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Research Article

Osteitis associated with endoscopic sinus surgery. A murine experimental model

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Abstract. There is increasing evidence supporting the high prevalence of osteitis in chronic rhinosinusitis, moreover regarding the association of osteitis with severe sino- nasal inflammation. However, the underlying mechanism, gradation, clinical and functional significance, remain controversial and further studies on new experimental models are needed. Endoscopic sinus surgery is the gold standard for patients with chronic rhinosinusitis. Sinus surgery involves several physiologic and geometrical changes which are likely to have an impact on wound healing, mucociliary function, and biofilms and moreover, is associated with recalcitrant chronic rhinosinusitis and high incidence of osteitis. Summarizing the current state of knowledge regarding the specific role of inflammation in bone remodeling in chronic rhinosinusitis, the application of our research was to develop a suitable murine experimental model that mimics nasal endoscopy surgery lesions, to better understand the pathogenesis of osteitis after surgery and its effect on bone remodeling.

Key Words: endoscopic sinus surgery (ESS); chronic rhinosinusitis; tissue injury; murine experimental model; osteitis- neo-osteogenesis.

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Introduction

According to (Snidvongs et al 2019) and (Lee et al 2006), osteitis in the paranasal sinus area is well-defined as the process of bone remodeling and is characterized by the simultaneous presence of following hallmarks: bony resorption, new bone formation, periosteal thickening, and fibrosis.

Snidvongs et al (2014) reported the incidence of osteitis around 51% in chronic rhinosinusitis (CRS) patients, with much higher prevalence (76%) in those with history of previous sinus surgery. Diagnosis was established upon imagistic criteria and histopathological findings. Moreover, the positive correlation between osteitis and inflammation severity has been clearly demonstrated. There is increasing evidence supporting the impact of neo-osteogenesis on the pathophysiology of CRS. Additionally, recurrent CRS is generated by inflammation and ostial stenosis triggered by osteitis.

Several animal models of CRS are currently available and few on subsequent neo-osteogenesis after nasal wounding. Nevertheless, the underlying role of neo-osteogenesis in CRS and additionally following endoscopic sinus surgery (ESS) remain unclear due to the lack of suitable experimental models (Khalmuratrova et al 2021; Khalmuratrova et al 2019; Kim et al 2017). A thorough understanding of the process of wound healing following ESS is required to avoid postoperative complications and recurrent disease.

In this regard, the purpose of our research was to create a murine experimental model of bone remodeling following nasal mucosa trauma. A mouse model of nasal wound healing following manual injury has been previously described (Joo et al 2019). Starting from this well-defined murine model on nasal wound healing and based on the Khalmuratrova et al (2019; 2020; 2021) research and results on neo-osteogenesis, we have established a new model for osteitis.

This pilot study aims to validate this murine model on inflammation and neo-osteogenesis induced by mechanical lesion that simulates tissue trauma following endoscopic sinus surgery. Because of the existing evidence of severity and recurrency of CRS in patients with prior sinus surgery (Sacks et al 2013; Snidvongs et al 2012; Georgalas et al 2010) our future research will study the relationship between inflammation and neo-osteogenesis and will focus on the development of new strategies aiming to impede the development of osteitis.

Materials and methods

Murine model. A pilot study protocol.

This experimental study was conducted at the Center for Experimental Medicine of the University of Medicine and Pharmacy 'Iuliu- Hatieganu', Cluj-Napoca, Romania. All experiments were approved by our Veterinary Institutional Ethics Committee (235/ 4.11.2020) and were performed in accordance with the Helsinki Declaration, legally regulations and international guidelines on animal experimentation.

The study took place over a period of 12 weeks and included 20 adults male Wistar line rats, 16 weeks old, weighing about

Fig. 1. Micro-CT scan of a rat skull, showing right pansinusitis

353.02 g (± 21.34 standard deviation). The animals were housed and cared for according to standard rules. They were kept in controlled conditions: temperature 22±2 º C, humidity 50± 10%, light-dark cycle 12h-12 h, and had access to tap water and solid food ad libitum.

After prior weighing and numbering, anesthesia was performed individually, by intraperitoneal injection of Ketamine 80 mg / kg (0.04 ml Ketamine- Vetased) and Xylazine 8 mg. To rule out the pre-existing inflammation, all individuals underwent a detailed inspection of the nose to identify any secretions at nostrils level. By raising the tip of the nose, the mucosa of the nasal vestibule was visualized. All specimens included in the study had a normal appearance of the nasal mucosa and no mucous or mucopurulent secretions at nostrils level. In addition to the clinical evaluation (inspection and narinoscopy), the histopathology revealed inflammatory changes at the level of the wounded nasal fossa. Also, right pansinusitis was highlighted during skull micro- CT scan (Figure 1). Thus, we can conclude that the inflammation was procedural induced, and a possible pre-existing inflammation has been ruled out.

