The effect of gingival aging in diabetic and non-diabetic status. An experimental study

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ABSTRACT

Major gingival-periodontal changes according to age have been observed in both diabetic and non-diabetic rats. Male Wistar rats weighing 200-220 g were divided into two groups: 1) Non-diabetic (ND) and 2) Diabetic (D) by receiving an intraperitoneal (ip) dose of streptozotocin (STZ) (50 mg /kg). Animals from both groups (ND and D) were euthanized at 4, 8, 12, 17 y 25 weeks after treatment with saline solution or STZ. Glycemia values in ND rats were 5 to 6 mmol/L, while in D, glycemia increased progressively between weeks 4 and 25, with values ranging from 18.3±2.1 to 39.3±2.7 mmol/L. Oxidative stress differed significantly in gums of ND and D rats. ND: lipid peroxidation: Malondialdehyde (MDA): 8.52±1.2 to 15.5±2(nmol/mgP); superoxide dismutase (SOD): 37.1±4.2 to 21.2±1.3 (U/100mgP); D: MDA 13.1±1.6 to 22.9±2.7 (nmol/L); superoxide dismutase (SOD): 17.7±0.8 to 9.±0.2 (U/100mgP). Vascular permeability (VP) and gingival edema (E) showed significant changes between ND and D rats from 4 to 25 weeks. ND: PV: 10±0.2 to 16.1±1.3 (EB ug/g dry t); E: 0.9±0.1 to 4.1±1.3 ml; D: PV: 12±1.2 to 24.4±1.6 (EB ug/g dry t); E: 2.2±0.2 to 8.4±1.3 ml. Aging produced progressive natural changes in oxidative stress, VP and gingival E. In diabetic animals, changes in oxidative stress, VP and gingival E were observed early and were progressively more significant than for ND. According to these results, non-diabetic gingival modifications develop naturally with age, while in aging associated to diabetic disease, hyperglycemia increases progressively and early.

Key words: *Diabetes Mellitus, gingiva, periodontitis, oxidative stress, capillary permeability, edema.*

El efecto del envejecimiento gingival en el estado diabetico y no diabetico. Estudio experimental

RESUMEN

Se han observados importantes cambios gingivo-periodontales en función de la edad tanto en ratas no diabéticas como en ratas diabéticas. Ratas machos Wistar de 200-220 g de peso corporal fueron separadas en dos grupos: 1) No diabéticas(ND) ; 2) Diabéticas (D), por haber recibido una dosis intraperitoneal (ip) de estreptozotocina (STZ) (50 mg /kg). Ambos grupos de ratas (ND y D) fueron sacrificados a las 4, 8, 12, 17 y 25 semanas de edad después del tratamiento con solución salina o con STZ. En ratas ND las los valores de glucemia fueron de 5 a 6 mmol/L, en tanto que en las D las glucemias se observaron progresivamente aumentadas entre las 4 y las 25 semanas con valores entre 18.3±2.1 a 39.3±2.7 mmol/L. El estrés oxidativo mostró diferencias significativas entre las encías de animales ND respecto a los D; ND: peroxidacion lipidica: Malondihaldeido (MDA): 8.52±1.2 a 15.5±2(nmol/mgP);superoxido dismutasa (SOD):37.1±4.2 a 21.2±1.3 (U/100mgP); D : MDA 13.1±1.6 a 22.9±2.7 (nmol/L); Superoxidodismutasa :SOD 17.7±0.8 a 9.±0.2 (U/100mgP). La permeabilidad vascular(PV) y el edema(E) gingival mostraron cambios significativos entre las 4 y las 25 semanas de edad entre los animales ND respecto a los D : ND : PV : 10±0.2 a 16.1±1.3 (EB ug/g t seco); E :0.9±0.1 a 4.1±1.3 ml; D: PV :12±1.2 a 24.4±1.6 (EB ug/g t seco); E 2.2_/- 0.2 a 8.4± 1.3 ml. El envejecimiento produjo cambios progresivos naturales en el estrés oxidativo, PV y E gingival. En tanto que en el estado diabético los cambios del estrés oxidativo, PV y E gingival se observan temprano y fueron progresivamente más significativos comparados con los ND. De acuerdo a estos resultados las modificaciones gingivales no diabéticas se desarrollan naturalmente en función de la edad, en cambio en la senectud asociada con enfermedad diabética la hiperglucemia aumenta progresiva y tempranamente.

