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VEGF, MMP2, and Osteonectin levels in the rats

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Abstract

Background: The objective of this study was to investigate if long term formaldehyde inhalation may effect periodontal membrane and alveolar bone loss leading to periodontitis. The negative effects of formaldehyde was described using vascular endothelial growth factor (VEGF), matrix metallopeptidase 2 (MMP2) and Osteonectin antibodies involved in the extracellular matrix and angiogenetic development.

Materials and methods: Thirty adult Wistar albino rats were used in this study. Rats were divided into two groups, control (n:15) and formaldehyde administered group (n:15). Formaldehyde group was administered 10 ppm formaldehyde during 5 days a week for 8 hours by inhalation. Maxillary bone regions were dissected under anesthesia. After fixation in
10% formaldehyde solution, tissues were passed through graded ethanol series to obtain paraffin blocks. Five-micrometer histological sections were cut with RM2265 rotary microtome stained with Masson- Trichrome and VEGF, MMP2 and Osteonectin antibodies for examination under Olympus BH-2 light microscopy.

Results: The present study revealed that congestion in blood vessels, degeneration of collagen fibers and alveolar matrix around alveolar bone were observed to be more significant in formaldehyde group than the control group (P≤0.001). Interestingly, VEGF expression in the formaldehyde group was the most significant finding between the two groups (P<0.001). When compared inflammation, MMP2 and osteonectin expressions were significant (P<0.01) in the formaldehyde group.

Conclusions: It was suggested that formaldehyde toxicity decreased the expression of MMP2 and in osteoblasts as well as affecting the retention of MMP levels in tooth cavity, which is very low in collagen fibers. But, vise versa for the expression of VEGF in dilated vascular endothelial cells and osteocytes in alveolar bone. As a conclusion, formaldehyde disrupts the periodontal membrane and may cause collagen fibers degeneration by affecting the alveolar bone matrix.

Key words: periodontal membrane, alveolar bone, formaldehyde, immunohistochemistry

INTRODUCTION

Periodontal disease is caused by bacteria in dental plaque, the sticky substance that forms on your teeth a couple of hours after you have brushed. Interestingly, it is your body's response to the bacterial infection that causes most of the dental problems. In an effort to eliminate the bacteria, the cells of our immune system release substances that cause inflammation and destruction of the gums, periodontal ligament or alveolar bone. This leads to swollen, bleeding gums, signs of gingivitis (the earliest stage of periodontal disease), and loosening of the teeth, a sign of severe periodontitis. Studies on periodontitis stated that it is a chronic destructive disease characterized by inflammation of the supporting tissues of the teeth, resulting in periodontal tissue damage and alveolar bone loss (1,2). As periodontitis is one of the most prevalent diseases several studies were reported to investigate its pathogenesis through the combination of experimental models (3-8).
Formaldehyde is highly soluble in water, as well as in most organic solvents, and is a highly reactive molecule that can be irritating to tissues through direct contact. Formaldehyde causes cytotoxicity through the formation of strong DNA-protein cross-links, as well as cross-links with other molecules, e.g., amino acids (9). Formaldehyde is used in household products, glues and as industrial fungicide, germicide, and disinfectant. It is commonly used as a preservative in mortuaries and tissue fixation in medical laboratories. It is a highly endogenous chemical substance of which workers from different jobs in a broad perspective, are frequently exposed to. Exogenously, formaldehyde can be absorbed after inhalative, dermal and oral exposure and the amount of absorption is dependent on the route of exposure. The overall uptake of inhaled formaldehyde by the nasal passages at resting minute volume airflow rates has been predicted to be 90% in rats, 67% in monkeys and 76% in human (10). A study on workers suggested that longterm exposure to formaldehyde can cause leucopenia and another one reported that a significantly higher percentage of formaldehyde-exposed workers had blood cell abnormalities (leucopenia, thrombocytopenia, and depressed serum hemoglobin levels) compared with unexposed controls (11).

In periodontal disease, there is collagen degradation leading to gingival and supporting tissue destruction and finally deterioration of teeth occurs (12). Collagen fibers are composed of protein and proteins have the affinity to react with formaldehyde. Formaldehyde reacts with primary amines to form Schiff bases, with amides to form toxic hydroxymethyl compounds (13). The degradation of the gingival tissue in periodontitis may be a result of cell and cell and also cell and matrix interactions, including enzyme activities like endopeptidases. It is known that endopeptidases are responsible for the tissue degradative process. Matrix metalloproteinases (MMPs) are endopeptidases produced by different types of gingival cells and contribute tissue breakdown (14). MMP plays an important role in the inflammation during periodontal disease as it hydrolizes different proteoglycans and glycosaminoglycans, such as Syndecan 1 (15,16). Excessive production of MMP2 because of genetic polymorphisms may influence the manifestation and development of periodontal diseases (17). In particular resident, periodontal ligament and gingival fibroblasts was reported to secrete matrix metalloproteinase 2 and chemoattractants for epithelial cells (5).

