J Clin Periodontol 2005; 32: 98–103, doi: 10.1111/j.1600-051X.2004.00636.x

Journal of Clinical Periodontology

Histologic, histomorphometric and immunohistologic changes of the gingival tissues immediately following mandibular osteodistraction

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Kruse-Lösler B, Flören C, Stratmann U, Joos U, Meyer U: Histologic, histomorphometric and immunohistologic changes of the gingival tissues immediately following mandibular osteodistraction. J Clin Periodontol 2005; 32: 98–103. doi: 10.1111/j.1600-051X.2004.00636.x. © Blackwell Munksgaard, 2004.

Abstract

Objectives: Even though osteogenesis after osteodistraction has been investigated in numerous experimental studies, there is limited information focusing on the influence of well-defined mechanical distraction forces on the associated gingival tissues.

Material and Methods: In a study including 48 rabbits, mandibular osteodistraction was performed in vertically osteotomized mandibular body, using defined distraction protocols with physiologic, moderate and hyperphysiologic forces. The soft tissues overlying the distraction gap were harvested finally for histologic, immunohistologic and histomorphometric investigations.

Results: The control group without distraction showed the typical architecture and thickness of normal gingiva. In groups with distracted mandibles, an accelerating atrophy of gingiva depending on the degree of mechanical loading was obvious, characterized by decreasing thickness of epithelial layer, loss of rete ridges and disorganization of the different cell layers with a high number of apoptotic cells. In lamina propria collagen fibres were reduced and elastic fibres increased. Histomorphometric analysis revealed significant correlation between degree of distraction and atrophy in overlying soft tissues.

Conclusion: This rabbit model of mandibular lengthening shows an accelerating atrophy in the covering soft tissues following hyperphysiologic distraction. The long-term outcome of these distraction-related soft-tissue alterations remains unclear. The atrophic changes may likely be of temporary nature.

Key words: animal study; apoptosis; gingiva histology; interfragmentary strain; mandibular osteodistraction

Accepted for publication 27 April 2004

Osteodistraction is an established therapeutic concept for correction of craniofacial deformities (McCarthy et al. 1992, Califano et al. 1994, Bell et al. 1997, Stewart et al. 1998). The principle is based on the process of osteogenesis in the distraction gap between the osteotomized bone segments induced by the distraction procedure (Samchukov et al. 2001). A further indication for osteodistraction includes alveolar bone augmentation as an alternative approach instead of using allografts or autografts (Cope & Samchukov 2000). Osteogenesis and mechanisms of new bone formation have been investigated in numerous experimental studies (McCarthy et al. 1992, Califano et al. 1994, Stewart et al. 1998). The process of osteodistraction also induces traction forces to the surrounding soft tissues. Several studies have evaluated the effect of gradual bony distraction on the temporomandibular joint and the different associated tissues such as ligaments, epithelium, nerves and tendons (Fisher et al. 1997, Makarov et al. 1998). Generally, it is known that distraction in a slow incremental manner is associated with a sufficient

mineralization of new bone formation and physiologic bone remodelling during the consolidation period. Under these conditions, stretching forces to the surrounding soft tissues induce adaptive and regenerative changes (Shevtsov 1996, Makarov et al. 2001), but there is only limited information available focusing on the influence of well-defined mechanical distraction forces on the adjacent gingival tissues.

Already under physiological conditions of normal mastication, the soft tissues of oral cavity are exposed to abrasion and mechanical forces such as compression, shearing and stretching. The important function of gingival tissues and oral mucosa is protection of deeper tissues. In comparison with the other mucosal surfaces in oral cavity, the anatomical structure of gingiva includes numerous modifications in its epithelial lining and underlying connective tissue to compensate traumatic influences (Ten Cate 1998).

Epithelial cells build a functional unit because of their extensive intercellular attachments. These act as a mechanical linkage distributing local forces applied to the epithelial surface over a wider area. The rete ridges represent the border between the two layers of gingiva, configurated by irregular downward projections of epithelium and the upward projections of connective tissue papillae. Because of this structure, the interface is enlarged. Under these conditions, applied forces at the surface of epithelium can be dispersed over a greater area of connective tissue.