Palabras clave: *Diabetes mellitus, gingiva, periodontits, estrés oxidativo, permeabilidad capilar, edema.*

INTRODUCTION

According to Van der Velden, the tissues surrounding the periodontium undergo major changes with aging[1]. Aging affects all tissues in the body, producing major anatomical and structural changes, and is associated to a decline in the control of tissue homeostasis with progressive deterioration and loss of ability to repair[2,3]. Diabetes mellitus (DM) is a well-known metabolic disorder due to which chronic inflammatory reactions affect and/or

modulate the structures and function of different tissues in patients[4,5]. DM occurs in various forms; however, all forms of DM are characterized by hyperglycemia and abnormal glucose metabolism, resulting in a deficit in insulin production or insulin action. When the prevalence of diabetic disease is associated to aging, there is an increase in susceptibility to a number of autoimmune and infectious diseases. Diabetes has been suspected of contributing to the deterioration of oral and systemic health because there is higher prevalence of infections in diabetics than in non-diabetics. Moreover, DM and periodontal disease are common chronic diseases -especially in the elderly- and are related to each other. Old age alone is not considered to be a risk factor for the development of gingival-periodontal pathology[6,7], but alterations in the gingival tissue may be caused by nutritional factors, metabolic factors and/or systemic diseases which determine gingival-periodontal alterations[8,9]. When the local oral system is modified, there may be gingival-periodontal inflammatory alterations according to age, while in the associated diabetic condition, old age fosters early production of inflammatory cytokines as an effect of hyperglycemia. Considering old age and diabetic status, our hypothesis is that individuals with gingival-periodontal disease may be at high risk of developing other systemic inflammatory diseases beyond gingival-periodontal disease.

MATERIALS AND METHODS

Male Wistar rats weighing 200-220 g were obtained from the Animal Facility at Instituto Malbran (Buenos Aires, Argentina). Animals were placed in boxes with controlled room temperature (23±2 º C) and a 12-hour light cycle. They were fed *ad libitum* on standard pellets and water. All experiments were conducted in agreement with the Ethics Committee of Universidad Argentina John F Kennedy (Code 621).

Treatment of animals

The animals were divided into 2 groups: 1) non-diabetic (ND) and 2) diabetic (D) by streptozotocin (STZ) (50 mg/kg). Five control animals and 6 diabetic animals were used for each experimental time. They were euthanized at 4, 8, 12, 17 and 25 weeks from the beginning of the experiment. Diabetic status was confirmed as from 76 hours after STZ injection and throughout the experimental period by means of an automatic glycemia analyzer (Contour TS, Bayer Diagnostic, BA, Argentina). Gingival tissue was obtained at the level of lower incisors and molar zone, to be used in biochemical determinations, vascular permeability and edema.

Experimental procedure

Control and diabetic rats were anesthetized ip with urethane (200mg/kg). Extracted tissue was placed in 50 mM tris-HCl buffer (Ph 7) at 4 ºC, dried on filter paper and homogenized in 10 mM cold Tris-HCl buffer pH 7.2. Samples were centrifuged at 10.000 rpm for 30 minutes. The supernatant was used for biochemical determinations.