Further on, we induced a single, unilateral mechanical nasal mucosa lesion, at the level of the nasal turbinate of the right nasal fossa. We performed 3 rotations, clockwise, using a 10 mm length interdental brush (Figure 2 A).

We controlled the dimension and the depth of the lesion using identical brushes and same induction procedure, to produce similar lesions in all specimens.

A 16-week-old rat weighting approximatively 300 g has a nasal length of 9.1 mm (± 0.3 mm standard deviation). This length was represented by the distance between an anterior point, defined as the posterior surface of the incisor tooth, and a posterior point, defined as the most anterior section with an incomplete septum. The most anterior plane close to the nostrils it is followed by the nasal passages. Onwards, the rat nasal turbinates create more curved spaces with large surface area (Gross et al 1982, Dong et al 2018).

Postprocedural, a minor epistaxis from the right nasal fossa was noticed in all subjects, that was spontaneously controlled after approximatively 2 minutes. After the induction of the nasal lesion, the rat heads were maintained in a downward position to prevent blood influx into the lungs.

At the end of the experiment, the rats were sacrificed by intramuscular administration of an anesthetic overdose, respectively Ketamine (Vetased), at day 2,14, 30 and 80, then, after cessation of cardiac activity, they were decapitated dislocating the joint in the cervical region. Consequently, the skulls were harvested. The specimens submitted for histological analysis consisted of rat heads (bone and overlying mucosa samples). After fixation in 10% neutral-buffered formalin (7 -14 days), the samples were decalcified in 8% HCl admixed with 8 % H2CO2 (in a 1/1 volume), using the protocol previously described by (Prophet et al 1992). After complete decalcification (7 days) the soft tissues, and the lower jaw, were removed and the splanchnocranium was cut into standard, serial coronal sections according to the bellow schema (Figure 3). Each coronal section was placed with the rostral face down into a standard histological cassette and embedded in paraffin wax following a previously described protocol. Briefly, all samples were washed in tap water for 30 minutes, submitted to graduate chemical dehydration in ascending baths of ethanol (70%, 90%, 95%, and 100%), cleared with 100% xylene, and embedded in high-melting temperature paraffin wax. Paraffin blocks were chilled at -4°C, and seriate tissue sections were cut at 3 µm with a rotary microtome. Finally, histological sections were stained with Mayer's Hematoxylin-Eosin and examined under the Olympus BX51 microscope. All specimens were reviewed by a single unblinded pathologist. Histological micrographs were obtained with an Olympus SP 350 digital camera and further processed by Olympus Cell B and Image J programs.

Results

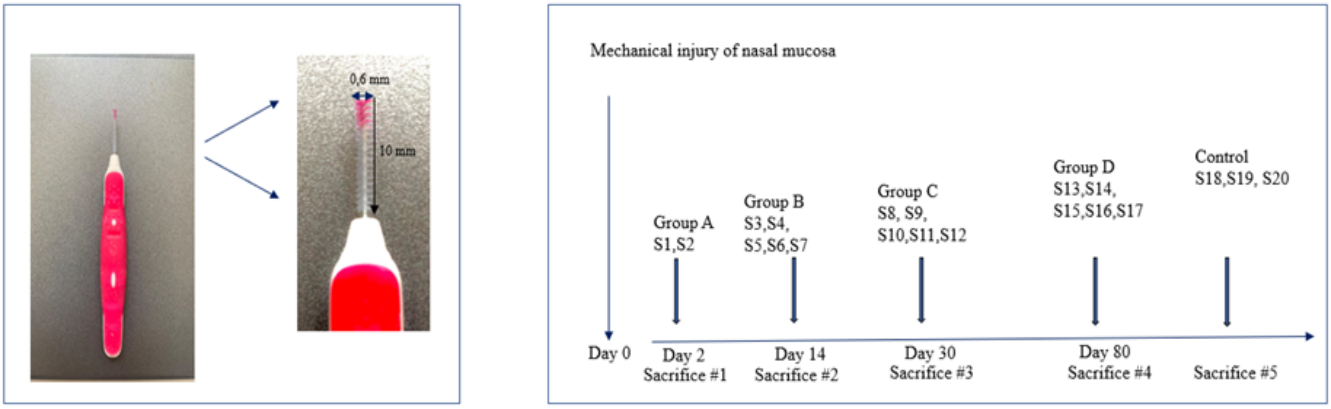
In our protocol we included 17 rats in the experimental group. As we microscopically evaluated the specimens (Hematoxylin and Eosin (HE) stain, x 10), the expected results of osteitis in the underlying turbinate bone after brushing procedure, were exceeded. As we observed, most of the inflammatory lesions were located at the head of the maxillo- turbinate, thus, we considered the importance of including in our study protocol, of 3 rats as a control group, so we can exclude any possible anatomical features of the breed or any incidental findings.

