THE EFFECT OF VITAMIN E SUPPLEMENTATION ON ARTICULAR CHONDROCYTES & BONE MARROW OF TEMPROMANDIBULAR JOINT IN DIABETIC RATS. (HISTOLOGICAL, IMMUNOHISTOCHEMICAL AND HISTOMORPHOMETRIC STUDIES)

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ABSTRACT

Objective: The aim of the present study was to investigate the effect of vitamin E supplement; in the management of Diabetes mellitus (DM) complications on the articular chondrocytes and bone marrow of tempromandibular joint (TMJ) of the diabetic rats.

Design: Thirty healthy adult male albino rats weighing 170 ± 10 grams were randomized into three equal groups, control (group I), Streptozotocin group (group II), and diabetic group treated with vitamin E (group III). The rats were sacrificed after 8 weeks. The TMJ was examined histologically, immunohistochemically and histomorphometrically.

Results: Group II revealed degradation in the articular surface, denudation of the chondrocytes. Regeneration of the fibrocartilagenous layer was evident in group III, chondrocytes clusters were present in discrete areas of the articular cartilage particularly in the superficial and mid zones. Histomorphometric analysis showed a difference in the chondrocytes number between the three studied groups ($p < 0.001$). The least thickness of the condyler cartilage was in group II, this difference was statistically significant ($p<0.001$). The optical density(OD) of CD31 was evident in the bone marrow of group II followed by group III, with the least expression in the control group I .The difference in CD31 expression was found to be statistically significant ($p<0.001$).

Conclusions: supplementation with vitamin E as an antioxidant may be an effective therapy for the management of the chronic complications of DM on the TMJ articular cartilage.

KEY WORDS: vitamin E; chondrocytes; tempromandibular joint; Diabetes mellitus.

1. INTRODUCTION

Diabetes mellitus (DM) is a multi-system disease characterized by persistent hyperglycemia that has both acute and chronic biochemical and anatomical sequelae.(1)

Diabetes mellitus (DM) may affect the musculoskeletal system in a variety of ways. The metabolic pertubarations in diabetes (including glycosylation of proteins, microvascular abnormalities with damage to blood vessels and nerves; and collagen accumulation in skin and periarticular structures) result in changes in the connective tissue.(2)

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The most frequently encountered musculoskeletal disorders in diabetic patients are osteoarthritis and enthesopathy (mainly flexor tenosynovitis).[3] The joints affected by osteoarthritis are not only weight-bearing joints of the lower extremities but equally frequently joints of the upper extremities.[2]

Some studies have suggested that enhanced oxidation is the underlying abnormality responsible for some of the complications of diabetes. Oxidative stress is defined as excessive production of reactive oxygen species (ROS) in the presence of diminished antioxidant substance. It has been shown that oxidative stress has an adverse effect on glucose metabolism.[4] Kang [5] suggested that chondrocytes could be damaged by endogenous free radicals and these free radicals may be related to the pathogenesis of cartilage aging, degeneration, degenerative joint disease and arthritis.

Other studies, on the other hand, have discussed whether the ingestion of antioxidant vitamins could retard or perhaps reverse the oxidative damage.[6]

Vitamin E (α-Tocopherol) is a major lipid soluble antioxidant found in human plasma, erythrocytes and tissue. The efficacy of vitamin E has been studied in a variety of rheumatological disorders, including rheumatoid arthritis (RA) and osteoarthritis. If α -Tocopherol has a peripheral anti- inflammatory effect this could be related to inhibition of the arachidonic acid pathway or scavenging of free radicals.[7]

Up to our knowledge, the effect of vitamin E supplement; in the management of DM complications on the articular chondrocytes and bone marrow of TMJ of the diabetic rats has not been discussed before. It is thus the aim of this research to study this effect histologically, immunohistochemically and histomorphometrically.

