ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Experimental animal model in a histological study of drug-induced gingival overgrowth

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Abstract

Gingival overgrowth (GO) is a pathology with important aesthetic and functional implications and with a multifactorial pathogenesis. Incriminated etiological factors include antihypertensive, antiepileptic and immunosuppressant medication. We aimed to evaluate the induction of gingival overgrowth on experimental rats, depending on the drug type, dose and duration. In the research conducted by us, the increase in gingival tissue production occurred gradually, depending on the administered medication and the time elapsed after its start. The study conducted shows that experimentally induced gingival overgrowth of the administered drugs is made possible by altering tissue homeostasis through altering the fibrocyte cell populations involved in the tissular turnover as well as those involved in the inflammatory process. A better understanding of the pathogenesis of this undesirable effect may lead to the development of improved management strategies for preventing it, or reducing it through non-surgical methods.

Keywords: outgrowing gingival, experimental model, acanthosis, acantholysis.

Introduction

Gingival overgrowth (GO) is a pathology manifested by an abnormal, excessive augmentation of the volume of the extracellular matrix found in the normal periodontal tissue, associated with an increase in the number of cells of the gingival mucosa (a process of hyperplasia) and an increase in the volume of these cells (a process of hypertrophy). Many etiological factors have been incriminated. Hyperplasia of the gums can be caused by local inflammation due to the existence of plaque, as well as by a certain medication (most frequently incriminated are antihypertensive, antiepileptic and immunosuppressant drugs), systemic causes, physiological ones (heredity, hormonal imbalances occurring in puberty or pregnancy) or pathologic causes (leukemia), with the involvement of growth factors, and heredity apparently may hold one particularly important role. This side effect of the administration of drugs causes aesthetic changes and clinical symptoms including pain, tenderness, bleeding, slurred speech, abnormal tooth mobility, occlusion problems, increased tooth decay and periodontal disorders [1, 2].

The understanding of gum overgrowth pathogenesis is not complete. However, the development of new techniques for prevention and new therapeutic approaches on gingival overgrowth requires a systematic approach. This should include the development and use of experimental animal models and cell cultures for GO that can be cross-correlated with studies on the human gingival mucosa. The complexity of events that contribute to overgrowth of the gum have not yet been fully elucidated, requiring approaches at a molecular level to clearly determine the pathogenesis of gum proliferation and to provide new information to assess future preventive and therapeutic possibilities [3, 4].

We proposed a study of the experimental drug-induced gingival overgrowth in order to assess its production mechanism, the variability caused by different classes of drugs incriminated in producing this pathology and the involvement of certain therapies in reducing GO. The experimental induction of drug-linked GO has been tried by many researchers, but was unsuccessful in most species of animals (monkey, cat, ferret, dog) because of the need for prolonged medication administration and high costs. The rat is more useful due to the availability of the animals, small differences in reaction to the drugs for various species and, in particular, due to the reproducibility of the experimental results [5].

Materials and Methods

The studied material was represented by 25 Wistar rats of the same age (three months old), with a body weight of 360–400 g, held in the same environment conditions, with a constant temperature between 20–24^{0}C, and receiving the same diet. The drug administration was performed in all animals for two months.

The animals were divided into four groups:

▪ Group I: Subcutaneous (s.c.) injected ×2/day with Phenytoin (PHT) sodium suspended in 0.5% Tween 80 solution (Richter Phenytoin 100 mg tablets). The doses were per kg/day and were increased every week for preventing the toxic effects up to 120 mg/kg per day (1 mL/100 g) of PHT in the first week. For preventing lethal toxic values, the PHT value was increased by

10 mg/kg every week. The body weight was measured every day, before the injection.

▪ Group II: There was administered Nifedipine solution 250 mg/kg/day – Nifedipine Terapia 20 mg tablets (125 mg/mL obtained by dissolving the Nifedipine powder into dimethyl sulfoxide – DMSO).

▪ Group III: S.c. injected with Cyclosporine 30 mg/kg/day – Equoral solution 100 mg/mL.

▪ Group IV: The control group received the same diet, without any medication, the rats being daily s.c. injected with 9‰ sodium chloride solution (saline).

The animals in the four groups were weighed at the beginning of the experiment and at the end of every week, for the administrating the drug dose according to the rat weight.