The objective of this study was to evaluate in a rabbit model the histologic, histomorphometric and immunohistologic changes in gingival tissues immediately following mandibular osteodistraction.

Material and Methods

Animal care

The experimental study was performed on 48 immature white female rabbits, each weighing between 2600 and 3400 g. All study protocols were reviewed and approved by the Münster University Animal Research Ethics Committee. The rabbits were kept in an animal holding facility under veterinary supervision. They received food and water ad libitum. Every day, body weight and food intake were monitored.

Anaesthesia and surgical procedure

Forty-two of the animals were surgically prepared for osteodistraction. The surgeries were carried out under general anaesthesia by intramuscular injection of xylazine 3 mg/kg, ketamine 20 mg/kg, and atropine 0.25 mg/kg. Keeping aseptic conditions, a longitudinal incision was performed along the inferior border of the right mandible. After reflecting the platysma muscle in the supraperiosteal plane, the periosteum was incised along the lateral side of the mandibular bone. Saving the mental nerve, a corticotomy was performed on the buccal aspect with a water-cooled drill (diameter 1 mm) immediately anterior to the first molar. A unilateral distraction device (Type Vazquez-Diner; Howmedica Leibinger GmbH, Freiburg, Germany) was positioned along the axis of the mandibular body in a plane perpendicular to the corticotomy surfaces, using self-tapping screws. The device was orientated parallel to the mandibular body to allow direct force transduction through both segments. The osteotomy was then completed on the lingual side of the mandible (Fig. 1A). The periosteal flaps were carefully repositioned and the wound was closed in layers. The entire distraction device was located subcutaneously, with the activation arm penetrating the skin. All animals began to masticate in the immediate postoperative period.

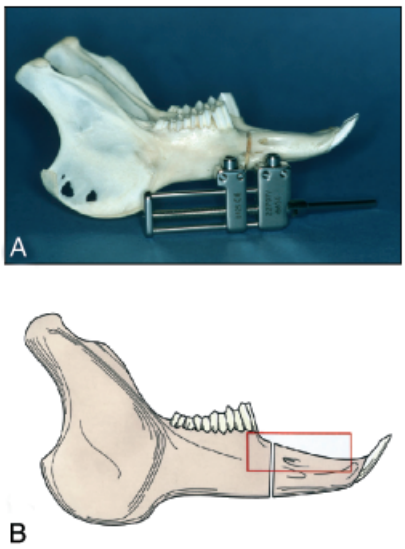

Fig. 1. (A) Distraction device inserted in mandibular body. (B) Graphically illustrated localization of the harvested gingival tissues overlying distraction gap.

Distraction osteogenesis

The animals were divided into groups of six exposed to intermittent distraction forces of 2000, 20,000, 200,000 and 300,000 microstrains, respectively, or used as sham-surgery (no distraction forces applied) or non-operated controls (Table 1). After allowing the animals to recover for 4 days, distraction was started and continued for 10 days at various strain magnitudes and frequencies. The lengthening procedure was performed allowing a calculated schedule for each strain magnitude, based on the ratio between the increment of lengthening and the initial gap size (1 mm). The interfragmentary strain (IFS) was calculated by the interfragmentary length (IFL) and the gap size (L) following the definition of strain by IFS = IFL/L (Meyer et al. 1999). Distraction was performed intermittently using one loading cycle per day (2000, 20,000, 200,000 and 300,000 microstrains), respectively, or 10 loading cycles per day (2000, 20,000 microstrains). Non-operated animals served as controls (group 1). Group 2 included sham-operated animals that underwent frame applications and corticotomy without distraction. Following the 14-day period, the animals were sacrified, the mandibles including the covering gingiva immediately harvested. The