2. MATERIALS AND METHODS

2.1 Animals: Thirty healthy adult male albino rats weighing 170 ± 10 grams were utilized in the study. The rats were obtained and housed in the animal house in the faculty of oral and dental medicine, Cairo University. Animals were kept under constant conditions for food, water, and temperature throughout the experimental period. All animals received human care in compliance with the national institutes of health criteria for care of laboratory animals

2.2 Chemicals

2.2.1.Streptozotocin STZ : Diabetes was induced by a single injection of STZ (Sigma Aldrich Chemical CO. St. Louis, MO 63103 USA) (40 mg/kg, i.p., 4% w/v in 0.1 M cold citrate buffer, pH 4.5).[8]

2.2.2.Vitamin E: provided by (Sigma Aldrich Chemical CO. St. Louis, MO 63178 USA) (100 mg/kg/day, i.p., 10% w/v in olive oil). [8]

2.3. Experimental groups:

The animals were divided into three equal groups (10 rats each) as follows:

2.3.1.Group I (control group):

Consisted of 10 healthy untreated animals.

2.3.2.Group II (STZ group):

Consisted of 10 animals, each received a single intra-peritoneal injection of STZ solution.

After 3 days, rats with blood glucose levels greater than 300mg/dL were accepted to be diabetic.

2.3.3.Group III (diabetic group treated with vitamin E):

Consisted of 10 animals, each received a single i.p. injection of STZ. Two weeks after STZ induced diabetes, the rats received daily (at 12:00 pm) a single i.p. injection of vitamin E for a period of 6 weeks.

The rats of the three experimental groups were sacrificed after 8 weeks

2.4. Light microscopic examination

The heads were cleaned from the surrounding soft tissues and specimens were immediately fixed in 10 % neutral formalin for 48 h., and then rinsed in distilled water. The heads were then decalcified in a solution containing equal parts of concentrated formic acid and 20% sodium citrate. After decalcification was completed, the tempro-mandibular joints were dissected out, dehydrated in alcohol and embedded in paraffin. 20-30 sections of 5um were cut from each specimen. The sections were subjected to haematoxylin and eosin stain according to the conventional method. Histopathologic examination was performed using light microscopy.

2.5. Immunohistochemistry (IHC)

Sections of the TMJ were mounted over optiplus slides. Tissue sections were deparaffinized by incubating them in xylene over night. The sections were then incubated in the oven at 56˚c for 20 minutes. Tissue sections were rinsed several times in phosphate buffer saline (PBS), and then immersed in 0.3% hydrogen peroxide to block endogenous peroxidase. To eliminate non specific antibody binding, normal goat serum blocking solution was applied for 10 minutes. The primary antibody used was the monoclonal mouse anti- human CD31 (Dako, catalog. MO 887). The tissue sections were incubated with the primary antibody overnight in moist chamber at 4˚c then rinsed with PBS, 3 times, 2 minutes each. The sections were labeled with a streptavidin- biotin method using Dako- LAB vision (catalog CA 94539). The tissue sections were visualized with freshly prepared solution containing 3,3 diaminobenzidin (DAB). The TMJ sections were finally counterstained with methylgreen and viewed under light microscopy.

2.6 Histomorphometric analyses

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.

2.6.1. Chondrocytes counting, and thickness of condyler cartilage

The image analyzer system was used to count the number of chondrocytes per unit area (Fig.1). The image analyser was also used to measure the thickness of the condyler head (Fig.2)

2.6.2. Optical density

The Optical density (OD) of CD 31 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. Mean values were obtained for each specimen (Fig.3).

Data obtained from histomorphometrical analysis were statistically described in terms of range, mean standard deviation (SD). 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 0.001 was considered statistically significant. All statistical calculations 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.

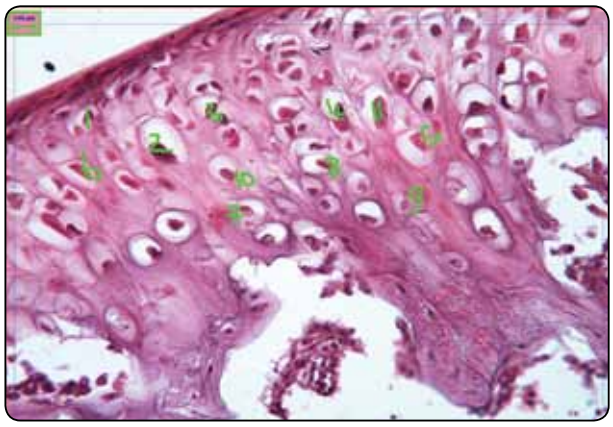

Fig. (1) A light micrograph showing the counting of the chondrocytes using the image computer analysis system.