The processing of the material for the microscopic examination was done by paraffin-embedding histological technique, the sections obtained being stained with Hematoxylin–Eosin (HE) and Goldner–Szekely (GS) trichrome, within the Department of Histology, University of Medicine and Pharmacy of Craiova, Romania.

The experiment received the approval (No. 134/12.06.2015) from the Ethics and Academic Deontology Board of the University of Medicine and Pharmacy of Craiova. The animals were treated according to the principles of animal protection used for scientific purposes (Directive No. 2010/63/UE of the European Parliament and Council on September 22, 2010; Act No. 43/2014 published in the Official Monitor, Part I No. 326/May 2014).

Results

The microscopic examination of sections belonging to experimental groups compared with sections from the control group show the presence of obvious changes and sometimes-common ones but of different degrees, depending on the drug administered. These changes can be induced by the drugs administered or may be due to the inflammatory process secondary to drug administration, which disturbs the balance of cytokines, inflammatory mediators and growth factors.

The changes occurred at both the epithelial level (increased thickness, different thicknesses, and sometimes acantholysis, acanthosis, hyperkeratosis and areas parakeratinization) as well as upon the lamina propria of the gingival mucosa (collagen deposition, varying degrees of chronic inflammation). These are non-specific, for they can possibly be seen in gingival overgrowth regardless of etiology, as well as other periodontal diseases.

In the research conducted by us, increased gingival growth occurred gradually, depending on the time elapsed since the start of drug administration. It appeared in experimental groups after about four weeks of treatment and continued until the 8th week. There were differences in the appearance of the gingival mucosa in the experimental groups. In the control group, there was no significant increase in tissue.

Histopathology results obtained in the group injected with Phenytoin

Compared with the control group, the sampled tissues from the group treated with Phenytoin presented obvious microscopic changes, characterized by an extensive gingival hypertrophy. The squamous cell layer presented an increase in thickness, sometimes even manifesting in focal bloating of the epithelial cells, with the presence of a thin layer of keratin on the surface. Also present was acanthosis, to varying degrees, caused by hyperplasia of the spinous cell layer of the gingival mucosa epithelium. On some sections, at the level of the spinous cell layer we have met different degrees of acantholysis (Figure 1, A and B). By proliferating, the basal and Malpighian layers caused "glove finger form" long and thin epithelial extensions, sometimes bifurcated at their ends (Figure 1, C and D). The lamina propria is thickened, presents numerical increase of fibroblasts, which also explains the existence of a well represented fibrillary component, leading to a fibrous transformation of the gingival lamina propria (Figure 1C). The collagen fibers show different thicknesses and are arranged compactly or in intersecting strands, which determine areas of different sizes, at the level of which intercellular matrix with many fibroblasts and moderate vascularization is present (Figure 1E). Sometimes, at a subepithelial level, we have identified some increased capillary number sections, which sustain the nourishment necessary for epithelium proliferation. Alongside the fibrillary component, there also were present chronic inflammatory cells, particularly lymphocytes and plasma cells, macrophages, arranged in many areas but predominantly perivascular (Figure 1F).

Histopathology results obtained in the group treated with Nifedipine

In the cases of the group to which Nifedipine was administered, proliferation tissue was found. The identified histological changes were the epithelial hyperplasia, the increase in thickness of the lamina propria without subepithelial collagen deposition, a reduction of cellularity and a rich vascularity. The parakeratinized stratified squamous epithelium presented areas of hyperplasia alternating with areas of normal appearance (Figure 2A). At the epithelial–conjunctival interface, the epithelial ridges are prolonged, deep and interdigitated, penetrating deep into the lamina propria, and this aspect is determined by the proliferation of the basal epithelial layer. In the chorion, there are numerous fibroblasts and collagen fibers arranged in bundles (Figure 2B). The connective tissue of the lamina propria presents a chronic, diffuse inflammatory infiltrate, sometimes with a micronodular aspect, composed of mononuclear cells, lymphocytes and plasma cells (Figure 2, C and D). The number of inflammatory cells was higher during the experiment, compared with the control group, identifying a population of inflammatory cells, arranged especially perivascular. Also, on the sections, capillaries were present in increased numbers.

