IN VIVO EVIDENCE OF ALOE VERA ROLE ON CELL PROLIFERATION AND APOPTOSIS IN LIGATURE INDUCED PERIODONTITIS

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ABSTRACT

Objective: The purpose of this study is to evaluate the proliferative and apoptotic effect of Aloe Verain the treatment of induced periodontitis in Wistar rats.

Design: Thirty adult male Wistar rats with an average weight 200-250 g were used in this study. Sterile 4/0 silk ligatures were placed around the maxillary central incisors to induce periodontitis. The 30 animals were divided randomly into 3 groups, 10 rats in each group. Group I (periodontitis group), group II (Oral group) were given Aloe Vera extract (300mg/kg) by oral gavage, while in-group III (intraperitoneal group) rats were given Aloe Vera intraperitoneally in the dose of (300mg/kg). The animals were sacrificed at the end of 30 days. The maxillae of each group (10 specimens each) were dissected around theincisors. The specimens were stained with heamatoxylin and eosin. PCNA and Caspase- 3 were used for detecting proliferative and apoptotic changes respectively.

Results: Histological results of both groups II & III revealed thicker bone trabeculae formation, and enhanced angiogenesis. Histomorphometric analysis revealed an increase in the area percentage of bone in both groups II &III, compared to group I with a statistically significant difference between the three studied groups (P=0.0016). Group II revealed the greatest PCNA area percentage, but witha statistically non-significant difference between the 3 studied groups (P=0.221). The highest Caspase-3 immunoreactivity was revealed in Group I. A statistically significant difference of Caspase-3 area percentage was present between the three groups ($p \leq 0.0001$).

Conclusions: Aloe-Vera administration proved to be effective in the treatment of periodontitis by enhancing proliferation and decreasing apoptosis of alveolar bone cells.

INTRODUCTION

Periodontitisis a set of inflammatory diseases affecting the periodontium i.e. the tissues that surround, and support the teeth. Periodontitis involves progressive loss of the alveolar bone around the teeth, and if left untreated, can lead to the loosening and subsequent loss of teeth. [1] The primary etiology of gingivitis is poor oral hygiene, which leads to the accumulation of a mycotic [2,3,4] and bacterial matrix at the gum line (dental plaque). Other contributors are poor nutrition and underlying medical issues such as diabetes.[5] Removal of

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microbic plaque and calculus is necessary to establish periodontal health. The first step in the treatment of periodontitis involves non-surgical cleaning below the gumline with a procedure called scaling and debridement. [6]

If non-surgical therapy is found to have been unsuccessful in managing signs of disease activity, periodontal surgery may be needed to stop progressive bone loss and regenerate lost bone where possible. There are many surgical approaches used in treatment of advanced periodontitis, including open flap debridement, osseous surgery, as well as guided tissue regeneration and bone grafting. The goal of periodontal surgery is access for definitive calculus removal and surgical management of bony irregularities, which have resulted from the disease process to reduce pockets as much as possible.[7]

Most alternative "at-home" gum disease treatments involve injecting anti-microbial solutions, such as hydrogen peroxide, into periodontal pockets via slender applicators or oral irrigators. This process disrupts anaerobic microorganism colonies and is effective at reducing infections and inflammation when used daily. A number of potions and elixirs that are functionally equivalent to hydrogen peroxide are commercially available but at substantially higher cost. [8]

Aloe Vera is a stemless, drought- resisting succulent of liliaceous family; which are known to have medical properties. [9] Aloe Vera has been used in several medical uses such as skin and wound healing, diabetes, peptic ulcer, anti-inflammatory, anti cancer and antioxidant. Current scientific investigations is producing evidence about Aloe Vera multiple beneficial effects at the topical level but apart from this, in vitro and in vivo tests have demonstrated diverse therapeutic effects after oral consumption.[10]

Most studies have examined the efficacy of Aloe Vera in vitro cell culture systems, with in vivo studies using intraperitoneal or intramuscular administration. This study was thus designed to examine the proliferative as well as the apoptotic effect of Aloe Vera on periodontitis after both oral and intraperitoneal administration.