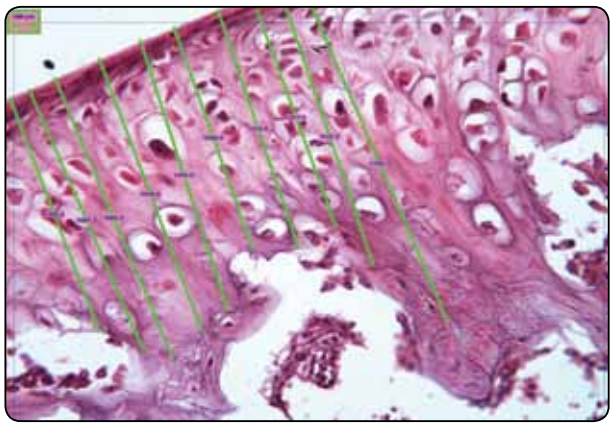

Fig. (2) A light micrograph showing the measurement of the condyler cartilage thickness, from the fibrous articular layer down to the entire thickness of the cartilage, using the image computer analysis.

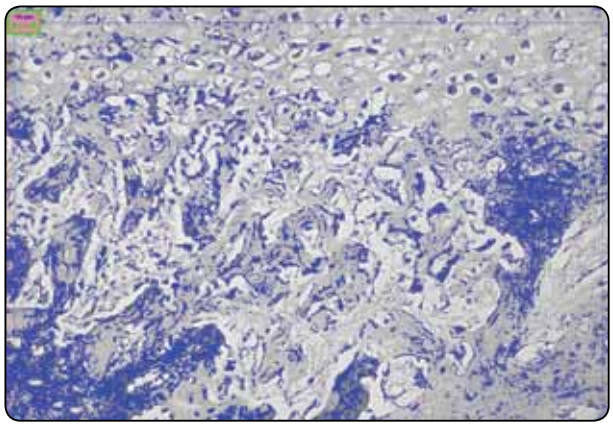

Fig. (3) Areas of positive immuno-reactivity of CD 31 in articular cartilage masked by a blue binary color

3. RESULTS

3.1. Histological results

3.1.1. Group I

Histological analysis of the control group I showed normal patterns of the articular cartilage. The articular cartilage revealed intact superficial, mid and deep zones. Chondrocytes were evenly distributed in the articular cartilage (Fig.4).

3.1.2. Group II

Group II revealed degradation in the articular surface in the form of delamination of the fibrocartilagenous layer. Denudation of the chondrocytes was evident particularly in the mid and deep zones. A change in the subarticular bone was also exhibited in the form of thinning of the bone trabeculae, and widening of the marrow spaces in between (fig.5).

3.1.3. Group III

Regeneration of the fibrocartilagenous layer was evident in group III sections, with evidence of chondrocytes columns arrangement in some areas of the articular cartilage. Chondrocytes clusters were present in discrete areas of the articular cartilage particularly in the superficial and mid zones. Resuming of normal subchondral bone architecture could be also observed (fig.6).

3.2 Immuno-histochemical results:

3.2.1. Group I

Examination of sections of the TMJ of rat control group incubated with CD31 antibody demonstrated the presence of CD31 immunostaining with weak, hardly detectable distribution throughout the bone marrow cells (fig.7).

3.2.2. Group II

CD31 immunostaining was distributed throughout the whole TMJ structures but strong immunostaining intensity was detected in aggregated platelets of the bone marrow (fig.8).

3.2.3. Group III

Examination of the immunostained sections of diabetic group treated by vitamin E showed mod-

erate homogenous membranous and cytoplasmic CD31 staining in all bone marrow cells (fig.9).

3.3. Morphometric analysis

The number of chondrocytes present in the condyler cartilage in the three studied groups is summarized by means, standard deviation and median in (Table 1). The highest number of chondrocytes was found to be in the control group I followed by group III, Group II, on the other hand, represented the least number of chondrocytes. The difference in the chondrocytes number between the three studied groups was statistically significant ($p< 0.001$).