Histopathology results obtained in the group injected with Cyclosporine

The examination of the sections from this group indicates an increase in epithelial tissue, in the form of epithelial extensions proliferating towards the lamina propria. The epithelial hyperplasia is sometimes uneven, and there are areas in which the thickness of the normal epithelium alternates with the deep peaks present in the

"glove finger" areas (Figure 3, A–C). The epithelium presents marked ortho- and parakeratinization and interdigitations at the conjunctival–epithelial level interface (Figure 3D). In other cases, epithelial hyperplasia was moderate. At the level of connective tissue, collagen fibers are numerous, arranged in perpendicular bundles between which there can be seen a chronic type of inflammatory infiltrate, lymphocytes and plasma cells (Figure 3E). In our study, we found the presence in this group of increased blood supply, located especially subepithelial, and this location is justified by the increased needs of the proliferating epithelium (Figure 3F). Sometimes, in the sections examined dilated blood vessels were present which support the development of the extracellular component, through the increased income of substances that are necessary to satisfy the needs, which appear during the augmentation of synthesis that occurs at this level.

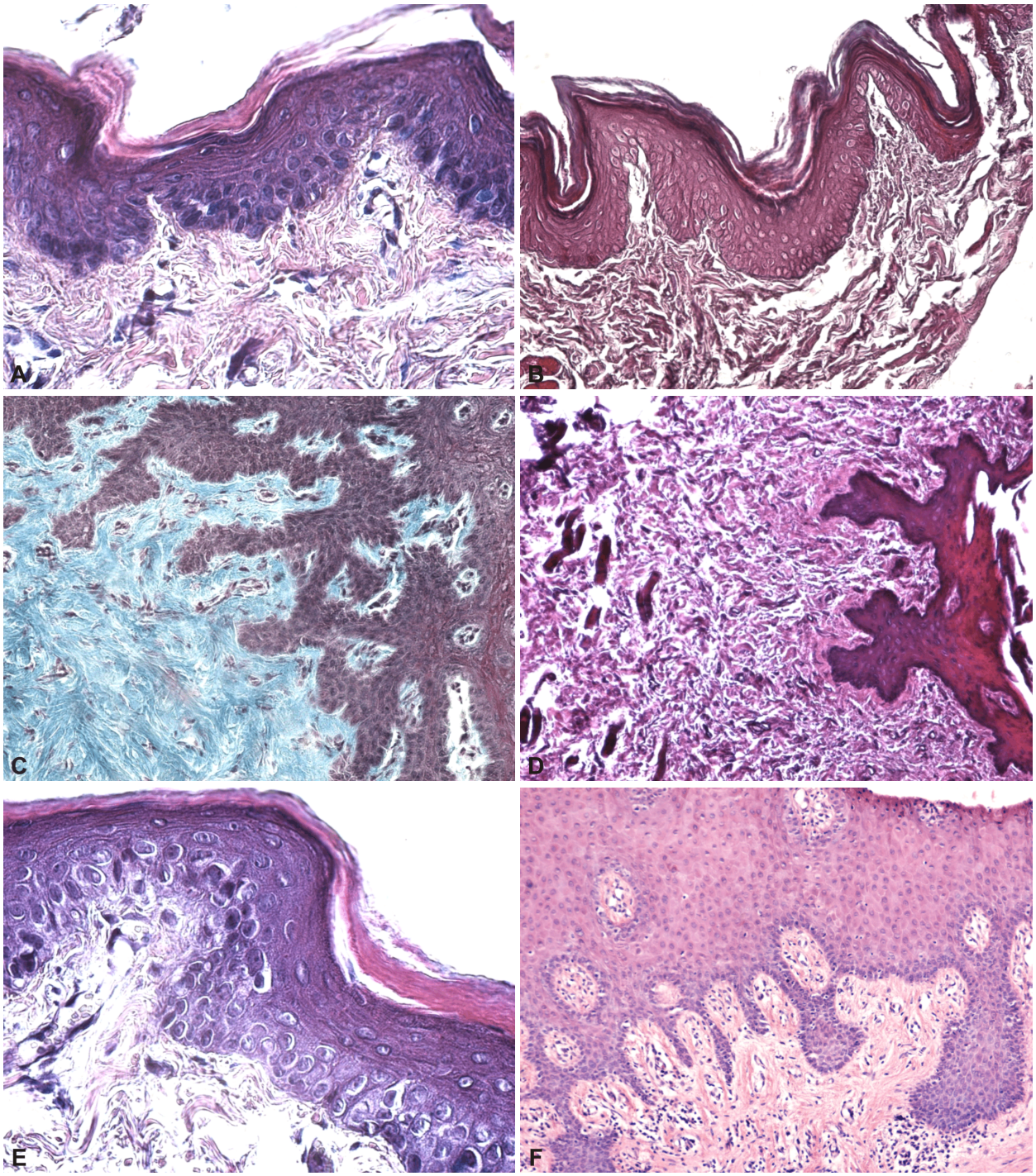

Figure 1 – ***(A) Parakeratosis, mild acantholysis. In the lamina propria, collagenous fibers placed in fascicles, moderate vascularization (HE staining, ×100); (B) Parakeratosis, mild acantholysis, increase of collagen fibrillary component in the lamina propria (HE staining, ×100); (C) Epithelial growths with a "glove finger" aspect, in the lamina propria, development of collagen fibrillary component (HE staining, ×100); (D) Epithelial prolongations in the form of "glove finger" branched at the end (HE staining, ×100); (E) Parakeratosis, acantholysis, moderate vascularization of the lamina propria (HE staining, ×200); (F) Discrete lympho-plasmocytary inflammatory infiltrate associated to the collagen fibers fascicles (HE staining, ×100).***

