5	65.5±6.1	64.5±4.0
Group 3	1	55.9±3.5	58.1±4.3	55.7±4.2
	3	63.7±5.7	65.1±4.0	65.4±4.3
	6	64.8±5.2	65.8±4.7	66.4± 4.8
Group 4	1	64.5±4.4	63.9±4.3	65.2±4.4
	3	65.8±4.7	66.4±6.4	66.2±4.8
	6	62.1±5.0	62.5±4.9	62.7±3.9

Data presented as Mean±SD, time /month, P-values <0.05 are considered significant.

Table 2: Fibroblast cells count in different positions of the alveolar bone after different applications of ozone

Applications	Time	Cervical	Middle	Apical
Group 1	1	98.9±2.3	98.2±3.4	97.1±3.8
	3	97.7±5.4	97.4±4.0	96.9±2.1
	6	99.1±6.1	100.3±4.9	98.3±4.5
Group 2	1	111.9±4.7	112.8±3.2	110.8±4.1
	3	118.3±4.2	119.1±3.7	119.2±5.0
	6	124.6±1.8	127.6±1.8	127.3±1.5
Group 3	1	124.3±4.9	129.9±4.8	129.8±4.6
	3	129.6±2.8	132.1±2.6	133.5±3.2
	6	133.2±2.1	136.4±2.3	136.3±2.8
Group 4	1	127.2±4.5	131.3±4.9	135.9±3.9
	3	124.6±1.4	129.6±1.4	132.6±1.6
	6	121.6±6.5	127.1±4.6	130.8±6.0

Data presented as Mean±SD, time /month P-values <0.05 are considered significant.

The data showed a significant difference in the number of bone cells of the three measured positions as compared to the control group ($P = 0.012$). The interaction between the number of applications and the time period showed significant change in the number of bone cells as compared with the different control groups ($P = 0.001$). The number of bone cells of the group that received one and two ozone application/week for different periods showed no significant difference in the cervical, middle and apical parts between the 3rd and 6th months of ozone application. However, there is a significant difference between the 1st month of ozone application and both the 3rd and 6th months of the application.

The counting the number of bone cells of the group which received three ozone applications for the different periods showed that there was no significant difference in the number of bone cells between the 1st and 3rd month at the cervical part. There was a significant difference

between 3rd and 6th month. On the other hand, there was a significant difference between the number of bone cells in the middle part between the 6th month and 3rd month. There was no significant difference between the 1st and both the 3rd and 6th months of ozone application. On looking to the number of bone cells at the apical part, the results showed no significant difference between the 1st and both 3rd and 6 thmonths periods. There was a significant difference between 3rd and 6th months application.

Fibroblasts Number: Table 2 shows the fibroblast cells number of the three different positions of periodontal ligament [cervical, middle and apical parts] after different numbers of applications (1, 2 and 3/week) for the different periods (1, 3 and 6 months). The results showed a significant difference in the number of fibroblast cells in the three measured positions (cervical, middle and apical) as compared to the control group. Also the interaction between the number of applications and the time period showed significant change in the number of fibroblast cells as compared with the different control groups ($P < 0.01$).The results obtained from the measurements of fibroblast cells for the group that received one ozone application for different periods indicated a significant difference could be found between the 1st, 3rd and 6th months in the cervical, middle and apical positions ($P < 0.001$).On looking to the results obtained from the measurements of fibroblast cells of the group, which received two ozone applications for different periods a significant difference can be seen between the 1st, 3rd and 6th months in the cervical part. In the middle portion, there was no significant difference between the 1st and 3rd months, but in the 6th month, there was a significant difference detected. Apically there was no significant difference between the 1stand 3rd months of ozone application, $P=0.543$ and no significant difference between the 3rd and 6th months of ozone application, $P=0.323$. There was a significant difference obtained between the 1st and 6th month with $P=0.011$.

The results obtained from the measurements of fibroblast cells of the group, which received three ozone applications for different periods showed no significant difference could be found between the 1st and 3rd months in the cervical, middle and apical positions.