Biochemical determinations

Protein concentration was determined using Bradford's method[10]. Lipid peroxidase (MDA) concentrations were determined following Okhawa et al [11]. Lipid peroxidase levels were measured by thiobarbituric acid (TBA) reaction. Tissue supernatant (50 ul) was added to tubes containing 2 ul butylated hydroxytoluene (BHT) in methanol. Then 50 ul 1 M phosphoric acid were added and finally 100 ul 0.8% TBA aqueous solution. The mixture was incubated for 60 min at 60 ºC. Absorbance was measured at 532 nm. TBA levels were expressed in nmol/mg P. The following reagents were used to determine superoxide dismutase (SOD): 0.3mM xanthine oxidase, 0.6 mM diethylenetriamine-penta acetic acid (DETAPAC), 150 uM nitroblue tetrazolium (NBT), 400 nM sodium carbonate and 1g/L BSA. The principle of the method is based on the inhibition of NBT reduced by the superoxide radicals produced by the xanthine oxidase/xanthine system. For the assay, 10 ul supernatant were added to 200 ul NBT diluted in 1.9 ml 50mM tris_HCl, pH 8.0, 0.1 mM DETAPAC and 0.1 mM hypoxanthine. Finally, 20 ul xanthine oxidase were added in 20 sec. It was incubated at 25 ºC for 20 min. At the end of the reaction, 0.8 mM cupric chloride was added and it was read at 560 nm. SOD activity was expressed in U/100mg[12].

Vascular permeability and tissue edema

Similar groups of control rats (n=5) and diabetic rats (n=6) were prepared with the same time sequence to measure vascular permeability and determine gingival edema, following Simard et al[13]. Animals in both groups, not anesthetized, were

injected with Evans Blue (EB) (20 mg/kg) through the tail vein. After 20 min the animals were euthanized, the thoracic cavity opened and 15 ml saline solution –heparin perfused through the right ventricle. Extracted tissues were divided into two portions and weighed. The first portion was desiccated at 60 ºC for 24 hs, while the second portion was immersed in 2 ml formamide for 24 h at 24 ºC to extract the dye (EB). Dye concentration was measured at 620 nm against an EB standard. The second portion was used to determine tissue edema, calculated by the difference between wet weight and dry weight, which reflects the amount of water (g) retained and calculated per ml (1g=1ml).

Statistical analysis

Statistical data were analyzed as means ± standard error of the mean (SEM). Analysis of variance (ANOVA) was accompanied by the Student-Newman-Keuls test for multiple comparisons, which was used to evaluate at a significance of $p<0.05$.

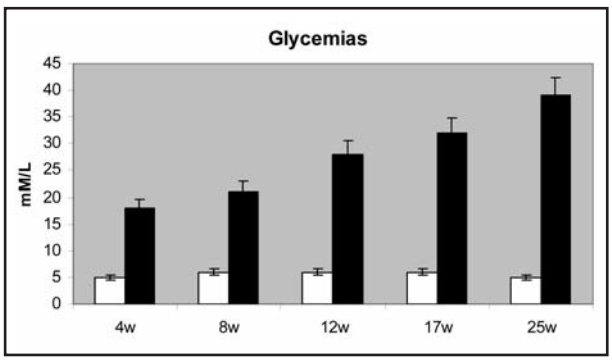

Fig. 1: Changes in blood glucose levels (mmol/L) in non-diabetic rats (White bars) and diabetic rats (Black bars) from 4 to 25 weeks. Data represent mean ±SEM of 5-6 rats. (Black bars vs. white bars) p< 0.05.

RESULTS

Blood glucose level was found to increase progressively in diabetic animals compared to non-diabetic animals from week 4 to week 25 (Fig. 1). At 4 weeks of diabetes, the increase was greater than 300%, while at 25 weeks it was 6.5 times higher than in controls ($p<0.05$). MDA concentrations in gingival tissue at 4 and 25 weeks were also significantly higher in D than in ND, with values of 53% at weeks 4 and 25 of diabetes ($p<0.01$) (Fig. 2). On the other hand, superoxide dismutase (SOD) was significantly lower in diabetic rats than in controls at week 4 (-53.1 %) and week 25 (-57.6 %) ($p<0.05$ and 0.01) (Fig. 3). STZ induces diabetes associated to marked alterations of vascular permeability over 4 to 25 weeks. Fig. 4 shows that permeability to EB bound to protein increased by over 100% in diabetic rats compared to non-diabetic controls ($p<0.05$). Gingival edema also showed alterations at weeks 4 and 25. Fig. 5 shows the progressive increase in gingival edema in diabetic rats to over 100% compared to non-diabetic rats.