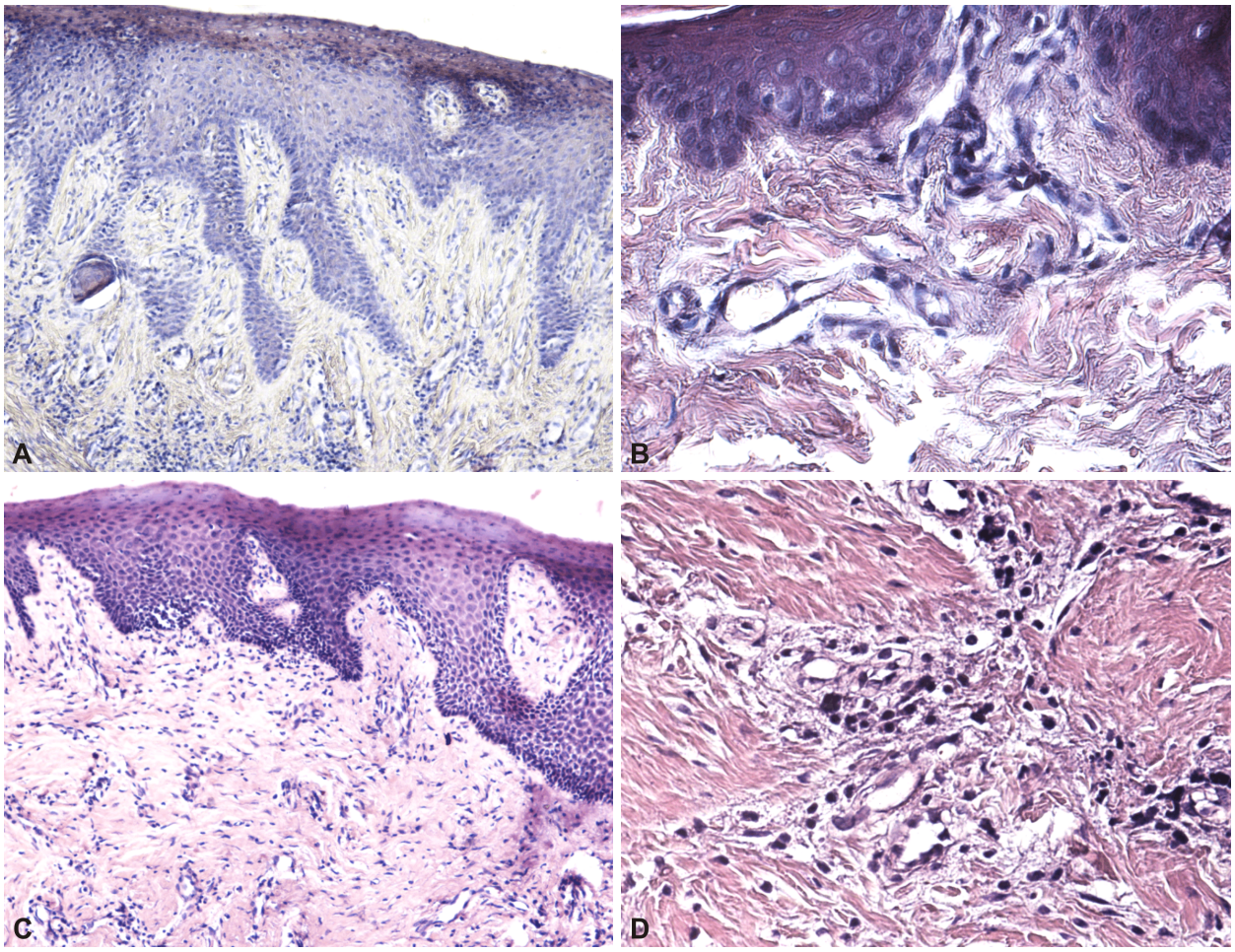

Figure 2 – ***(A) Parakeratinization epithelium, chronic moderate inflammatory infiltrate, inter-digitations in the epithelial–conjunctive interface (HE staining, ×100); (B) Development of the collagen fibrillary component in fascicles (HE staining, ×200); (C) Lympho-plasmocytary and macrophage inflammatory infiltrate