Histopathological Results: The gingiva of the control rats showed the normal histological features of the surface epithelium and lamina propria. The surface epithelium was of the keratinized stratified squamous type characterized by numerous folding towards the underlying connective tissue of the lamina propria forming numerous slender long epithelial ridges. The epithelium showed four categories of cells, the basal cell layer formed of a single row of low columnar cells resting on the basement membrane, prickle cell layer formed of several rows of polyhedral cells. Then the granular cell layer formed of 2-3 rows of flattened granular cells and then the most superficial keratinous layer with its eosinophilic amorphous appearance. The lamina propria showed the two indistinct layers, the papillary layer characterized by having thin collagen fibers and small sized blood capillaries and the reticular layer having coarse collagen fibers and larger blood vessels. Few chronic inflammatory cells were sometimes encountered. The cementum appears as homogenous eosinophilic linearly area. The periodontal ligament consists of numerous thick dense collagen fibers arranged in bundles. The alveolar bone appeared as homogeneous eosinophilic materials with numerous lacunae of osteoblasts as shown in Fig. 1.

Figure 2 shows the gingival of the group, which received one ozone application for one month showing almost the same histological features of the surface epithelium of the gingiva of the control rats. The lamina propria showed the two indistinct layers, the papillary layer, which contains thin collagen fibers and the reticular layer containing coarse fibers and large blood vessels with numerous chronic inflammatory cells. The cementum also appeared as homogenous eosinophilic line and the periodontal ligament had the same histological features as the control group with slight increase in the number of fibroblasts. There is slight increase in the number of osteoblasts lacunae of alveolar bone. The gingiva of the group, which received two ozone applications for one month, showed almost the same histological features of the surface epithelium of the control group. The underlying lamina propria revealed increased number of chronic inflammatory cells with slight degeneration of the collagen fibers. There are also numerous dilated blood vessels. No change in cementum was observed. The periodontal ligament showed slight increase in condensation of collagen fibers arranged in bundles with increased number of fibroblasts. There are numerous lacunae of osteoblasts as shown in Fig. 3.

The histological examination of the gingiva of rats, which received three ozone applications for one month, revealed some structural changes in the surface epithelium presented as slight hyperkeratosis with the epithelial ridges appearing broad with some flattening. The underlying lamina propria showed increased degeneration of collagen fibers with few fibroblasts present. The periodontal ligament was thickened with an increase in the number of fibroblasts (Fig. 4).

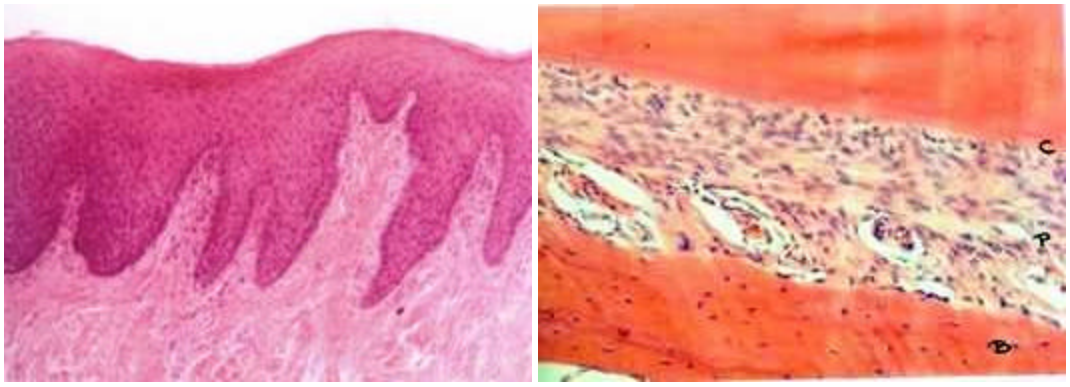

Fig.1: [a] shows the normal histological features of the gingiva of control rat. [b] shows normal cementum [C], periodontal ligament [P] and alveolar bone [B] of control rat [H& E X 200].