The thickness of the condyler cartilage of group I was thicker than both groups II and III, while the least thickness was the diabetic group II, this difference was statistically significant ($p<0.001$). (Table 2)

The OD of areas occupied by active CD31 of the control and experimental groups is summarized by means, standard deviation and median in (Table 3). An increase in the OD of CD31 was evident in the bone marrow of group II followed by group III, the least expression of CD31 was exhibited in the control group I . The difference in CD31 expression was found to be statistically significant ($p<0.001$).

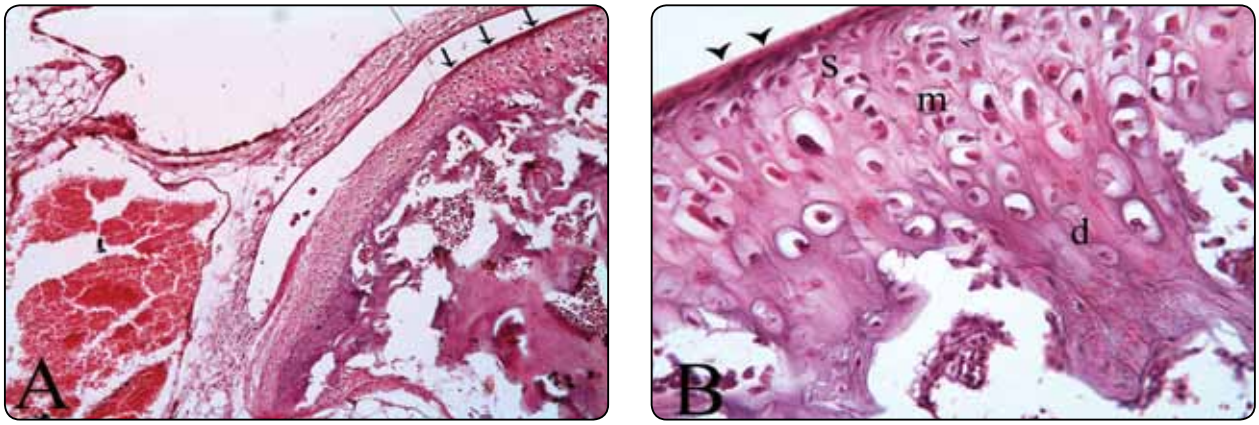

Fig. (4) A photomicrograph of the control group I showing :(A) normal pattern of the articular cartilage (arrows). (H&EX100). (B) higher magnification revealing a healthy articular cartilage having intact superficial (s), mid(m) and deep (d)zones. Intact fibrous articular layer (arrow heads) (H&E X400)