Osteonectin is a 32 kDa phosphorylated glycoprotein. Evidence that SPARC/osteonectin contributes to human periodontal disease is supported by significant increases in SPARC/osteonectin expression detected in the gingival crevicular fluid of
patients with periodontal disease (7). Vascular endothelial growth factor potently regulates the formation of new blood vessels through VEGF receptors and has been reported to be a key factor in periodontal angiogenesis during tooth movement (18).

The purpose of this study is to investigate the effects of formaldehyde on periodontal membrane and alveolar bone which may lead to periodontitis when inhaled, using immunohistochemical methods for VEGF, MMP2 and osteonectin observed in extracellular matrix composition and angiogenetic development.

MATERIALS AND METHODS

Animals and experimental design

All surgical procedures and the subsequent care and treatment of the animals used in this study were in strict accordance with the National Institutes of Health (NIH) guidelines for animal care (NIH Publication no. 85-23, revised 1996). All procedures performed in this experiment were approved by the Ethics Committee for the Treatment of Experimental Animals (Faculty of Medicine, University of Dicle, Turkey). Thirty Wistar Albino rats (9 weeks old, 180–200 g) were maintained under 22±1°C and 12 h light/dark cycles with ad libitum access to standard pelleted food and water. The 30 rats were divided into 2 groups as control and formaldehyde groups. All the animals were individually housed in stainless steel cages at room temperature. The rats of the control (n=15) were only administered 1.5 ml physiologic saline solution subcutaneously. The formaldehyde group was administered with 10 ppm formaldehyde 5 days a week for 8 hours by inhalation method. Formaldehyde vapor in the environment, with a special air pump ventilation constant volume, pressure and temperature achieved (19). The rats of all groups were maintained in the same conditions. All rats at the end of the experiment were healthy and no difference in food/water consumption and body weight gain between experimental and control rats were observed. At the end of the study, the animals were sacrificed using a decapitator (Harvard Apparatus, Holliston, MA, USA).

Histologic examinations

The maxillary regions were dissected under ketamine hydrochloride anesthesia and placed in 10% formaldehyde solution. They were placed in paraffin inclusion melted at 58°C after treatment with xylool, the 4-6µm sections were taken by rotary microtome (Rotatory Microtome, Leica, RM 2265, Germany) and the sections were stained with Trichrom-Masson.
Periodontal membrane and alveolar bone of maxilla sections were examined histopathologically and immunohistochemically.

**Immunohistochemical staining**

Sections were brought to distilled water and washed in 3x5 min Phosphate Buffered Saline (PBS). Catalog number 10010023, Thermo Fischer Scientific Fremont, CA, USA. Antigen retrieval was done in microwave (Bosch®, 700 watt) for 3min x 90°C. They were subjected to a heating process in a microwave oven at 700 watts in a citrate buffer (pH 6) solution for proteolysis. Sections were washed in 3x5 min PBS and incubated with hydrogen peroxide [K-40677109, 64271 Hydrogen peroxide (H2O2) Dortmund+Germany, MERCK] (3ml %30 Hydrogen peroxide (H2O2) + 27ml methanol) for 20 min. Sections were washed in 3x5 min PBS min and blocked with Ultra V Block (lot: PHL150128, Thermo Fischer, Fremont, CA, USA) for 8 min. After draining, primary antibodies were directly applied to sections distinctly (Vascular Endothelial Growht Factor (VEGF), 1:100, lot#MA5-12184, Thermo Fischer, Fremont, CA, USA. Matrix metallopeptidase 2 (MMP2) monoclonal antibody 1:100, CA719E3C Thermo Fischer, Fremont, CA, USA. Osteonectin (SPARC), Catalog #:33-5500, 1:100, Thermo Fischer, Fremont, CA, USA. Sections were incubated and left overnight at 4°C. Sections were washed in 3x5 min PBS and then incubated with Biotinylated Secondary Antibody (lot: PHL150128, Thermo Fischer, Fremont, CA, USA) for 14 min. After washing with PBS, Streptavidin Peroxidase (lot: PHL150128, Thermo Fischer, Fremont, CA, USA) was applied to sections for 15 min. Sections were washed in 3x5 min PBS and DAB (lot: HD36221, Thermo Fischer, Fremont, CA, USA) were applied to sections up to 10 min. Slides showing reaction was stopped in PBS. Counter staining was done with Harris’s Haematoxylin for 45 sec, dehydrated through ascending alcohol and cleared in xylene. Product Number: HHS32 SIGMA, Hematoxylin Solution, Harris Modified, Sigma-Aldrich, 3050 Spruce Street, Saint Louis, MO 63103, USA. Slides were mounted with Entellan® (lot: 107961, Sigma-Aldrich, St. Louis, MO, United States) and examined under Olympus BH-2 light microscopy.