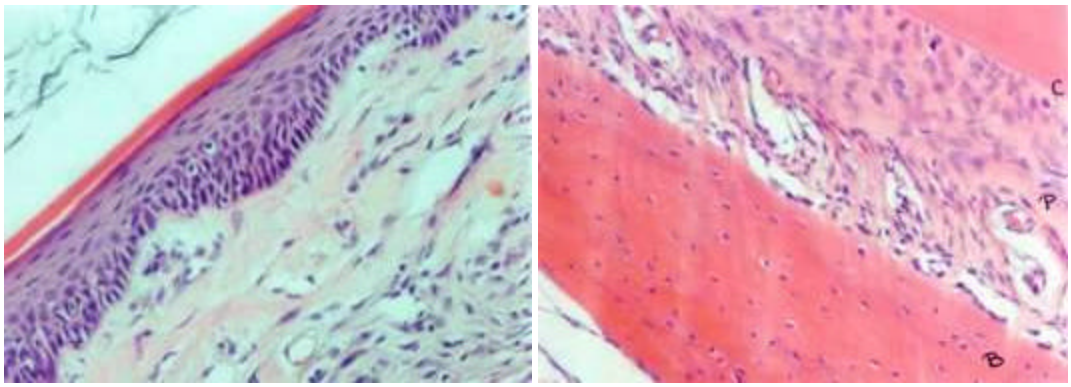

Fig. 2: [a] the gingiva of rat, which received one ozone application for one month showing normal histological features of the surface epithelium with the slight increase in the number of inflammatory cells. [b] shows cementum [C], periodontal ligament [P], and alveolar bone [B] showing mild increase in the number of fibroblasts [H & E X 200]

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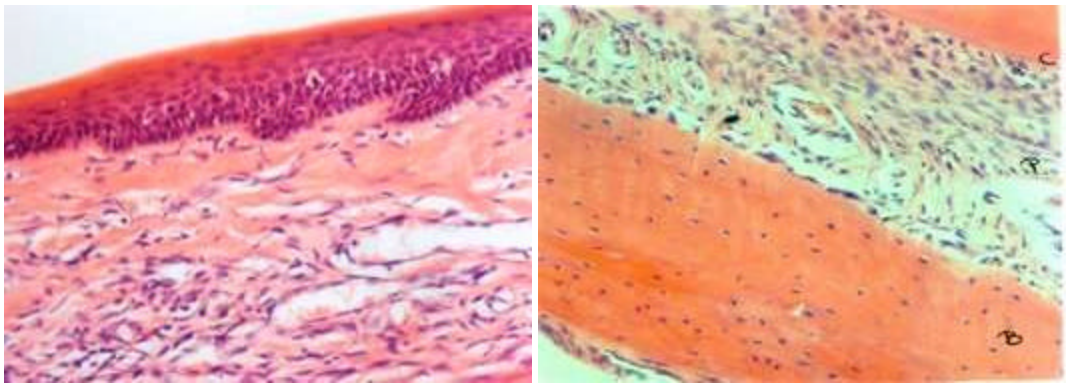

Fig. 3: [a] the gingiva of rat which received two ozone applications for one month showing degeneration of the collagen fibers, increased number of chronic inflammatory cells and numerous dilated blood vessels. [b]: cementum [C], periodontal ligament [P] and alveolar bone [B] showing increased number of fibroblasts [H & E X 200]

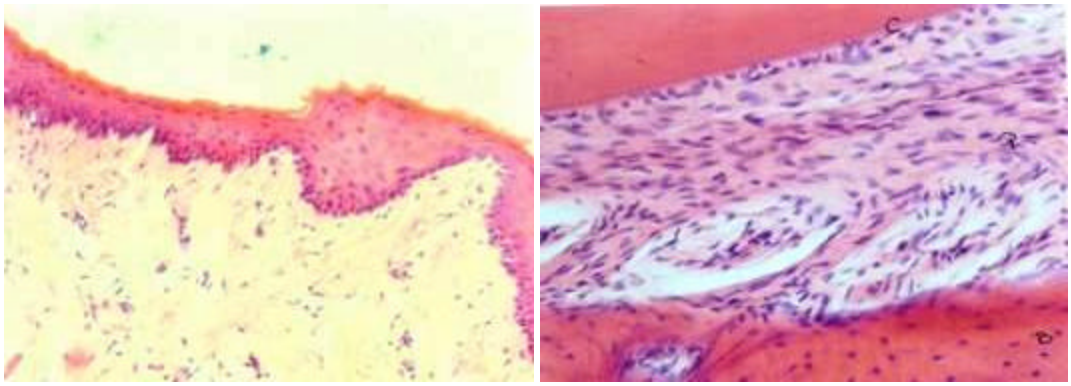

Fig 4: [a] The gingiva of rat, which received three ozone applications for one month showing slight hyperkeratosis with dissociation of collagen fibers and broad & flat epithelial ridges, [b] cementum [C], thickened periodontal ligament [P] with increased number of fibroblasts and alveolar bone[B][H&E X 200]