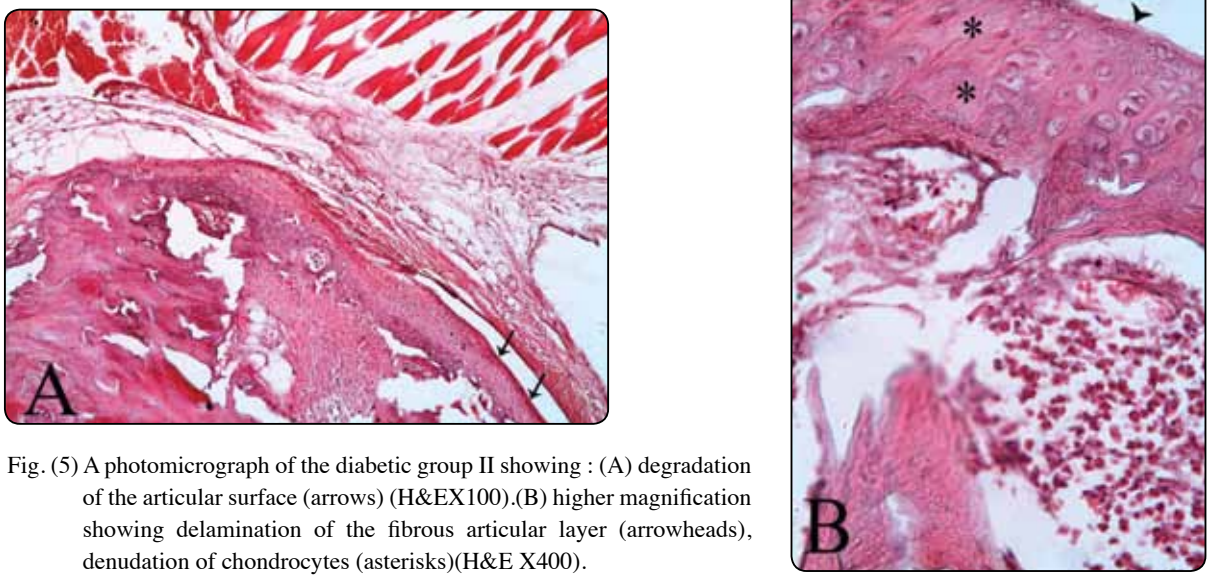

Fig. (5) A photomicrograph of the diabetic group II showing : (A) degradation of the articular surface (arrows) (H&EX100).(B) higher magnification showing delamination of the fibrous articular layer (arrowheads), denudation of chondrocytes (asterisks)(H&E X400).

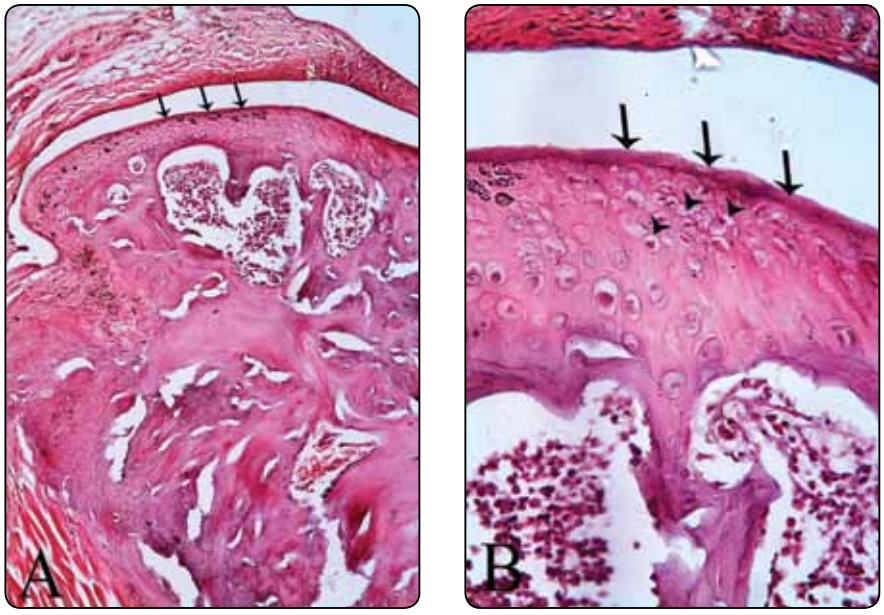

Fig. (6) A photomicrograph of group III showing : (A) renewal of articular cartilage cells arrangement (arrows) (H & EX100). (B) higher magnification revealing evidence of chondrocytes regeneration, chondrocytes clusters particularly in the superficial and mid zones (arrow heads), note the renewal of the fibrous articular layer (arrows) (H&EX400) .

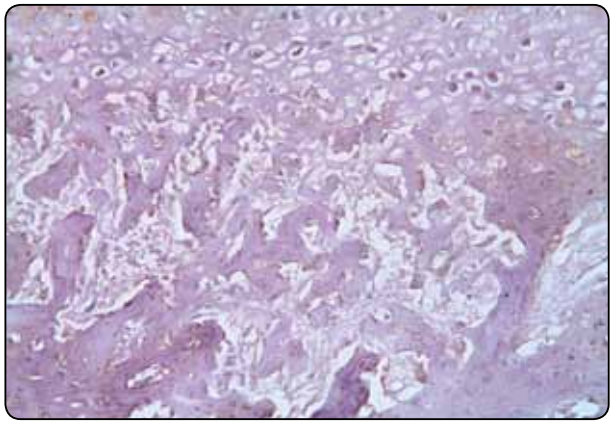

Fig. (7) A photomicrograph of the control group I showing: a weak CD31 immunoreactivity in the bone marrow (LAB- SA. X 200).