MATERIALS AND METHODS

Experimental procedure

Thirty adult male Wistar rats with an average weight 200-250 g were used. The animals were housed in individual cages at a controlled room temperature of 20–25˚C and relative humidity of 70–80%. They were kept under 12 hrs. dark/light cycles and fed with standard pellets diet and tap water adlibitum. The animals were acclimatized to the housing conditions for four weeks. The study protocol was reviewed and approved by the Ethics Committee of the Faculty of Oral and Dental Medicine, Cairo University, Egypt.

All the rats were assigned to ligature. All procedures of periodontitis induction were performed under general anesthesia by intramuscular injection of a combination of 0.1 mL ketamine hydrochloride (50 mg/mL); and 0.05 mL Xylazinehydrochloride (2 g/100 mL) for each 100 g body weight. After anesthesia, sterile 4/0 silk ligatures were placed around the maxillary central incisors.

The 30 animals were divided randomly into 3 groups, 10 rats in each group. Group I (periodontitisgroup), group II (Oral group) were given Aloe Vera extract (300mg/kg) by oral gavage (Freeze dried Aloe- Vera, Eva Cosmetics), while rats in-group III (intraperitoneal group) were given Aloe Vera intraperitoneally in the dose of (300mg/kg). Aloe Vera administration in both groups II & III started one day before the induction of periodontitis and continued for a total of 1 month. The animals were sacrificed with cervical dislocation at the end of 30 days.

Light microscopic examination

The maxillaeof each group (10 specimens each) were dissected around the 2 incisors andwere

immediately fixed in 10% neutral formalin for 48 h, washed and soaked in 10% EDTA for decalcification for 4 weeks, and then rinsed in distilled water. Specimens were dehydrated in ascending grades of alcohol and embedded in paraffin.The paraffin blocks were serially cut in a mesiodistal direction along the long axis of the teeth to provide 4-5µm-thick sections. Care was taken to obtain histological sections in which the 2 central incisors, the interproximal alveolar bone crest and the coronal and root pulp chambers were clearly identified. The sections were subjected to haematoxylin and eosin stain according to the conventional method. Histopathologic examination was performed using light microscopy.

Immunohistochemical examination

The sections were incubated for 2 hours at 56°C, deparaffinized in xylene, and rehydrated by ethanol series ending with pure H_2O (Millipore Corporation, Temecula, CA, USA). After 5-minute washing in PBS, sections were incubated in 0.05 mg/ml proteinase K in 0.05 M Tris-HCl, 0.01 M EDTA, and 0.01 M NaCl, pH 7.8 for 10 minutes at 37°C. After two washes with PBS, unmasking of the antigens was carried out using antigen retrieval citrate buffer solution for 10 min in boiling water. Then the sections were placed in a humid chamber and the endogenous tissue peroxidase was blocked with 3% hydrogen peroxide for 5 min. Incubation with bovine serum albumin for 20 min was performed to reduce unwanted nonspecific reactions. Without washing, the sections were incubated with the Primary antibodies overnight at 4°C. The Primary antibodies used were polyclonal anti- PCNA (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-caspase-3 active form (1: 100; Millipore Corporation, Temecula, CA, USA). In the next day, after washing in PBS, the sections were incubated with secondary universal antibody (Vectastain Universal Elite ABC- peroxidase kit, Vector Laboratories) and then with the Avidin-Biotin complex (ABC) (Vectastain Universal Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol. The substrate DAB was applied until development of desired brown color (2-10 min). Finally, the sections were counter-stained with Mayer's hematoxylin (Sigma, St. Louis, MO, USA) for 30 sec. to visualize tissue topography. The negative control was obtained by omitting the primary antibody from the protocol outlined above.

Histomorphometric analysis

The data were obtained using Leica Qwin 500 image analyzer computer system (England). The image analyzer consisted of a coloured video camera, coloured monitor, hard disc of IBM personal computer connected to the microscope, and controlled by Leica Qwin 500 software.

The area and the area percentage of bone trabeculae were measured using an objective lens of magnification 20x (total magnification of 200). Using the color detect, areas were masked by a blue binary color. (Fig. 1) The area percentage of PCNA and Caspase -3 was measured using an objective lens of magnification 40 x, i.e. of a total magnification of 400. Ten fields were measured for each specimen. After grey calibration, the image was transformed into a grey delineated image to choose areas exhibiting positive reactivity with accumulation of all grades of reactivity (minimum, maximum and median grey). Areas of positive reaction were then masked by a blue binary color. The area percentage was calculated in relation to a standard measuring frame of area 118476.6µm2. Mean values were obtained for each specimen.