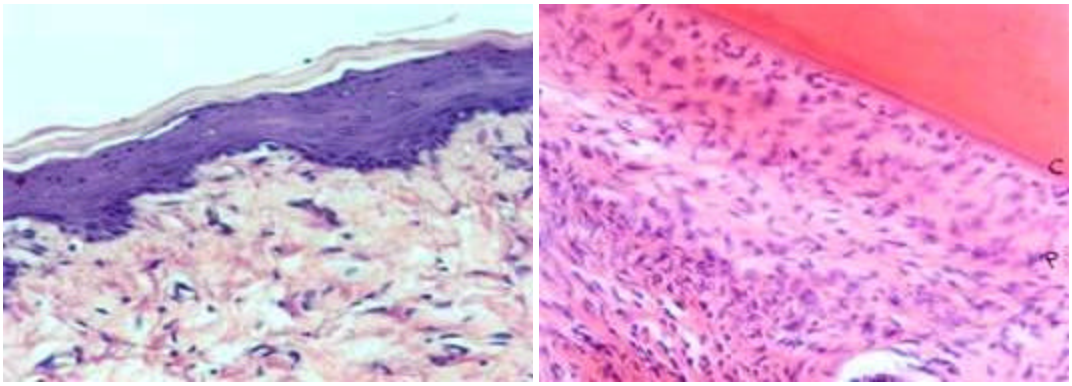

Fig. 5: [a] the gingiva of rat, which received one ozone application for three months showing broad epithelial ridges and mild increase in chronic inflammatory cells. [b] cementum [C] and thickened periodontal ligament [P] with increased number of fibroblasts were seen [H&E X 200]

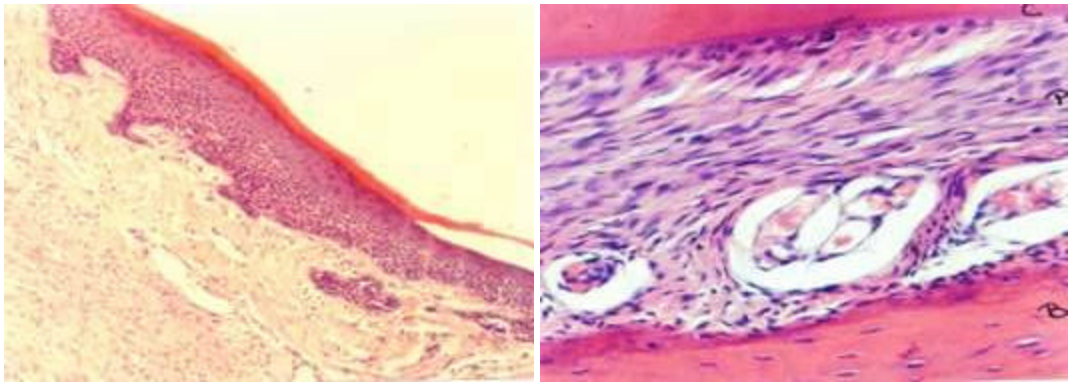

Fig. 6: [a] the gingiva of rat, which received two ozone applications for three months showing hyperkeratosis, epithelial hyperplasia and loss of the characteristic pattern of epithelial ridges with the mild increase in chronic inflammatory cells. [b] shows cementum [C], thickened periodontal ligament [P] with increased number of fibroblasts in the alveolar bone [B] [H & E X 200]