in the lamina propria (HE staining, ×100); (D) Micronodular aspect of the inflammatory infiltrate (HE staining, ×200).***

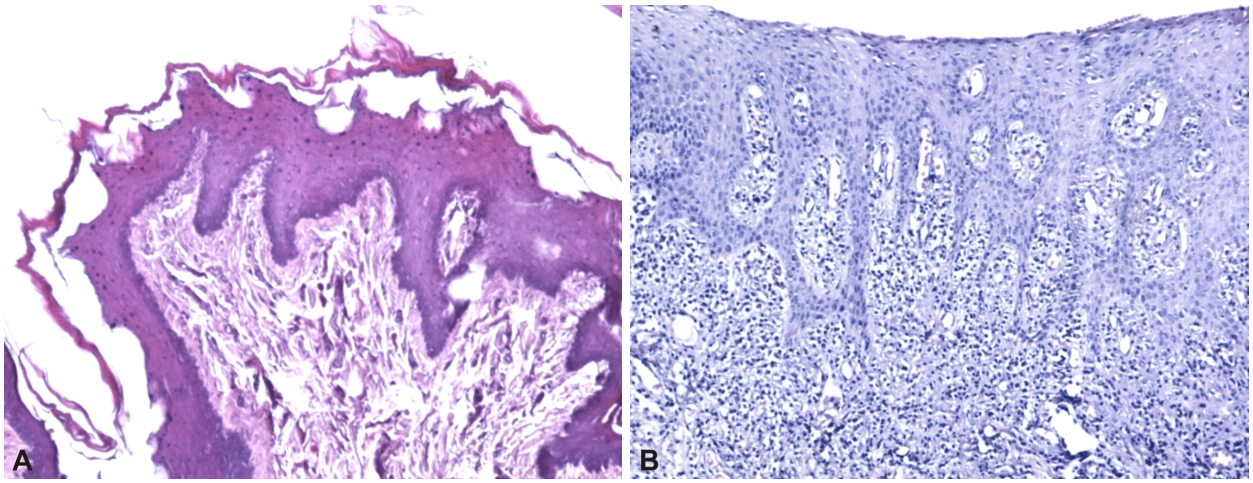

Figure 3 – ***(A) Epithelial hyperplasia, epithelium areas of different thickness (HE staining, ×200); (B) Epithelial proliferation with deep and branched epithelial growths (HE staining, ×100).***