Data obtained from histomorphometrical analysis were statistically described in terms of range, mean ± standard deviation (± SD), and median. Comparison between the studied groups was done using Kruskal Wallis analysis of variance (ANOVA) test with Conover-Inman test for independent samples as post hoc multiple 2-group comparisons. A probability value (p value) less than 0.05 were considered statistically significant. All statistical calculations

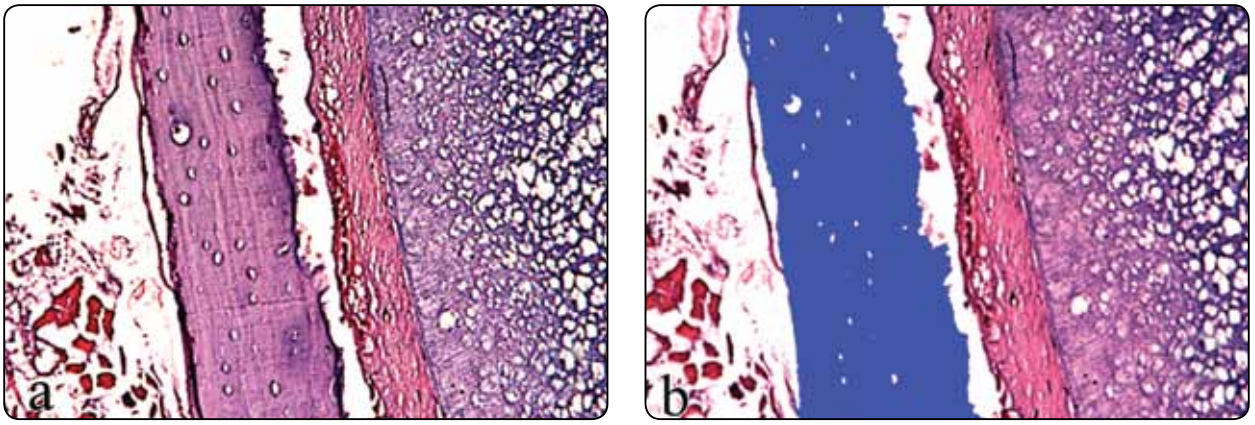


FIG. (1) (a) Bone trabeculae of the alveolar process, (b) bone trabeculae masked by blue binary color.

were done using computer programs Microsoft Excel 2003 (Microsoft Corporation, NY, USA) and Stats Direct statistical software version 2.7.2 for MS Windows, Stats Direct Ltd., Cheshire, UK.

RESULTS

Histological results

The periodontitis group (Gp. I)

The alveolar process of the periodontitis group revealed disoriented thin lacerated bone trabeculae that lacked the normal architecture of bone. Some resorptive bays were detected in few sequestered bone spicules. (Fig.2)

Aloe Vera treated group (oral route) (Gp II)

Well-organized bone trabeculae were revealed. The trabeculae were thick, homogenous, enclosing osteocytes in their lacunae. Marrow spaces filled with red marrow were present in between the bone trabeculae. (Fig.3)

Aloe Vera treated group (intraperitoneal route) (Gp III)

Interconnecting bone trabeculae were found, however they were thinner than those revealed in Gp II. Abundant, large marrow spaces were observed containing numerous lymphatic and blood vessels. (Fig.4)

Histomorphometric results

The area percentage of bone trabeculae of the three studied groups are summarized by means, standard deviation and median in (table 1)