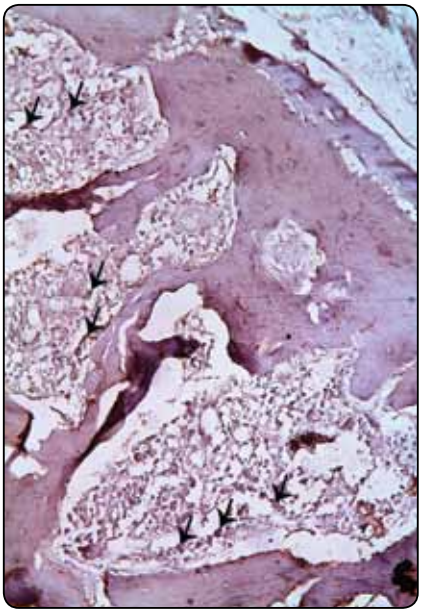

Fig. (8) A photomicrograph of the diabetic group II showing: a more intense CD31 reactivity in the bone marrow cells (arrows). (LAB- SA. X200).

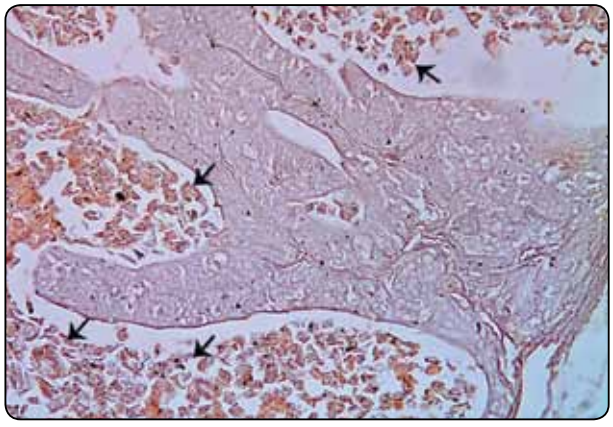

Fig. (9) A photomicrograph of group III showing: a moderate reactivity of CD31in the bone marrow cells (arrows) (LAB- SA. X200).

TABLE (I) Difference in mean chondrocyte counting between different groups using ANOVA statistical test

Group	mean chondrocyte counting	
	M±Sd	p-Value
GpI	83.0±2.58	0.000**
GpII	41.0±1.58	
Gp III	57.0 ±1.22	

***High Significant difference, (p<0.001).*

TABLE (II) Difference in mean thickness of condyler cartilage between different groups using ANOVA statistical test.

Group	mean thickness of condyler head	
	M±Sd	p-Value
Gp I	146±16.9	0.000**
Gp II	77.5±4.45	
Gp III	96.4 ±6.2	

***High Significant difference, (p<0.001).*

TABLE (III) Difference in mean CD31 optical density between different groups using ANOVA statistical test.

Group	mean CD31 optical density	
	M±Sd	p-Value
Gp I	36.4 ±1.66	0.000**
Gp II	82.4±1.93	
Gp III	51.5 ± 1.98	

***High Significant difference, (p<0.001).*

4. DISCUSSION

The precise etiology of most of the musculoskeletal disorders -such as osteoarthritis- and diabetes mellitus is not clear, neither is the reason for their higher prevalence in type I and type II DM.[9&10]

In the present study, Streptozotocin was used to induce diabetes in rats. Streptozotocin can induce insulin deficiency (type I DM) by destruction of pancreatic β-cells, and act through a mechanism that involves free radical generation.[11]

Articular cartilage is an avascular, aneural and alymphatic connective tissue designed to distribute mechanical load, and provide a wear resistant surface to articulating joints. [12] Chondrocytes are the only cells found within the articular cartilage and they are responsible for synthesizing and degrading extra cellular matrix and type II collagen, non collagenous proteins and proteoglycans. [13]

In the herein study, the histological results in group II showed delamination of the fibrous articular layer, as well as denudation of the chondrocytes. Separation of the articular cartilage, and its break off of the underlying subchondral bone.