Table 1. Experimental schedule

Group	Animals (n)	Corticotomy	Applied strain			Bone elongation (mm)*
1	6	no	–			0
2	6	yes	0	microstrains		0 ± 0.01
3	6	yes	2000	microstrains	1 cycle/day	0.02 ± 0.01
4	6	yes	2000	microstrains	10 cycles/day	0.22 ± 0.02
5	6	yes	20,000	microstrains	1 cycle/day	0.20 ± 0.02
6	6	yes	20,000	microstrains	10 cycles/day	7.31 ± 0.20
7	6	yes	200,000	microstrains	1 cycle/day	6.20 ± 0.20
8	6	yes	300,000	microstrains	1 cycle/day	13.70 ± 0.40

*Mean value ± Standard deviation

gingiva overlying the regenerated bone was removed (Fig. 1B). Samples were prepared for further histologic, immunhistologic and histomorphometric analysis.

Histologic and histomorphometric evaluation

The diameter of the distraction gap was measured by histologic evaluation of the harvested mandibular bone and calculated from the data of the six animals in each group as mean value ± standard deviation (Table 1). For histologic and histomorphometric investigations of the overlying soft tissues, the gingiva and mucosa from the six animals in every group were fixed in 4% phosphate-buffered paraformaldehyde, pH 7.2, for 4 h at room temperature. The specimens were then embedded in paraffin, serially cut into 7 μm sections and stained with haematoxylin and eosin. Serial histologic sections were analysed for histomorphologic changes in structure, cell population, thickness of the tissue layers and integrity of the interface between epithelial layer and lamina propria. The parameters were measured in 10 random high-power fields (magnification × 250) using an image analysing system (Image Tool for Windows, Version 3.00, The University of Texas Health Science Center, San Antonio, TX, USA), which has been previously calibrated with a 0.01 mm objective micrometer (Ernst Leitz, Wetzlar, Germany). The measurements were performed using conventional light microscopy (Zeiss photomicroscope III, Carl Zeiss, Oberkochem, Germany) with the images being transmitted to a high-resolution colour video monitor. These data were logged to a spreadsheet for analysis. All histologic evaluations were conducted in such a way that the investigator performing the analysis did not know to which experimental group the specimens belonged. The quantitative measurements for histomorphometric analysis were carried out by two investigators, counting cells or other relevant structures in 10 representative high-power fields for each specimen as described below. Out of the count rates determined by the two investigators, mean values were calculated for each animal and each group ± SEM. The following parameters were determined by histomorphometric analysis: thickness and structure of the epithelium layer and the lamina propria, number of epithelium cells and fibroblasts, number of capillaries and lymphocytes in the lamina propria. The detection of elastic fibres was performed in specimens stained with orcein. As measure for the interface area, the papillar index was established, which was calculated as a quotient from the measured length of the basal lamina including all papillae and rete ridges and mean length of the epithelium line within the analysed image area.

Immunohistologic investigation

DNA fragmentation occuring in gingiva overlying the osteodistraction site was analysed by a modified terminal transferase-mediated digoxigenin-labelled nick end labelling (TUNEL) method, similar to that described by Gavrieli et al. 1992. Briefly, this assay is based on the fact that during programmed cell death a multitude of new 3′-OH DNA ends are generated by nuclear endonucleases, which digest genomic DNA at linker sites between nucleosomes into fragments of multiples of approximately 200 bp. In order to analyse areas of similar size, sections from the gingiva were cut from the centre of the distraction gap. Paraformaldehyde-fixed tissue sections were deparaffinized by immersion in xylene and a graded series of alcohols. After washing in phosphate-buffered saline (PBS), the tissue samples were digested with 40 μg/ml proteinase K (Boehringer, Mannheim, Germany) for 15 min at room temperature. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide in PBS followed by two rinses with PBS. Prior to the labelling, the sections were pre-incubated with potassium cacodylate-containing equilibration buffer followed by the terminal deoxynucleotidyl transferase reaction, using the ApopTag plus in situ apoptosis detection kit (Oncor, Gaithersburg, MD, USA). The slides were incubated at 37°C in a reaction mixture containing digoxigenin-labelled deoxynucleotides and terminal deoxynucleotidyl transferase. After 1 h, the reaction was stopped and labelled deoxynucleotides catalytically added to the ends of DNA were detected by the use of anti-digoxigenin F_{ab} fragments conjugated to peroxidase. Sections were developed for 6 min with diaminobenzidine used as chromogenic substrate and finally counterstained with methyl green for 5 min. Controls were treated in the same way except that terminal deoxynucleotidyl transferase was substituted. The number of apoptotic cells was quantified within 10 randomized high-power fields (magnification × 250).