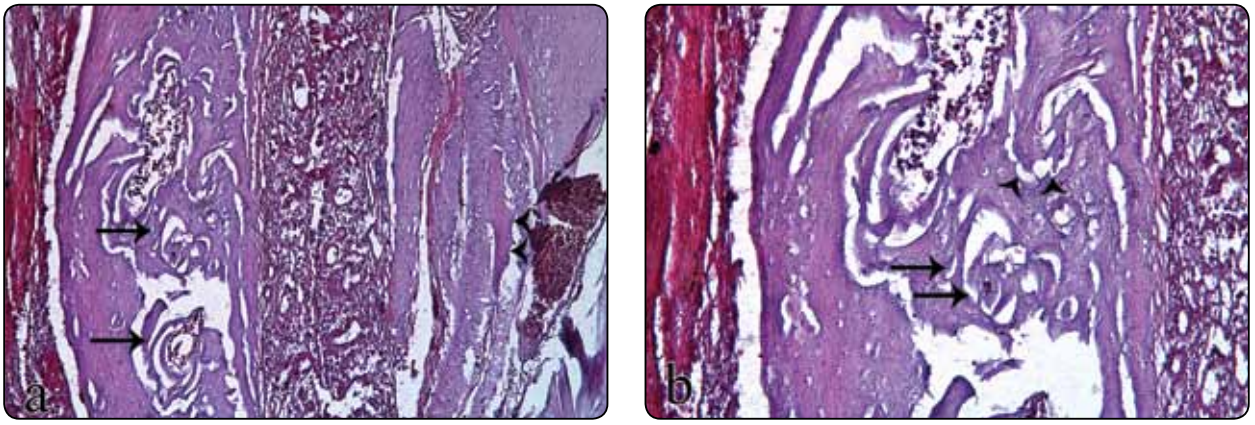


FIG. (2) A photomicrograph of (Gp I periodontitis group)showing: the alveolar process with lacerated disoriented bone trabeculae (arrows), resorptive bays in sequestered bone spicules (arrow heads). H&E (a) 100 X, (b) 200 X

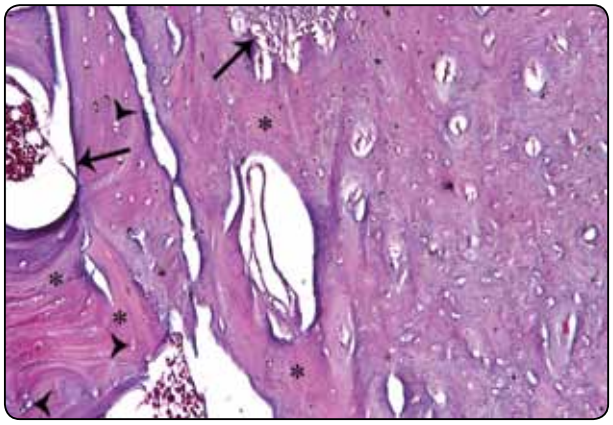


FIG. (3) A photomicrograph of (Gp II Aloe Vera via oral route) showing: well-organized, thick bone trabeculae (asteriscs) enclosing osteocytes in their lacunae (arrow heads). Marrow spaces (arrows) filled with red marrow were present in between the bone trabeculae. H&E200X

The bone trabeculation area percentage revealed a high significant difference between the three studied groups (P=0.0016). Moreover, there has been a significant difference between groups I &II and between groups II & III (P= 0.0034, P≤0.0001 respectively). On the other hand, there has been a non-significant difference between groups I & III (P= 0.3186).

Immunohistochemical results

Proliferating cell nuclear antigen (PCNA)

The PCNA positive cells were expressed in all specimens. The PCNA marked cells were present on the peripheries of bone trabeculae and spicules, they were also seen lining the marrow cavities. The PCNA cells were often found solitary and discrete in the groups I and III (fig.5a&c respectively). In Gp II the PCNA positive cells were revealed as dark irregularly dispersed knots on the bone surface (fig.5b). The area percentage of the PCNA positive cells of the three studied groups are summarized by means, standard deviation and median in (table2). Despite that gp II revealed the greatest PCNA area percentage, but a statistically non-significant difference between the 3 studied groups has been recorded (P=0.221).