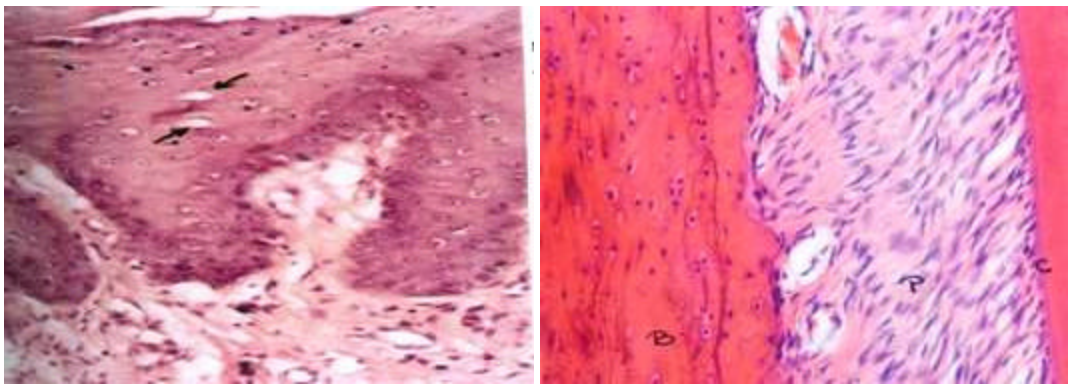

Fig. 7: [a] the gingiva of rat that received three ozone applications for three months showing hyperkeratosis degenerative intracyotplasmic vacuolization in the prickle cells layer. The underlying lamina propria revealed some degree of dissociation and degeneration of collagen fibers; [b] shows alveolar bone [B], periodontal ligament [P] and cementum [C] with mild increase in the number of fibroblasts & osteoblasts [H & E x 200]

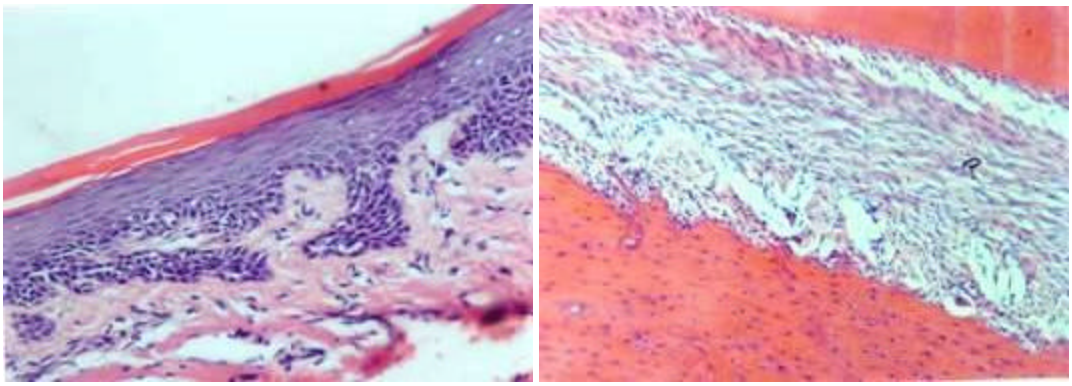

Fig. 8: [a] the gingiva of rat, which received one ozone application for 6 months showing hyperkeratosis and loss of the characteristic pattern of the epithelial ridges.[b]shows dissociation of the collagen fibers of the periodontal ligament [P] [H & E x100]

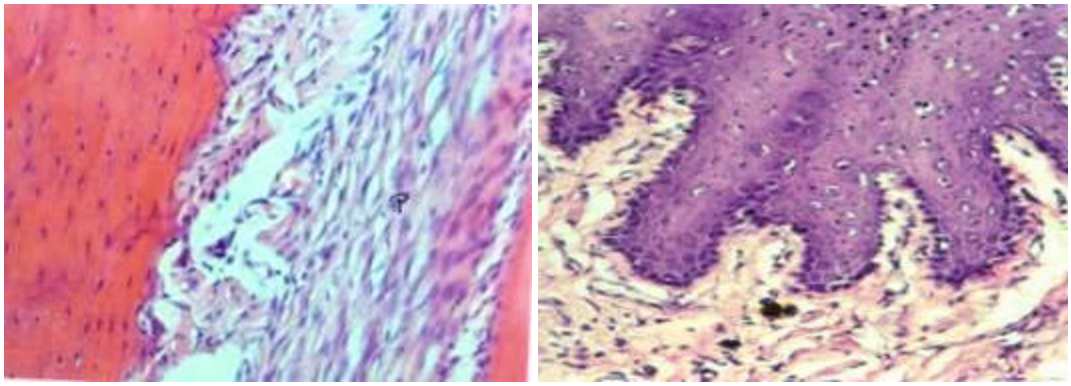

Fig. 9: [a] the gingiva of rat, which received two ozone applications for six months showing hyperplasia and degeneration of the collagen fibers of the lamina propria.[b]shows dissociation of the collagen fibers of the periodontal ligament [P] [H & E X 200]