All morphological changes; congestion in blood vessels, inflammation, damage of collagen fibers, alveolar matrix, VEGF, osteonectin and MMP2 expression were noted. The intensity of these changes were graded from 0 to 6 (0: no change, 1: low, 2: intermoderate, 3-4: moderate, 5-6: intense) (20).

**Statistical analysis**
Statistical analysis was performed with the Statistical Package for the Social Sciences for Windows (version 15.0, SPSS Inc., Chicago, IL, USA). The Mann–Whitney U test was used for the statistics as indicated, test and results were expressed as mean ±SD. P values below 0.05 were considered to indicate statistical significant.

RESULTS

Histological analysis

The histopathological results of the present study were evaluated under light microscope. There were no histopathological changes in the control group. We compared histopathological findings and primer antibodies expressions in the control and formaldehyde groups (Table 1 and Table 2).

Results of the study indicated that congestion in blood vessels, degeneration of collagen fibers and alveolar matrix around alveolar bone were observed to be more significant in formaldehyde group than the control group (P≤0.001). Interestingly, VEGF expression in the formaldehyde group was the most significant finding between the two groups (P<0.001). When compared, inflammation, MMP2 and osteonectin expressions were statistically significant (P<0.01) in the formaldehyde group.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=15) (Mean±SD)</th>
<th>Formaldehyde (n=15) (Mean±SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congestion in blood vessels</td>
<td>0.01±0.02</td>
<td>6.0±0.01</td>
<td>≤0.001**</td>
</tr>
<tr>
<td>Inflation</td>
<td>0.18±0.31</td>
<td>4.80±0.50</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Damage of collagen fibers</td>
<td>0.0±0.0</td>
<td>2.70±0.10</td>
<td>≤0.001**</td>
</tr>
<tr>
<td>Alveolar matrix</td>
<td>0.01±0.02</td>
<td>3.0±0.02</td>
<td>≤0.001**</td>
</tr>
<tr>
<td>VEGF expression</td>
<td>1.53±0.5</td>
<td>4.53±0.61</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>MMP2 expression</td>
<td>3.62±0.3</td>
<td>0.82±0.4</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Osteonectin expression</td>
<td>2.62±0.3</td>
<td>0.74±0.6</td>
<td>&lt;0.01*</td>
</tr>
</tbody>
</table>

Table 1. Histopathological scoring of control and formaldehyde group.

Values are represented as mean ± SD. Mann-Whitney U test was performed.

*** P<0.001, versus control

** P≤0.001, versus control

* P<0.01, versus control
Table 2. Graphic showing histopathological difference and primer antibodies expressions in control and formaldehyde groups (Scoring was determined in the periodontal membrane and alveolar process of maxilla sections in 15 different regions within the microscope field).

In the transversal section taken from the maxillary region, particularly in the periodontal ligament extending to the alveolar bone of molar teeth, fusiform fibroblast cells parallel occupied collagen fibers and no increase in the amount of connective tissue, and the capillary structure was observed to be normal (Figure 1a). In the formaldehyde group, both thickening and degeneration of collagen fibers around alveolar bone were observed. Hyperplasic fibroblast cells, congested blood vessels and mononuclear cell filtration around vessels were observed (Figure 1b). In the control group, VEGF expression in the vascular endothelial cells of the periodontal membrane was positive while it was negative in the osteocytes of the alveolar bone (Figure 2a). In the negative control of the same section, expression was negative in endothelial cells and osteocytes(Figure 2a*). In the formaldehyde group, VEGF expression in dilated vascular endothelial cells was increased while VEGF was also positively expressed observed in osteocytes in alveolar bone (Figure 2b, Table 2). In the negative control of the same section, expression was negative in osteocytes of alveolar bone (Figure 2b*). Therefore, it was thought formaldehyde can induce angiogenesis. When matrix metallopeptidase 2 levels were examined in the control group, MMP2 expression was positively observed in the collagen fibrils and fibroblast cells of the periodontal membrane (Figure 3a, Table 2). In the negative control of the same section, expression was negative in the collagen fibrils and fibroblast cells of the periodontal membrane (Figure 3a*). In formaldehyde group, no MMP-2 expression in the collagen fibers of periodontal membrane and fibroblast cells with degenerative fibers was remarkable (Figure 3b). The negative control of the same section was shown in Figure 3b*.