Caspase-3 immunoreactivity

Caspase-3 positive cells were detected in the three experimental groups. The Caspase -3 reaction was exhibited along the borders of the bone trabeculae as well as in the lacunae of osteocytes. The highest Caspase- 3 immunoreactivity was found in-group I, followed by group III and then group II (fig.6). The area percentage of the Caspase -3 positive cells is summarized by means, standard deviation and median in (table3). A statistically significant difference of Caspase-3 area percentage was present between the three groups (p≤0.0001). A high significant difference was present between groups I & II and groups II and III (p≤0.0001). However, there was no significant difference between groups I & III (p = 0.0538)

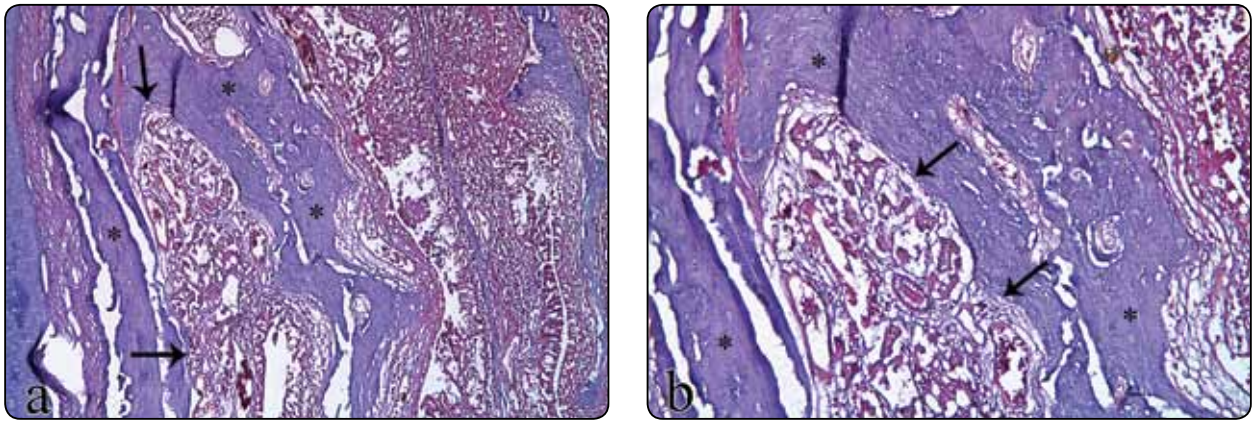


FIG. (4) A photomicrograph of (Gp III Aloe Vera via intraperitoneal route) showing: Interconnecting bone trabeculae (asteriscs) but thinner than those revealed in Gp II. Abundant, large marrow spaces (arrows) containing numerous lymphatic vessels and engorged blood vessels. H&E (a) 100 X, (b) 200 X

TABLE (1) Mean values & standard deviation of the area percentage of bone in groups I, II& III.

Variables	Area percentage Gp I	Area percentage Gp II	Area percentage Gp III
Number of samples	10	10	10
Mean	23.686	29.652 a	25.487 b
Standard deviation	5.314	1.758	1.608
Maximum	29	31.99	28.15
Median	22.21	29.94	24.58
Minimum	17.78	27.01	24
P value	0.0016*		

**** statistically significant difference***

a statistically significant with Group I ***b statistically significant with Group II***

TABLE (2) Mean values & standard deviation of the area percentage of the PCNA positive cells in groups I, II& III.

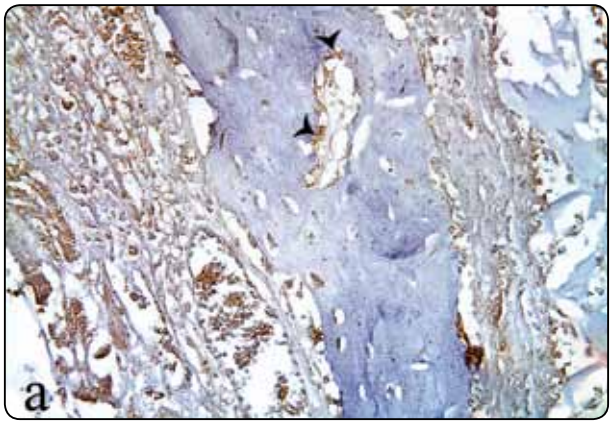
Variables	Area percentage Gp I	Area percentage Gp II	Area percentage Gp III
Number of samples	10	10	10
Mean	0.697	1.220	1.042
Standard deviation	0.674	0.467	0.810
Maximum	1.9	2	2.27
Median	0.375	0.99	0.655
Minimum	0.1	0.77	0.13
P value	0.2210		