DISCUSSION

Aging is a continuous, complex process that gradually affects various body tissues. Aging is associated to a progressive decline in the ability to maintain tissue homeostasis and alterations in the composition of the extracellular matrix[14,15]. Aging also causes abnormal functioning as well as qualitative and quantitative modifications and structural changes in morphology[16]. Old age produces aging changes with atrophies and reduction of the junction between epithelium and

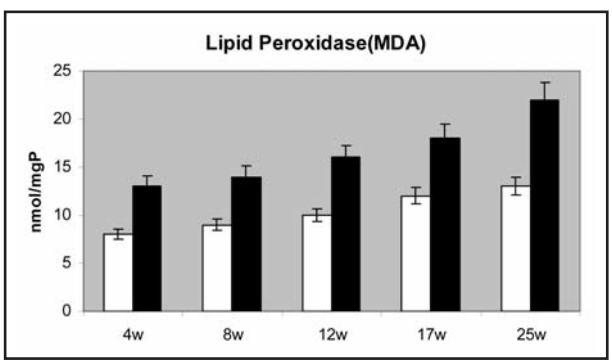

Fig. 2: Age-related changes in gingival lipid peroxidation (MDA) in non-diabetic rats (white bars) and diabetic rats (black bars) from 4 to 25 weeks. Data represent mean ± SEM of 5-6 rats. (Black bars vs. white bars) p< 0.05.

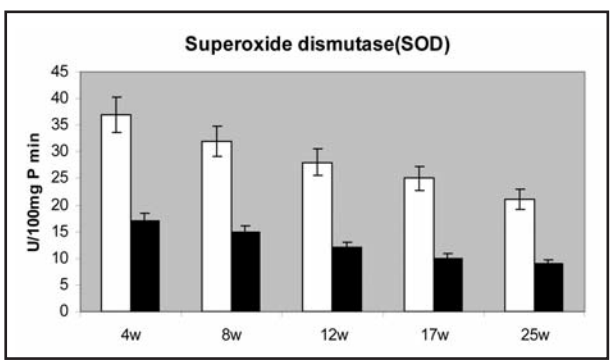

Fig. 3: Antioxidative enzyme (SOD) activity in gingival tissue in non-diabetic rats (white bars) and diabetic rats (black bars) from 4 to 25 weeks. Data represent mean ± SEM of 5-6 rats (Black bars vs. white bars) p< 0.05.

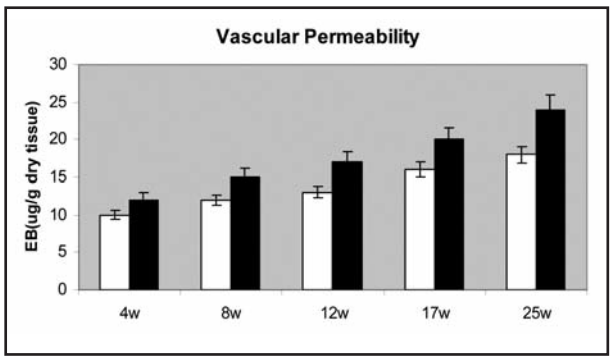

Fig. 4: Changes in vascular permeability (EB) in gingival tissue in non-diabetic rats (white bars) and diabetic rats (black bars) from 4 to 25 weeks. Data represent mean ± SEM in each group(5-6 rats). Black bars vs. white bars) p< 0.05.

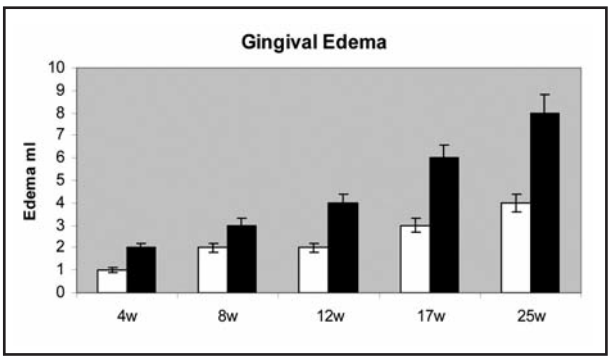

Fig. 5: Edema induced in gingival tissue of non-diabetic rats (white bars) and diabetic rats (black bars) from 4 to 25 weeks. Data represent mean ± SEM of 5-6 rats in each group (black bars vs. white bars) p< 0.05.