Statistical methods

The data are presented as mean ± standard deviation (SD). To determine if the treated groups were significantly different from each other, non-parametric one-way analysis of variance (Kruskal–Wallis test) was performed. If this indicated a significant result, paired comparisons were performed on control and experimental groups (group 1 versus groups 2–8) at a p value of <0.05. Further analysis was also performed using Student's t-test for non-paired samples. A level of $p<0.05$ was established as significant difference between the control and experimental groups. The data were analysed using SPSS software (SPSS Inc., Chicago, IL, USA; 11.0 Edition for Windows). Histograms were drawn using the mean ± standard error of the mean (SEM) for each group.

Results

All of the animals tolerated the surgery and the different distraction regimens well. Body weight and food intake were stable. After lengthening of the mandible, gross clinical observation showed an expected lengthening of the right mandible dependent on the daily strain magnitude and frequency (Table 1). The total length achieved ranged from 1 mm in non-distracted samples (osteotomy width) to 13.7 mm in samples distracted with hyperphysiologic strain (300,000 microstrains/1 cycle/day).

Histologic and histomorphometric analysis

The soft tissues covering the distraction gap displayed characteristics apparently related to the magnitude of the applied distraction force. In non-operated and sham-surgery controls, the histologic evaluation showed the typical morphology of normal gingiva with well-defined borders between epithelial layers and the stratum basale and a harmonic configuration of the rete ridges and the interdigitating connective tissue papillae (Fig. 2A). The epithelium displayed normal thickness, cell maturation and

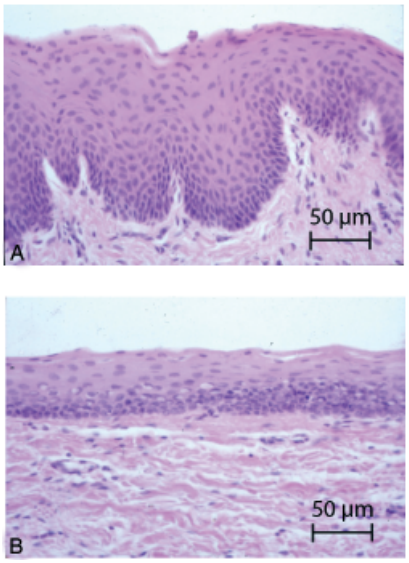

Fig. 2. Histological features of gingival tissues (H&E staining, magnification × 250). (A) Non-operated control. (B) After hyperphysiologic distraction (300,000 microstrains/1 cycle/day); note atrophic changes with flattening of epithelium layer and loss of rete ridges.

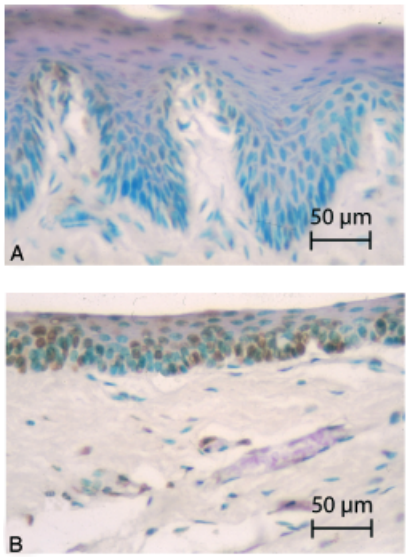

Fig. 4. Detection of nuclear DNA fragmentation by TUNEL-procedure in gingival tissues (magnification × 250). (A) Non-operated control. (B) After hyperphysiologic distraction (300,000 microstrains/1 cycle/day), numerous apoptotic cells (stained red-brown) in atrophic epithelium layer.