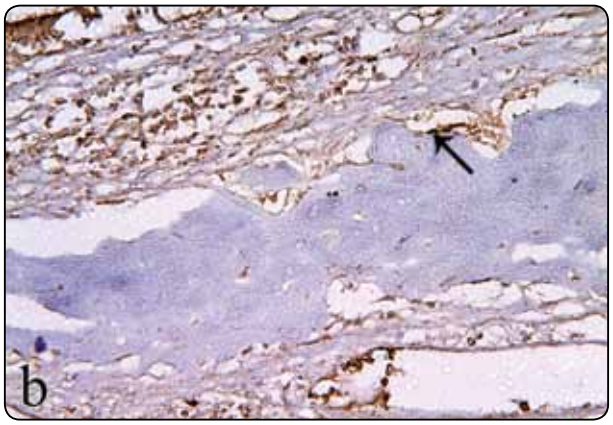
TABLE (3) Mean values & standard deviation of the area percentage of Caspase-3 activity in groups I, II& III

Variables	Area percentage Gp I	Area percentage Gp II	Area percentage Gp III
Number of samples	10	10	10
Mean	3.016	1.291a	2.360b
Standard deviation	0.946	0.489	0.341
Maximum	4.28	2	2.7
Median	3.115	1.185	2.52
Minimum	1.1	0.73	1.8
P value	<0.0001*		

**** statistically significant difference***

a statistically significant with Group I ***b statistically significant with Group II***





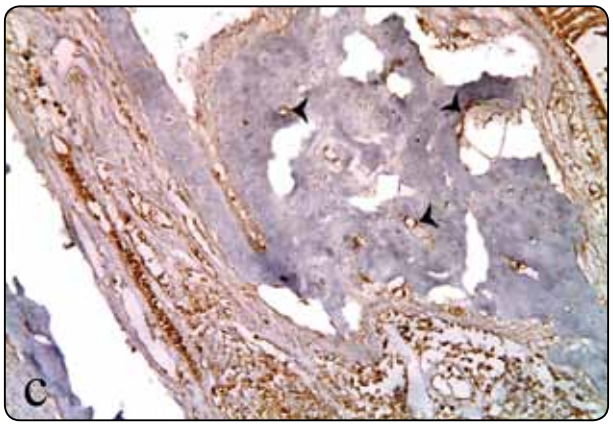
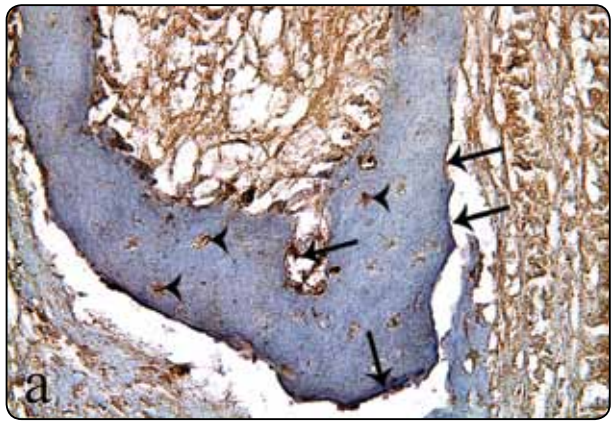
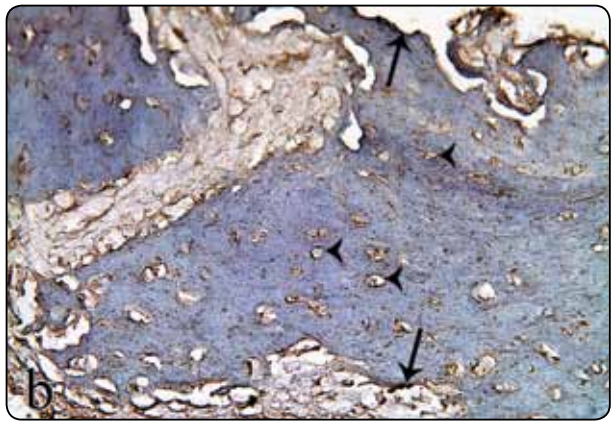