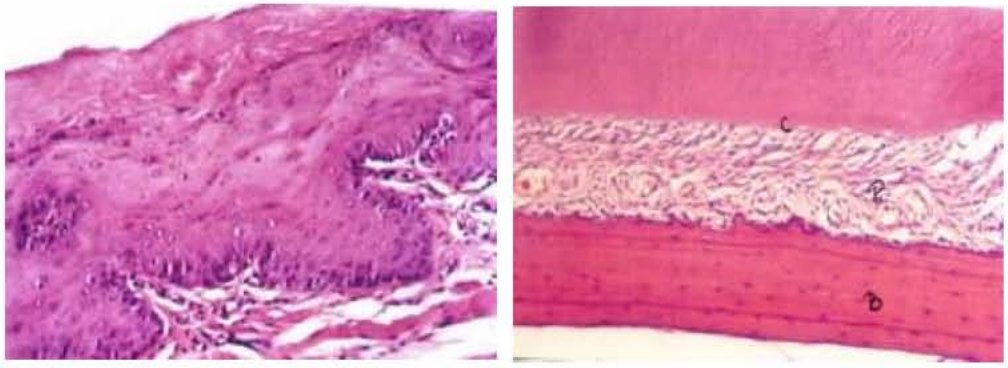

Fig. 10: [a] the gingiva of rat which received three ozone applications for six months showing marked hyperkeratosis and dissociation of collagen fibers with marked decrease of fibroblasts. [b] shows cementum [C], dissociation of the collagen fibers of the periodontal ligament [P] and alveolar bone [B] [H & E x100]

In rats, which received one ozone application for three months period, the histological examination of the gingiva revealed slight hyperkeratosis with broad epithelial ridges showing some degree of flattening. The underlying lamina propria showed mild increase in chronic inflammatory cells. In the periodontal ligament, there is marked thickening and increased number of fibroblasts. The cementum layer appeared as homogenous eosinophilic line as shown in Fig. 5.

As shown in Fig. 6 the gingiva of the group, which received two ozone applications for a period of three months, showed slight hyperkeratosis and loss of the characteristic pattern of the epithelial ridges. There was hyperplasia of the epithelial cells. There was some dissociation of collagen fibers. Increase in number of chronic inflammatory cells can be also noticed. The periodontal ligament showed increase in thickness and condensation of collagen fibers with increased fibroblasts number.

The surface epithelium of the gingiva of rats, which received three ozone applications for three months period, showed hyperkeratosis. Some of the epithelial cells showed degenerative intracyotplasmic vacuolization in the prickle cells layer. The underlying lamina propria revealed some degree of degeneration of collagen fibers with decease of fibroblasts. There is some loss of orientation of the basal cell layer with intact basement membrane present. There is mild increase in the number of fibroblasts in the periodontal ligament. There is also mild increased number of lacunae of osteoblasts of the alveolar bone as shown in Fig. 7.

Fig. 8 represents the histological examination of gingiva of group of rats subjected to one ozone application for six months revealed marked structural changes in the surface epithelium presented as hyperkeratosis and loss of the characteristic pattern of the epithelial ridges where it become broad and bulging deeper into the underlying connective tissues. Some of the epithelial cells show cytoplasmic vacuolization. The underlying lamina propria showed some degeneration of collagen fibers with decreased number of fibroblasts. The periodontal ligament showed dissociation of collagen fibers. There is also mild increased number of lacunae of osteoblasts of the alveolar bone.

In the group, which received two ozone applications for six months, Fig.9 shows the surface epithelium of the gingiva with presence of hyperplasia. There is dissociation and degeneration of the collagen fibers of the lamina propria with apparent decrease in the number of fibroblasts and presence of mild increase in chronic inflammatory cells. There is dissociation and degeneration of the collagen fibers in the periodontal ligament.

In the group, which received three ozone applications for six months, there is marked hyperkeratosis. There is dissociation of collagen fibers with marked decrease of fibroblasts. The periodontal ligament showed less condensation of collagen fibers and less number of fibroblasts as shown in Fig.10. There is also decreased number of osteoblasts.

DISCUSSION

Various treatment modalities were performed aiming at controlling and reducing the infection caused by different oral microorganisms found in dental plaque, which is considered as the main etiological factor of almost all periodontal problems [5, 7, 10].

Alternative medicine is now introduced as a new trend in therapy to overcome any failure or side effects of other lines of treatment. Among this trend,

ozone has been used in a variety of disorders showing high degree of efficacy, almost unrecorded side effects and good results [11]. Several researchers have investigated the use of ozone in management of inflammatory periodontal diseases. Local ozone therapy was found to reduce plaque accumulations with reported antibacterial and anti-inflammatory effects [12-14]. Local application of ozonized water as subgingival irrigation in management of inflammatory periodontal diseases has long been used with no recorded clear data regarding the direct effect of ozone on the oral tissues [11].