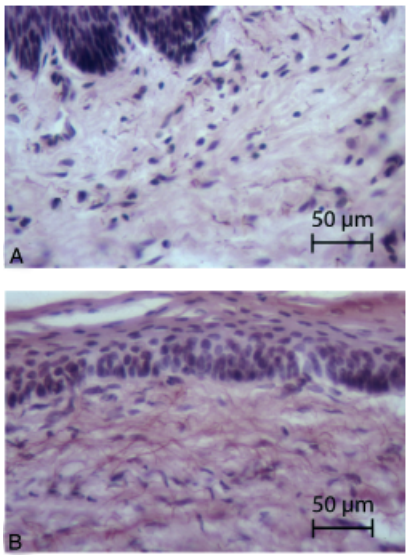

Fig. 3. Proof of elastic fibres in gingival tissues (Orcein staining, magnification × 250). (A) Non-operated control. (B) After hyperphysiologic distraction (300,000 microstrains/1 cycle/day), elastic fibres (stained dark red) in lamina propria increased.

scattered apoptotic cells (Fig. 4A). Within the lamina propria, the collagen and elastic fibres appeared normal in form and number as well as the blood vessels (Fig. 3A). No inflammatory infiltrate was evident. In animals exposed to physiologic distraction forces (2000 microstrains), the histologic features were similar to those in the controls. However, among animals distracted at elevated (20,000 microstrains/1 or 10 cycle(s)/day) or high distraction forces (200,000 and 300,000 microstrains/1 cycle/day) an increasing atrophy of the gingival tissues apparently correlating to the degree of mechanical load was observed. In these animals, the histologic changes were characterized by a decreasing thickness of the epithelium, loss of rete ridges and a corresponding flattening of the interdigitating connective tissue papillae (Figs 2B and 5A). The number of epithelial cells was reduced in specimens of animals exposed to elevated and high distraction forces (Fig. 5C). A reduction in epithelial thickness and disorganization of cell layers and maturation was observed with increasing forces. The epithelial cells showed a broad variation in size and shape as well as featured loss of intercellular bridges. The papillar index was reduced in animals exposed to hyperphysiologic distraction forces (Fig. 5E). However, the lamina propria was not significantly flattened (Fig. 5B). In this zone, the collagen bundles were reduced to longitudinally orientated stretched collagen fibres without undulation. Fibrocytes were significantly reduced following hyperphysiologic distraction (Fig. 5D). In contrast, the number of elastic fibres stained with orcein was significantly elevated in specimens from animals receiving 300,000 microstrains/1 cycle/day (Figs 3B and 5H). A mild inflammatory, mainly lymphocytic infiltrate was observed below the basal lamina (Fig. 5F). Also, the number of blood vessels in the lamina propria increased with accelerating strain magnitudes (Fig. 5G). TUNEL-positive cells were frequently found in the epithelium of sections from sites distracted at high strain magnitudes (Figs 4B and 5I). Apoptotic cells, however, were not registered in the lamina propria following hyperphysiologic distraction (Fig. 5J). In summary, the results suggest a significant correlation between the distraction forces and atrophy of the associated gingival tissues.

Discussion

Osteodistraction is an established therapeutic concept for the correction of craniofacial deformities. Even though osteogenesis and mechanisms of bone formation have been investigated in numerous experimental studies (McCarthy et al. 1992, Bell et al. 1997), there is limited information available focusing on the influence of well-defined mechanical distraction forces to intra-oral soft tissues. In our animal experiment including 48 rabbits, different distraction protocols with physiologic, moderate hyperphysiologic or hyperphysiologic distraction procedures were performed to investigate histologic, immunohistologic and histomorphometric changes in the associated gingival tissues.