FIG. (5) Aphotomicrograph of (a) GpI , (b) Gp II & (c) Gp III showing: PCNA positive cells present on the peripheries of bone trabeculae and lining the marrow cavities (arrow heads). The PCNA cells were found solitary and discrete in the Gp I and III; they were dark &irregularly dispersed knots in Gp II (arrows). PCNA 400X





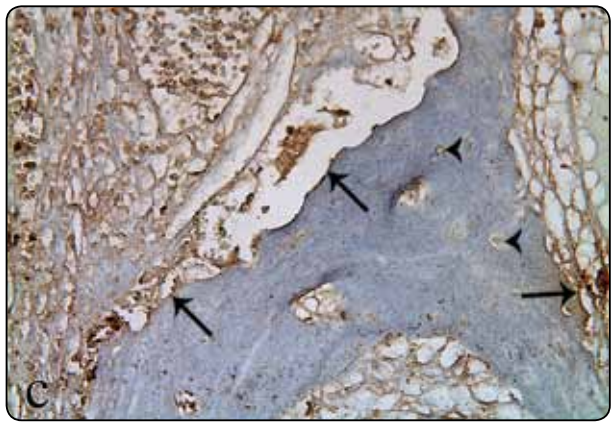


FIG. (6) A photomicrograph of (a) GpI, (b) Gp II & (c) Gp III showing:The Caspase -3 reaction exhibited along the borders of the bone trabeculae (arrows) as well as in the lacunae of osteocytes (arrow heads). The highest Caspase- 3 immunoreactivity was found in-Gp I, followed by Gp III and then Gp II. Caspase-3 400 X

DISCUSSION

Periodontal disease is the most frequent cause of tooth loss in humans and is the most prevalent disease associated with bone loss, including osteoporosis. [11] Most of the tissue damage that characterizes periodontal diseases is caused indirectly by the host response to infection rather than directly by the infectious agent.[12]

In the present research, the periodontitis group (Gp I) revealed thinner bone trabeculae than those in groups II and III. Howships lacunae were also observed and the bone architecture was generally disrupted.

Previous studies have attributed that the host-mediated response in periodontal disease involves the activation of the innate immunity, specifically by upregulation of proinflammatory cytokines from monocytes/polymorphonuclear leukocytes, and down regulation of growth factors from macrophages. [13] The host-mediated response in periodontal disease is also characterized by the production of inflammatory mediators.[14] One of the prominent inflammatory mediators associated with periodontal disease is Prostaglandin E2, which is a potent stimulator of bone loss that is the hallmark of periodontal disease.[15]

The usage of Aloe-Vera in the herein study in both groups II and III via oral and intraperitoneal routes respectively have shown the efficacy of Aloe- Vera administration when compared with the periodontitis group I. Thicker well organized bone trabeculae were observed in both groups II and III compared with group I. The bone area percentage of the three groups revealed a high significant difference. A significant difference was also present between groups I and II, and between groups II and III, however, a non significant difference was present between groups I and III. Concurrent with our results, Aloe- Vera anti- inflammatory effect has been associated with the inhibition of cyclooxygenase activity, which prevents the synthesis of prostaglandins that are the fundamental chemical mediators in inflammatory processes.[16]

Moreover, Davis et al. [17] demonstrated the effectiveness of Aloe Vera in treatment of wound and reduction of inflammation was due to the action of mannose-6- phosphate, a major sugar present in Aloe Vera.

In a study carried out by Jittapiromasak et al. [18], it was found that Acemannan, the major polysaccharide in AloeVera, significantly induced alkaline phosphatase activity, production of bone morphogenic protein and dentin sialoprotein and mineral deposition. This finding is consistent with the results of the current research as for the increase of the bone area percentage of both groups II and IIIcompared to that in-group I.

In the present study, a decrease in the angiogenesis process was clearly evident in-group I. By contrast, the Aloe-Vera administration was found to enhance angiogenesis, as evidenced by the presence of newly formed blood vessels in the marrow cavities of both groups II and III. Previous studies have shown that Aloe- Vera enhanced angiogenesis in the chick embryo. It has also been postulated that the b- sitosterol present in Aloe – Vera, is a potent angiogenic factor that stimulated the neovascularization and the healing of wounds[19] .