In the control group, osteonectin expression in the periodontal membrane and alveolar bone was observed positively (Figure 4a, Table 2),
and it was decreased especially in the bone matrix and colloidal fibers. The negative control of the same section, osteonectin expression was negative in the periodontal membrane and alveolar bone (Figure 4a*). This phenomenon was clearly observed between the alveolar bone and periodontium (Figure 4b). The negative control of the same section was shown in Figure 4b*.

**Figure 1a. Trichrom-Masson staining, control group.** Normal appearance of alveolar bone and periodontal ligament structure. Scale bar = 100µm.

**Figure 1b. Trichrom-Masson staining, formaldehyde group.** Degeneration of collagen fibers around alveolar bone (yellow arrow), dilatation and congestion in blood vessels, mononuclear infiltration in periodontal ligament (red arrow). Scale bar = 100µm.

**Figure 2a. VEGF immunostaining, control group.** Positive VEGF expression in the vascular endothelial cells of the periodontal membrane (yellow arrow), negative VEGF expression in the osteocytes of the alveolar bone (red arrow). Scale bar = 100µm.

**Figure 2a*. Negative control, Hematoxylene staining.** Scale bar = 100 µm.
Figure 2b. VEGF immunostaining, formaldehyde group. An increase VEGF expression in vascular endothelial cells (yellow arrow) positive VEGF expression in osteocytes in alveolar bone, (red arrow). Scale bar = 100µm.

Figure 2b*. Negative control, Hematoxylene staining. Scale bar = 100 µm.

Figure 3a. MMP2 immunostaining, control group. Positive MMP2 expression in the collagen fibers and fibroblast cells of the periodontal membrane (red arrow). Scale bar = 100µm.

Figure 3a*. Negative control, Hematoxylene staining. Scale bar = 100 µm.
Figure 3b. **MMP2 immunostaining, formaldehyde group.** Negative MMP-2 expression in the collagen fibers of periodontal membrane and fibroblast cells (yellow arrow). Scale bar = 100µm.

**Figure 3b*. Negative control, Hematoxyline staining.** Scale bar = 100 µm.

![Figure 3b](image)

Figure 4a. **Osteonectin immunostaining, control group.** Expression of osteonectin in fibroblast cells and collagen fibres of the periodontal membrane (yellow arrow), and osteonectin expression in the bone matrix (red arrow). Scale bar = 100µm.

**Figure 4a*. Negative control, Hematoxyline staining.** Scale bar = 100 µm.

![Figure 4a](image)

Figure 4b. **Osteonectin immunostaining, formaldehyde group.** Negative expression of osteonectin in the periodontal membrane (yellow arrow) negative expression of osteonectin in osteocyte cells of alveolar bone (red arrow). Scale bar = 100µm.

**Figure 4b*. Negative control, Hematoxyline staining.** Scale bar = 100 µm.

![Figure 4b](image)

**DISCUSSION**

Formaldehyde is a highly toxic substance, especially by inhalation, which affects the nasal and oral cavities. In a study of Bansal et al. (21) on rabbits, it was stated that even the
short term exposure (6 weeks) of concentrated vapors of formaldehyde (40% solution) cause an irritant effect on the respiratory tract and alters its functional activity and cellular morphology. Cell proliferation is increased by 5 days of inhalation at 6 ppm formaldehyde and microarray analysis shows the expression of 15 genes were altered by 5 days of inhalation at 2 ppm formaldehyde (22).