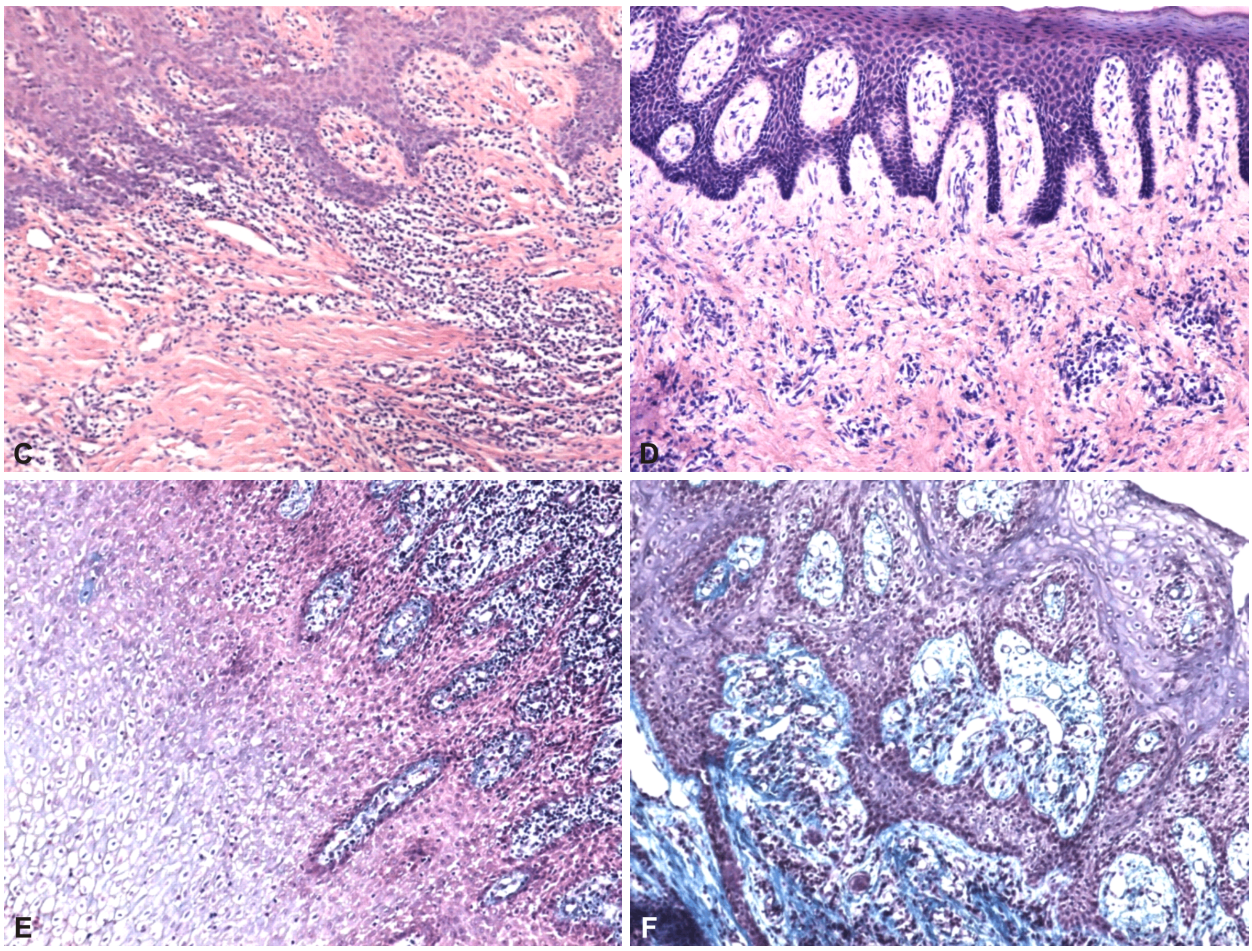

Figure 3 (continued) – ***(C) Epithelial proliferation with a "glove finger" aspect, with high cellularity in the lamina propria and subepithelial collagen deposit (HE staining, ×100); (D) Epithelium with ortho- and para-keratinization, proliferate as deep growths, lympho-plasmocytary inflammatory infiltrate (HE staining, ×100); (E) Epithelial proliferation, balloon-like cells, acanthosis and acantholysis. Inflammatory infiltrate and intense vascularization of the conjunctive papillae (HE staining, ×200); (F) Similar image to the previous one, in a cross-section (HE staining, ×200).***

Discussion

Many studies of experimental rats were extensively used to study the changes induced by certain drugs upon the gingival mucosa, as the effects of this outgrowing are similar to those in humans. In addition, the rat is convenient because the animals are easy to maintain, entailing significant costs, and the response in rats is more uniform than that in humans, as well as many other variables like: the genetic predisposition, gender, age, dose and duration of treatment, are better controlled [6–8].