Principally, soft tissues may respond in different ways to injury, depending on direction and degree of tissue irritation. Minimal injuries cause no inflammatory reaction, but they induce mild adaptive changes such as hyperkeratinization of the gingiva. If an injury is severe enough to result into an inflammatory reaction, it is followed by a healing response that may be a complete restoration of tissue architecture (regeneration) or restoration of soft-tissue continuity with scarring and distortion (repair) of soft tissue (Ten Cate 1998). As a result to distraction exposure, adaptive mechanisms are induced to keep the tissue integrity. Following the

distraction of mandibular bone, dependent on the degree of distraction, there may occur tissue irritation associated with an inflammatory reaction. According to the degree of inflammation caused by the distraction, the changes in the soft tissues may lead to degeneration and scars (repair) or restoration by cellular proliferation and regeneration (Shevtsov 1996). Under the stimulating effect of gradual distraction, the adaptive changes in the soft tissues are often associated with neohistogenesis (Shevtsov 1996).

In our rabbit study of mandibular lengthening, using distraction with elevated strain magnitudes (20,000 microstrains/10 cycles/day) and high strain magnitudes (200,000–300,000 microstrains/1 cycle/day) immediately at the end of distraction period, we found significant degenerative atrophic changes of the gingival tissues overlying the distraction gap. In these groups, an accelerating atrophy of the associated soft tissues depending on the degree of mechanical loading was obvious. Following the forced distraction, the histologic changes were characterized by a decreasing thickness of the epithelial layer with disorganization of the cell layer and an almost complete loss of the rete ridges. TUNEL-positive apoptotic cells were frequently detected in samples distracted at higher strain magnitudes (200,000–300,000 microstrains/1 cycle/day) especially in the epithelial layer. In the lamina propria, the collagen bundles appeared thinned with longitudinally stretched collagen fibres and lacked undulation. The blood vessels in the lamina propria were also stretched. The number of elastic fibres was significantly elevated mainly in the groups with hyperphysiologic distraction. A mild inflammatory infiltrate was registered below the lamina propria.