Some investigators affirmed that Acemannan, has an immunostimulative effect through activation of macrophages in the inflammatory phase. [20-22] Previous studies have also reported that Accemannan stimulated macrophage and dentritic cell cytokine production.[20,23] The immunomodulatory activity of orally administerd Aloe –Vera was also examined in a Candida albicans- infection showing that Aemannan mediated its activity mainly through activation of macrophages. [21] Macrophage is the prime source of several growth factors in wound healing. [24] Previous reports speculated that Aloe-Vera active components such as mannose-6- phosphate and acemannan bind with special

ligands on the cell surface of macrophages. After this binding the cells will be stimulated to produce growth factors and proliferate.[20,21&25] Taken together our histological and statistical data of the present study demonstrated the influence of Aloe Vera in treatment of periodontitis.

In the on going research, Immunohistochemical labeling indices for PCNA was used to further evaluate the effect of Aloe Vera in bone regeneration. The immunohistochemical results of the ongoing research have revealed the greatest PCNA area percentage, in gp II but with statistically non-significant difference between the 3 studied groups. The higher PCNA expression in groups II and III is directly correlated with rates of cellular proliferation and DNA synthesis, which indicates the presence of osteoblastic cells that are incorporated in bone regeneration. The higher proliferative expression could be attributed to the assumption that the glycoprotein fractions of Aloe- Vera together with bradykinase activity and stimulants of cellular proliferation were identified in nondialized fraction of the Aloe Vera from several species.[26]It has also been attributed that Aloe Vera contains glycoproteins(lectins)and polysaccharides, the lectins promote the growth of the normal cells and inhibit the growth of tumor cells.[27]

The primary line of defense of the host immune response against pathogenic microbes in periodontal diseases is Polymorphonuclear Luecocytes (PMN). PMN produce a range of antimicrobial factors, which include ROS, during phagocytosis of periodontopathic bacteria in inflammatory periodontal diseases.[28]

Caspase -3 immunohistochemical results of the current work revealed the highest Caspase – 3 area percentage in group I, followed by group III, while the least was in group II with a statistically high significant difference between the three groups.

The identification and characterization of connective tissue metabolites in gingival crevicular fluid resulting from the degradation of periodontal tissues, notably alveolar bone, provides evidence for a role for Reactive oxygen species (ROS) in tissue destruction associated with inflammatory periodontal diseases. [28] ROS are active in depolymerization of extracellular matrix components [29&30], lipid peroxidation [31], and increased apoptosis in deepest layer of sulcular pocket. [32]

The highest Caspase -3 immunoreactivity revealed in gpI of the present study could thus be attributed to the released ROS, culminating in heightened oxidative damage to alveolar bone.

Kim et al. 2001[33] have proved that nitric oxide, which is a form of ROS, activates apoptosis in many cells such as macrophages, pancreatic islets, neurons, and thymocytes. Nitric oxide also inhibits fibroblast proliferation and induces apoptosis, contributing to the imbalance of tissue destruction with tissue repair that is characteristic of periodontitis.

The Caspase -3 immunoreactivity in both groups II and III of the ongoing research showed a reduction in the immunoreactivity compared with that in-group I. The antioxidant activity of Aloe- Vera has been previously reported. [34] It was deduced that some compounds located in the cortex of the leaf were those responsible for most of the antioxidant capacity of Aloe Vera.[35] The possible mechanism of antioxidant activity of Aloe Vera has been attributed to its protection against lipid peroxidation. [36]

The antioxidant potency of Aloe Vera has also been proved in a study performed by Rajasekharan et al. [34] Furthermore, Anilakumar et al., 2010 attributed the reduced oxidative stress and toxicity observed in their study to barbaloin, glucmannans, acemannan, flavonoids and tannic acid present in Aloe Vera Gel.[37]

The histological, immunohistochemical and morphometric results of the present research have proved the efficacy of Aloe- Vera in the treatment of

periodontitis. However, oral administration of Aloe Vera proved to be more beneficial in the treatment of periodontits rather than administration of Aloe – Vera via intraperitoneal route. The AloeVera effect on periodontitis could be attributed to angiogenesis, immunomodulatory effect, anti-inflammatory and anti oxidant effect.

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