There are three classes of drugs that cause gingival outgrowing in most cases: anti-epileptics (Phenytoin), antihypertensive medications calcium channel blockers (Nifedipine), immunosuppressive medication (Cyclosporine). The literature indicates an incidence of occurrence GO between 10–50% for Phenytoin, between 8–70% for Cyclosporine and between 0.5–83% for Nifedipine [9]. The three classes of drugs have a common property, directly affecting the cellular calcium metabolism. Since the cellular production of collagenase is modulated by the inflow of calcium, fibroblasts from patients treated with these drugs may produce an inactive form of collagenase, resulting in an increased extracellular matrix [10].

Gingival overgrowth may be the result of inflammation, fibrosis, or a combination of both. Inflammatory overgrowth is determined by local and systemic causes and fibrous overgrowth is determined by the idiopathic or drug-induced gingival fibromatosis [11]. Several factors can influence the relationship between the different drugs involved and the components of gingival tissues: age, gender, genetic predisposition, pharmacokinetic variables, coadministration with other drugs, impaired homeostasis of the gingival tissue (periodontal factors) ultrastructural factors, changes in inflammatory growth factors, and so on [12]. Plaque-induced inflammatory changes will exacerbate drug-induced GO [13–15], while other studies show that there is a direct link between the two entities [16, 17]. However, it is difficult to tell if the plaque is a contributing factor to the GO or if it is a consequence of it.

To assess drug-induced GO mechanisms and to assess a new medication with fewer effects on gingival tissue growth, it is important to establish an animal model in which to reproduce GO secondary to administering certain drugs. The experimental induction of GO has been tried by many researchers, but was unsuccessful in most animal species. The rat is more useful because of the availability of the animal, the existence of small differences in response to drugs, as well as the reproducibility of experimental results [18]. Some authors indicate that young rats present a more severe outgrowing, as compared with adult rats [19–22].

Phenytoin-induced gingival overgrowth

Epilepsy is a neurological disease with disrupted neural activity, in which groups of neurons send signals causing abnormal sensations, emotions or strange behavior and sometimes convulsions, muscle spasms and loss of consciousness. In the long-term administration of anti-convulsant medication, secondary reactions occur with important clinical implications. Amongst the side effects of this medication, administration there is included gingival overgrowth, but not all patients develop it [22, 23].

Several mechanisms have been proposed to induce gum outgrowth after taking anticonvulsants, including the existence of Phenytoin sensitive fibroblast populations or Phenytoin stable populations [24–26]. Gingival overgrowth can be determined by an increase of fibroblast proliferation and synthesis, which causes an accumulation of connective tissue components, especially of collagen [27, 28]. Some authors indicate an increase in the inflammatory infiltrate as soon as the very start of the treatment, suggesting the importance of the inflammatory response in the occurrence of GO [25, 29, 30]. This finding differs from other studies showing the presence of very few inflammatory cells [31]. Thus, the association of the inflammatory process to the production of Phenytoin-linked GO remains uncertain. It has been suggested that the proliferative effect of Phenytoin is mainly expressed on the basal layer of the oral epithelium, thus increasing the conjunctival–epithelial tissue interface [32]. Moreover, the epithelium may have a fibroblast inducing quality, a process involving alkaline phosphatase [33]. In our experiment, we found the presence of epithelial proliferation of both the basal layer and spinous layer, with the aspect of deep epithelial ridges, penetrating deeply into the connective tissue, as "glove fingers".

Nifedipine-induced gingival overgrowth

Although there are many drugs used to treat hypertension, Ca^{2+} channel antagonists like Nifedipine, Verapamil and Diltiazem are frequently used as agents acting quickly, though causing, as a side effect, gingival outgrowth by increasing the collagen and the density of fibroblasts [34, 35]. In medical practice, since the pathogenic mechanism of GO linked to these drugs remains unknown, the treatment used is surgical. Thus, the development of treatment based on the cause is imposed.

Nifedipine is a calcium channel blocker used to treat cardiovascular diseases, which can induce gingival overgrowth. It inhibits the influx of calcium through the extracellular membrane of cardiac muscle and vascular smooth muscle cells without changing serum calcium levels, which reduces cardiac muscle contraction, reducing the use of oxygen of the myocardium. Gingival overgrowth occurs within 1–2 months from the start of medication and is reduced to one week after discontinuation [36, 37]. Our study shows that Nifedipine determines GO by fibroblast proliferation followed by an increase in the deposition of collagen and blood vessels, especially capillaries, which express an increase in number and have an enlarged lumen. The same issues are pointed-out by other authors [38]. Some studies have reported that Nifedipine induced angiogenesis in humans, which supports our results [39].