This provoked the idea of the present work to study the effect of ozone on the periodontal tissues in order to detect any abnormality caused by the use of the ozonized water. This is done with different numbers and periods of applications, since the accumulation of dental plaque. In the present study, histopathological results revealed that one or two ozone applications for one month showed no structural alterations in histological sections examined. The results of Valacchi [15] who used controlled ozone therapy is coinciding with the results obtained in the present study. Valacchi [15] confirmed this statement by an experiment conducted to study the effect of ozone on the skin proving that ozone cannot penetrate into the cutaneous tissues because it immediately reacts with the unsaturated fatty acids and traces of water overlaying the surface epithelium, generating reactive oxygen species [ROS] and lipoperoxides [LOP]. These generated ROS and LOPs can be either partially reduced the skin antioxidants or partially absorbed via the venous and lymphatic capillaries. In another study conducted using ozonated olive oil preparation as topical application, it was proved that this preparation was ideal in the treatment of chronically infected cutaneous and mucosal surfaces. The ozonated oil is now used topically for the treatment of gingivitis, herpetic infections, infected wounds, anaerobic infections, ulcers and burns, cellulites, abscesses, anal fissures, decubitus ulcers e.g. bed sores, fistulae, fungal infection and vulvovaginitis. This preparation remains stable for two years at 4°C with no reported side effects of its use [12].

Turk [16] examined the effect of local ozone therapy in treatment of stomatitis and gingivitis. The results showed considerable reduction in the number of bacteria, with positive influences on the disease, acceptance by the patients with no recorded side effects after performing a single application per week for one month using ozonized water. The results of Ramzy [14] are in agreement with the present study.

Stubinger [17] explained the therapeutic effect of ozone in management of periodontitis on the bases of its strong oxidation properties and its capability in directly destructing almost all pathogenic microorganisms.

Some structural alterations of the surface epithelium were recorded in the present study such as hyperplasia and hyperkeratosis observed in both groups that received two or three ozone applications for three months. These alterations can result from disturbance in the process of physiological turnover of the epithelium. These alterations are associated with change in the normal pattern of epithelial ridges showing loss of the slender pattern and sometimes-incomplete epithelial ridges. This can be noticed in the diabetic groups that received three ozone applications for three months. Some epithelial cells showed degenerative cytoplasmic vacuolization and the lamina propria suffered degeneration of collagen fibers with decreased fibroblasts as observed in the non-diabetic group which received three ozone applications for three months. This is in agreement with the results obtained in the present study explained on the bases that the effect of ozone therapy is dose dependent [18] and that the cumulative dose of ozone after a long exposure can kill the cells [11].

The increased numbers of inflammatory cells as recorded in the present study in groups received three applications for one month and the groups that received one and two ozone applications for three months. This could be due to the effect of ozone in activation of the immune system as explained in a study conducted by Paulesu [19]. The authors documented that ozone can activate monocytes and lymphocytes together with increased production of various cytokines such as interleukin 1, 2, 6, 7, 8, interferon-γ, tumor necrosis factor-α, granulocyte and macrophage colony-stimulating factors.

In addition to that the present study showed the periodontal ligament with increase in collagen fibers condensation and increased fibroblastic activity in groups which received one, two or three ozone applications for one and three months period. These results are coinciding with the results of Sforza and Sforza [20], which reported that ozone is capable of stimulating the fibroblastic activity initiating the repair process by stimulating deposition of collagen. This was also confirmed in a study conducted on extracted human teeth where ozonized water irrigation was done for 2 minutes resulting in increase in proliferating cell nuclear antigen of fibroblasts after this irrigation [21].

In addition, Silver and Glasgold [22] reported that ozone can stimulate the release of transforming growth factor β1 [TGF- β1] and fibroblast growth factor [FGF] with consequent increase in fibroblastic activity denoting that fibroblasts can initiate repair process by stimulating the deposition of collagen fibers [20]. These results are in similarity of the results obtained in the present study. On the other hand, the present work showed also decreased condensation of collagen fibers with decreased number of fibroblasts in the group, which received three ozone applications for three months and three ozone applications for six months. This result can be explained on the basis that the effect of ozone therapy is dose dependent as stated by Gornicki and Gutsze [18]. The cumulative dose of ozone after a long period of exposure is capable of killing the fibroblastic cells, which is in agreement with the results obtained by Bocci [11] and explains the results obtained in the present study.