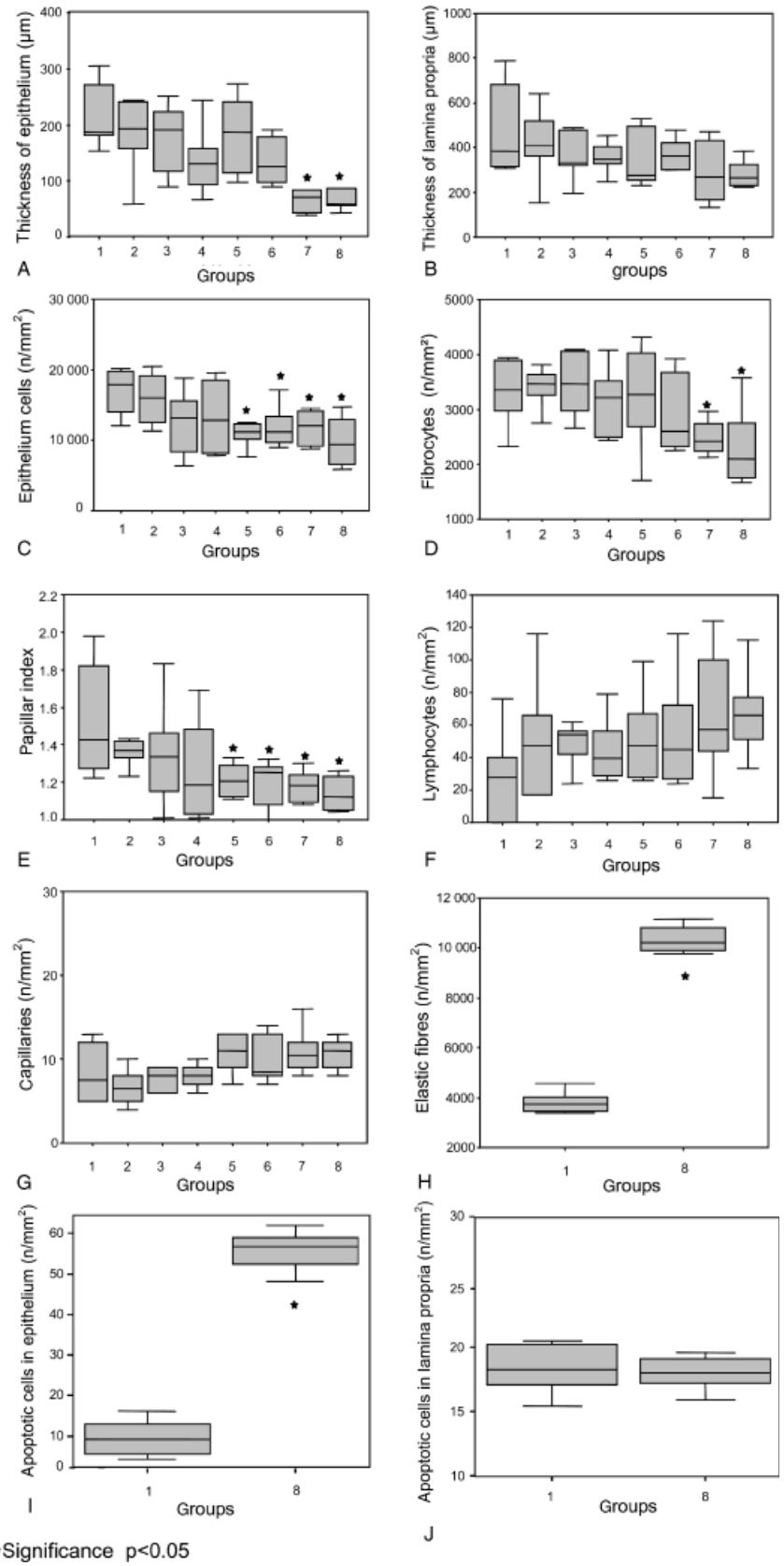

Fig. 5. Graphic illustration of histomorphometric analysis. Obvious changes in groups after elevated (groups 5 and 6) and hyperphysiologic distraction (groups 7 and 8). (A) Significant reduction of epithelium layer in groups 7 and 8. (B) Tendentious flattening of lamina propria in groups 7 and 8. (C) Significant reduction of epithelium cells/mm^2 in groups 5–8. (D) Significantly decreased fibrocytes in groups 7 and 8. (E) Papillar index significantly reduced in groups 5–8. (F) Tendentiously increased lymphocytes in groups 7 and 8. (G) Slight amount of capillaries in lamina propria in groups 5–8. (H) Significant increase of elastic fibres in group 8. (I) Significant amount of apoptotic cells in epithelium layer in group 8. (J) No evidence of apoptotic changes in lamina propria after hyperphysiologic distraction.

The study design did not include long-term outcome of soft-tissue alteration after distraction osteogenesis. Considering the variations of physiologic reaction in soft tissues after insult or injury, the gingiva probably will respond by regeneration as opposed to repair. This may be supported by the registered inflammatory response at the end of distraction and the fact of preserved continuity of gingival tissues instead of destroyed continuity or even scars. Similar findings are reported in other studies (Oda et al. 1998, Bell et al. 1999, Sawaki & Ueda 1999).

The documented apoptotic cell death and other degenerative changes in the upper gingival tissues might be compensated by adaptive regeneration in the postdistraction period. This should be the subject of long-term investigations including a prolonged postoperative period. Because of the regenerative capacity in gingival tissues, restitution with adequate long-term healing will be more likely than a degenerative process.

As our findings are gained from a rabbit study, which is serving as an excellent screening model it is not appropriate to be extrapolated to clinical outcome in humans. It may be possible that biologic events found in rabbits are more reactive than clinical cases. Actually, no clinical trials in humans have been performed to clarify gingival reaction following osteodistraction.

The histologic and histomorphometric data of our study showed a significant correlation between the degree of distraction and the atrophy including apoptotic cellular changes in the depending soft tissues. This may be because of cellular distraction and disorganization following hyperphysiologic distraction procedures, probably resulting in repair mechanisms. If hyperphysiologic mandibular distraction procedures in humans may lead to similar histologic findings, this should be clarified by clinical investigations.

Acknowledgments

The authors thank Mrs K. Cebulla and Mrs B. Strothbaum for their technical assistance during the study.

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