Chondrocytes are highly glycolytic and require a regular supply of glucose for optimal ATP production and cell homeostasis. [14&15] Since the steady supply and transport of physiological levels of glucose is critical for chondrocytes viability and matrix synthesis. The unstable glucose concentration in the extra cellular microenvironment of the chondrocytes could have impaired the anabolic activities and thus promoted the revealed joint pathologies in group II of the present study. Corroborating with our results, it was hypothesized that since the cartilage is highly dependent on glucose as a metabolic fuel and structural components, the extreme blood glucose (and hence tissue/ synovial fluid glucose) can cause complications for bones and joints. [16]

Oxidative stress has been implicated in the development of diabetic complications through mechanisms that are probably linked to both hyperglycemia and hyperinsulinaemia. [9,17&18] The role of ROS as a tissue damaging agent is currently suspected in arthritis. [19] In this condition, ROS are generated by mononuclear phagocytes in particular when these cells adhere to the cartilage in an attempt to engulf immune complex located beneath the articular surface and discharge ROS and other damaging agents [20]. Consistent with this hypothesis, it has been shown that ROS can interfere with various biological processes clarified in the revealed histological results of group II in the present research.

Alteration in the number of the articular cartilage chondrocytes revealed in the morphometric results of the current research, revealed a decrease in both the chondrocytes number as well as articular cartilage thickness between the three studied groups. The later results were statistically significant $p<0.001$. It is clearly evident that in group II of the herein study, the decrease in the number of chondrocytes was directly proportional to the thickness of the articular cartilage. Consistent with a previous study [20] ,the effect of ROS on the biosynthsis of proteoglycans molecules by rabbit articular cartilage seems to be biphasic, and dependent on their concentration. At low concentration they frequently stimulate, and at higher concentration they inhibit proteoglycans synthesis.

In the ongoing study, the potential benefit of the appropriate use of vitamin E with the diabetic rats (group III), showed a significantly improved histological picture than that revealed in (group II). Regeneration of the fibroaricular cartilage, chondrocytes clusters and the condyler cartilage were seen adherent to the subchondral bone.

On the other hand, immunohistochemical results of the herein research revealed a mild and moderate reactivity of CD 31 in groups I and III respectively. On the other hand a significant up regulation of CD31 was present in group II. The alteration in CD31 reactivity was statistically significant $p<0.001$.

CD31 is a cluster of differentiation molecule. It is also called PECAM-1 for Platelet Endothelial Cell Adhesion Molecule. CD31/ PECAM-1 is a member of the immunoglobulin gene superfamily found on platelets, macrophages, granulocytes, T / NK cells, lymphocytes, megakaryocytes, osteoclasts, neutrophils and endothelial cells. [21]

Although the precise function of CD31/ PECAM-1 is not known in all the cell types that express it, recent investigations have demonstrated its ability to function as a cellular adhesive molecule. It has both homophilic and heterophilic adhesive properties that potentially play significant roles in a variety of important processes such as leukocyte recruitment at inflammatory sites, regulation of release of bone marrow leukocytes. [22]

It is thus clearly evident that Diabetic group II of the ongoing study is associated with a hypercoaguable state driven by platelet hyperreactivity which has been revealed by the CD31 upregulation. The latter result is in accordance with that of Eibl et al.[23] In diabetic microangiopathy, increased platelet and leukocyte activation and heterotypic aggregation are present.[24,25,26] Leukocytes from diabetic subjects exhibit enhanced adhesion molecule expression increased aggregability as well as impaired cell deformability.[27]

Platelets are essential for haemostasis, and abnormalities of platelet function may cause vascular disease in diabetes. Diabetic patients have hyperreactive platelets with exaggerated adhesion, aggregation and thrombin generation. The entire coagulation cascade is dysfunctional in diabetes. [28, 29]

Vitamin E treatment in group III of the current research have shown that improved metabolic con-

trol can decrease platelet activation revealed by the significant decline of the platelet activation marker CD31. Eibl and coworkers [23] were able to show that improved metabolic control can decrease platelet activation in patients with type 2 diabetes. After three months of improved metabolic control, a significant decline of the platelet activation markers CD31, CD49b, CD62P and CD63 was noted. It was also suggested that vitamin E might exert a favorable effect by stabilization of platelet membranes and reduction in the low- density lipoprotein oxidation. [30]

In summary, there is convincing evidence for a strong association between oxidative stress and DM. Available data indicate that supplementation with vitamin E as an antioxidant may be an effective therapy for the management of the chronic complications of DM on the TMJ articular cartilage.

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