Yorgancilar et al. (22) reported that inhalation of formaldehyde resulted in nasal mucosa and connective tissue inflammation, as well as other structures in the nasal cavity, which may increase rhinitis. In our study, remarkable increased inflammation in the maxillary region, altered fibrous structure in the periodontal region and developed cellular infiltration were observed. In a previous study it was reported that 16-day and 13-week inhalation studies with glutaraldehyde in rats and mice, the nose was the primary target site (23). Lesions in the nasal cavity included hyperplasia, squamous metaplasia, necrosis, and acute inflammation. It was thought that all these could affect the alveolar bone structure by inducing periodontitis development. In periodontal disease, there is collagen degradation so deterioration develops in gingival tissue with the supporting apparatus (12). The collagen is the major extracellular matrix component of gingiva (24). So, collagen as a protein have the affinity to react with formaldehyde forming toxic hydroxymethyl compounds (13) means that being exposed to formaldehyde, gingival tissue with the binding structures for example; periodontal ligaments of teeth are degenerated. Our results also indicated the degeneration of collagen fibers around alveolar bone (Figure 1b) that may cause teeth loss ultimately.

SPARC/osteonectin production in bones is substantial and is also expressed in cementum, the outer layer of the tooth connecting to the Periodontal ligament (PDL). Periodontal disease is difficult to treat because of the associated bone loss that occurs when the PDL is degraded. Osteonectin production in bones is substantial and is also expressed in cementum, the outer layer of the tooth connecting to the PDL (25,26). In the current investigation, with immunohistochemical methods, it was identified to be decreased especially in the bone matrix and colloidal fibers. This phenomenon was clearly observed between the alveolar bone and periodontium.

Matrix metalloproteinases (MMPs), hydrolyze components of the extracellular matrix. Although the activity of MMPs has been shown to be essential in cell biological processes and many fundamental physiological events involving tissue remodelling, such as angiogenesis, bone development, wound healing and mammary involution (27).

Several MMPs have been identified in the inflamed gingival tissues: MMP-1, -2, -3, -8, -9, -13, produced by the keratinocytes, macrophages, polymorphonuclear leukocytes (19,27-29).
Their activity could be different depending on the severity of disease and the needs for extracellular matrix digestion. In some cases, MMP-1 expression extended to the lamina propria as inflammation progressed. MMP-1 increased activity could explain the change of collagen quality and quantity, since its preferred substrates are the type I and type III collagens (30). Several other researchers reported an intense MMP-1 collagenolytic activity in fibroblasts and macrophages resident in the periodontal tissue (31,32) and focused on the interrelation between MMP-1 and MMP-3 in order to amplify the proteolysis in chronic periodontitis (29). It has been reported that the induction of MMPs (such as MMP-2) in osteoblasts is essential for bone resorption. Excessive production of MMP-2, combined with the selective production of MMP-9, can lead to the acceleration of matrix degradation in pathological conditions such as periodontitis (33). In our study, no MMP-2 expression in the collagen fibers of periodontal membrane and fibroblast cells was observed in the formaldehyde administered group perhaps in the meaning of osteoblastic activity due to formaldehyde toxicity leading to matrix degeneration and impaired the collagen fiber structure which encouraged periodontitis.

VEGF is a key regulator of physiological and pathological angiogenesis, because it induces endothelial cell proliferation, stimulates angiogenesis and increases vascular permeability. Vascular Endothelial Growth Factor immunoreactivity was seen to express in vascular endothelial cells, osteoblasts, osteoclasts in resorption lacunae, in fibroblasts adjacent to hyalinized tissue, a local necrotic area in compressed zone, and in mononuclear cells in periodontal tissues of experimental animals (18). In periodontitis patients, VEGF was detected within vascular endothelial cells, neutrophils, plasma cells, and junctional, pocket and gingival epithelium (34,35). According to our examinations, VEGF expression was positive in the vascular endothelial cells of periodontal membrane but, negative in the osteocytes of the alveolar bone in the control group. On the other hand, in the formaldehyde group, VEGF expression was positive in dilated vascular endothelial cells and also in osteocytes in alveolar bone. These results suggested us that formaldehyde may induce angiogenesis.

CONCLUSIONS

In the formaldehyde group, VEGF expression was positive in two significant regions; in dilated vascular endothelial cells and in osteocytes of the alveolar bone, as a regulator of vasculogenesis and angiogenesis and a potent inducer of vascular permeability. Formaldehyde toxicity may be a consequence of decreased expression of MMP2 in osteoblasts as well as affecting the retention of MMP levels in tooth cavity, which is very low in colloid fibers. It is
thought that long term formaldehyde inhalation may disrupt the periodontal membrane and cause collagen fiber degeneration by affecting the alveolar bone matrix which may lead to periodontitis.

REFERENCES