Furthermore, the decreased condensation of collagen fibers and decreased number of fibroblasts found in the groups, which received two or three ozone applications for six months period. Bocci [11] and Gornicki and Gutsze [18] reported that the prolonged ozone exposure could kill the fibroblastic due to the dose dependency and cumulative effect. On looking to the number of bone cells, the present study showed significant increase in the number of bone cells as compared to the control groups in the groups, which received 1 or 2 ozone applications for 1 or 3 months. This can be explained on the basis that ozone could increase the osteoblastic activity after its application. In the group, which received three ozone applications for six months there, is starting of decrease of the number of bone cells, which can be related to dose dependency reported by Gornicki and Gutsze [18].

In the present study, the Leica Qwin image analyzer was used to measure the DNA content of the gingival epithelium. The image analysis system automatically expressed the DNA content of each individual cell measured then gave the percentage of each cell class out of the total number of cells examined and classifies the cells into four groups namely; normal diploid [2c], >1.5c, tetraploid [4c] and cells with more than 4c DNA content. As the Leica Qwin system calculates the percentage of cells with DNA values above the 4c level, this parameter was taken as a measure of aneuploidy. Cellular DNA content is abnormal at early stage of dysplasia and may even predate it. Increasing values of abnormal DNA was found related to the severity of dysplasia [23]. Bearzi [24] found a sharp increase in the degree of aneuploidy in severe dysplasia cases.

In the present work, the results showed the normal DNA content [2c] measurements in the control group [80.82%]. After one application of subgingival irrigation with ozonized water for one month the DNA content measurements changed to be 78.57 % proving that ozone therapy did not produce a significant harmful effect on the DNA content of the cells of theses groups. These results are in agreement with a study conducted by Brauner [12] in which it was reported that after one ozone application per week in a clinical four weeks study a significant reduction of plaque accumulation was recorded with decrease in gingival bleeding index and reduced amount of gingival crevicular fluid with no observed clinical side effects. Ramzy [15] conducted another study in order to manage aggressive periodontitis using ozonized water as subgingival irrigation one time per week proving a significant improvement regarding pocket depth, plaque index, gingival index and bacterial count. The results related to quadrants treated by scaling and root planning together with ozone application showed the most significant improvement as compared to other lines of treatments [oral hygiene instructions / oral hygiene instructions and ozone application/scaling and root planning]. Total bacterial viable count was reduced by 99.8% after scaling and root planning together with ozone application after the four weeks treatment period.

Calculations of the complications after using of ozone therapy used in patients suffering from diffused form of purulent peritonitis showed that the complications were 1.8 times less as compared with the control group where ozone was not used [25].Cruz [26] used ozonated olive oil in management of alveolitis and detected the effectiveness and safety of ozone as a therapy.It should be emphasized that if ozone is judiciously used according to precisely defined guidelines including correct time period of application and number of applications, it causes neither acute, nor chronic side effects which was also stated by Bocci [11].

On the other hand, further looking on the results of the present study when increasing the number of applications more than one application for a period more than one month abnormality of the DNA content of the cells starts to appear whereas the worst results obtained when ozone was applied three times per week for a period of six months. These results are in agreement with Ito et al [27] in a study conducted on experimental rats reporting that ozone in high doses is capable of inducing cleavage of deoxyreibose of double stranded DNA. In addition, Turrent *et al.* [28] reported that high concentrations of

inhaled ozone can cause lung damage as hyperplasia, metaplasia and dysplasia with toxicological effect on DNA producing cellular and epithelial damages in human subjects.

In another study by Basset [29] revealed that rats subjected to different high concentrations for different periods of ozone gas showed metaplasia in the nose and lungs with increase in the goblet cells of the respiratory epithelium with mild squamous metaplasia of the cuboidal epithelium. Nightingale [30] proved that inhalation of ozone for a prolonged period in normal human subjects caused neutrophilic inflammatory response in the airways.

CONCLUSION

These unusual complications suggest the necessity of further investigating the benefits and adverse effects of medical ozone therapy.

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