Our results indicate that experimentally induced GO by administration of the three categories of drugs is due to the alteration of tissue homeostasis. This is supported by changing the number of cells involved in the turnover of the connective tissue: macrophages, lymphocytes, plasmocytes. The numerical changes of these cells could affect cytokines and growth factors, which could influence the function of fibroblasts.

Cyclosporine-induced gingival overgrowth

Cyclosporine is an immunosuppressant used in organ transplantation to prevent rejection as well as in the treatment of other autoimmune diseases. Its immunosuppressive effect is selective, as it influences only certain specific components of the immune system, such as lymphocytes. Thus, Cyclosporine suppresses T-helper lymphocytes, leaving the humoral immune response uninfluenced, as B-lymphocyte activity is not influenced [40]. In addition to its systemic effects, Cyclosporine produces gingival overgrowth, but this does not develop in all patients who receive Cyclosporine. Depending on the different responses of the patients at the same therapy, there has been suggested that there are two groups of fibroblasts, responder and non-responder [41]. Gingival overgrowth is a side effect of long-term administration of Cyclosporine and may be influenced by other predisposing factors such as plaque. As in the case of therapy with Phenytoin, GO was found to occur more often in younger individuals than in adults. This hypothesis is supported by experiment Kitamura *et al.*, who reported 100% incidence in young Fischer rats [42]. Other authors, depending on the blood concentration and the degree GO do not prove there was any correlation between humans or rats, although other studies have opposite results present [42, 43]. Other studies state that proteoglycans and glycosaminoglycans in the rat gums decrease with age [44].

GO arising from the incidence of Cyclosporine therapy is reported as ranging between 25% and 80% in humans and 30% to 40% in dogs [45–47]. This variable incidence may be due to individual differences in susceptibility to the drug or may be dose-dependent [48]. In our experiment, the difference between the control group and the injected one consisted in the thickness of the stratified squamous epithelial and of cellular components of the lamina propria. The epithelium was significantly thicker in the experimental group due to ortho- and parakeratinization. Acanthosis was also present, and specialty literature believes that this is due to increase in life span rather than the proliferation of keratinocytes [49].

A recent study has suggested that the imbalance of cell proliferation and apoptosis may contribute to the pathogenesis observed in the Cyclosporine-induced gum hypercellularity overgrowth [50, 51]. Cyclosporine effect on human gingival fibroblasts proliferation is still controversial, ranging from absence, increase or decrease [52]. These differences were attributed to the heterogeneity of human gingival fibroblasts [53, 54] or differences in the experimental conditions applied [55].

As we ourselves found, Cyclosporine-induced outgrowing gingival tissues are highly inflamed, do not express high levels of TGF (transforming growth factor) or CTGF (connective tissue growth factor), and are not the most fibrotic tissues [56]. These findings are surprising

and indicate that oral bacteria and gum tissues and cells must interact in one way in subjects receiving Cyclosporine resulting in inflammation and greater cellularity compared to other forms of outgrowing gum. The biological mechanisms responsible for this phenomenon must be unique to cells and gingival tissue and are currently under investigation.

Conclusions

Gingival overgrowth, with its aesthetic and functional implications, is a serious concern for both patients and clinicians, which warrant a thorough morphological study, on animal models. In the research conducted by us, increased gingival growth occurred gradually, depending on the time elapsed since the start of drug administration. Gingival inflammation increases the incidence and severity of gingival outgrowth, which is supported by the results recorded in the group treated with Cyclosporine, case in which inflammation was more pronounced and mucosal overgrowth was higher compared to other groups. Our results suggest that experimentally induced gingival overgrowth with the administered drugs is determined by altering tissue homeostasis through altering the cell populations involved in the fibrocyte turnover tissue and of those involved in the inflammatory process. A better understanding of the pathogenesis of this undesirable effect may lead to the development of improved management strategies, strategies for preventing it or for its non-surgical reduction.

Conflict of interests

The authors declare that they have no conflict of interests.

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Received: December 10, 2015

Accepted: September 5, 2016