connective tissue. It has been established that microvascular changes in gums and alveolar mucosa are similar to those in other organs and tissues affected by DM[17]. The association of age produces differential expression of oxidative stress on gingival tissues in non-diabetic animals compared to diabetic animals of the same age. Gingival tissue deteriorates under persistent oxidative stress, inducing an inflammatory reaction due to the presence of microflora in the oral cavity[18]. The loss of homeostatic balance between proteolytic enzymes and their inhibitors and reactive oxygen species (ROS) and the reduction of the antioxidant defense system which protects and repairs living tissues, cells and molecular components, are the main factors in tissue damage. In the aging process, gingival antioxidant defenses decline late, but when aging is associated to diabetes, the two factors contribute to early amplification of the response on tissue damage. Glucose intolerance is a major risk factor for endothelial inflammation in diabetic subjects, compromising microvasculature and altering wound healing[19]. Natural aging in ND rat models shows that old age produces alterations in lipid peroxidation (MDA) as one of the main free radicals. It is interesting to note that our results showed that SOD, as an antioxidant defense system, declines significantly in ND rats as from week 12, regardless of their glycemia status. Vascular permeability and edema showed a significant late increase in ND rats. Together, these results document the fact that phenomena related to aging produce modifications in the oxidative stress of the gingival microvascular system. Other evidence in animal models confirms that high levels of lipid peroxidation and oxidative DNA reduce the synthesis of collagen in the gums of diabetic rats[20,21]. Previous studies have shown antioxidant reduction in diabetic condition[22] and reduction of antioxidant defenses[23]. Age associated to differential expression of inflammation in ND gingival tissue may specifically reflect the influence of age status by changes dependant on global age. Diabetic hyperglycemia measured over long-term diabetes is associated to damage and complications in several organs[24]. Moreover, early hyperglycemia (4 weeks) induces early oxidative stress in gums and increases vascular permeability and edema. The regulation of vascular permeability in normal conditions or pathophysiological conditions such as diabetes is related to the release of certain agents (NO, eicosanoids, bradykinin, free radicals or ROS) which affect microvascular homeostasis. There is an important relationship between levels of hyperglycemia and progression of gingivitis in diabetic patients[25]. Natural aging reduces defense capacity and develops inflammatory problems. Diabetes and aging produce immunosenescence, and chronic periodontal disease exhibits a two-way relationship centered by a local increase and a systemic inflammatory response with severity and rapid progression of the gingival-periodontal tissue, attributed to the possible bacterial increase and host contribution[24]. Hyperglycemia induces a pre-inflammatory state in microvasculature and increases vascular permeability and edema. Tissue and/or organ homeostasis in health is maintained by the continuous process of microcirculation, defined as the exchange between blood and tissue

fluids. This process includes the fluid and its spatial distribution, capillary pressure and permeability of the wall and the potential exchange area. If oxidative stress is altered in diabetic condition, one of the earliest signs of inflammation with increase in fluid filtration from capillaries to tissue, microfiltration and capillary vasodilation increase[26]. These assessments are consistent with hyperfiltration producing alterations in the microcirculation of gingival tissue in diabetic patients[27]. Hyperglycemia causes intravascular accumulation of polyols, leading to an increase in osmolarity with intramural water retention[28]. Moreover, the potential role of an immune receptor or molecular signal in the gingival tissue during the inflammatory process is also suggested as important in aging and progression of diabetic disease. The increase in the expression of C5a and TREM in old age contributes to elevated gingival-periodontal inflammation because these receptors participate actively in the amplification of the host inflammatory response[29,30].

To conclude, this study showed that with age, the activity of free radicals, vascular permeability and edema increase more significantly and earlier in diabetics than in non-diabetics. Aging and diabetes have greater impact on the gingival tissue of diabetics than non-diabetics. In both cases, oxidative stress is present, but long periods of diabetic disease produce early modifications and serious damage, with declining capacity to maintain tissue homeostasis.

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