Of the 17 subjects from the experimental group, we observed inclusion criteria for osteitis (neo- osteogenesis, bone resorption, bone thickening), in different stages, from mild (borderline) to moderate, in 6 rats, accounting a percentage of 35.3 %. The turbinate bones of these individuals were irregularly expanded and extensively lined by a continuum border of hypertrophic, polygonal osteoblasts, producing woven bone with variable degrees of mineralization. Occasionally, the turbinate bones were lined by active osteoclasts located in resorption lacunae.

Regarding the inflammatory mucosal changes (edema, decreased number of ciliated cells, and goblet cell hyperplasia), we identified the specific features in 11 of the 17 rats (64.7 %), for the experimental group. The respiratory meatus was partially occupied by purulent exudate, the lamina propria of the respiratory mucosa was diffusely infiltrated by many lymphocytes admixed with plasma cells, neutrophils, and edema.

In none of the 3 controls, inflammatory and osteitis changes was found, except a thin blade of inflammatory catthar, at the level of the nasal meatus, associated with a molar periapical abscess.

The histological analysis in light miscrosocpy was performed by a single unblinded pathologist, using different high-powered



A B

Figure 2 A. The interdental brush. The brush (radius 0.6 mm, length 10 mm) was used to mechanically injure the rat nasal mucosa; B. Study protocol. Nasal mucosal injury was induced using the aforementioned brush, followed by sacrification at 2, 14, 30 and 80 days after injury.

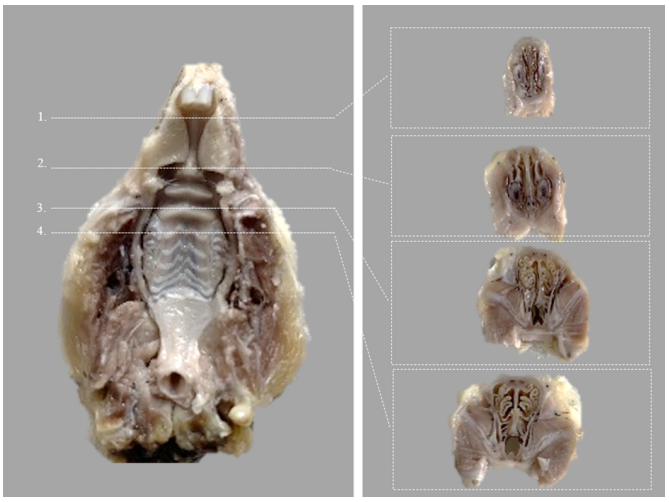


Fig. 3. Ventral view of the rat hard palate region, with the lower jaw removed, indicating the four tissue slices (Modified after (Renne et al 2009)). Corresponding transverse incisions localizations are: 1. Posterior part of upper incisor teeth; 2. Incisive papilla; 3. Second palatal ridge; 4. First upper molar teeth.

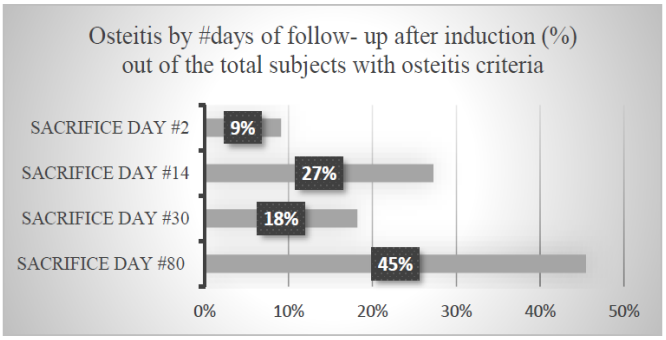


Fig. 4. Mucosal inflammation histopathological changes observed (%), by #days from induction to sacrifice, out of the total subjects (64.7 %) with inflammation criteria

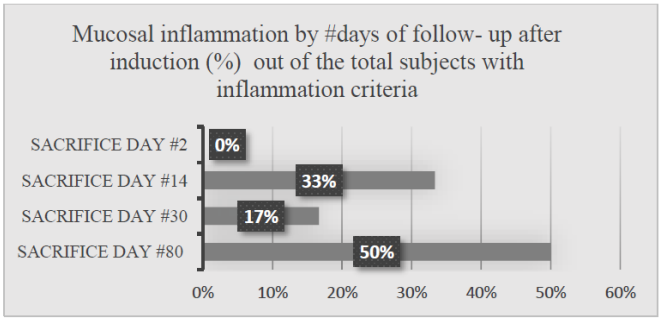


Fig. 5. Underlying bone osteitis histopathological changes observed (%), by #days from induction to sacrifice, out of the total subjects (35.3 %) with osteitis criteria

fields, with an Olympus BX51 microscope model. The histopathologic changes observed in the mucosa and underlying bone are summarized in the Figure 6.

Discussion

According to Khalmuratrova et al (2021), the overlying inflammatorysino- nasal mucosa plays a critical role in the initiation of osteitis, in patients with CRS, however the molecular mechanism and functional significance remains unclear. Withal, according to (de Campos et al 2015), through the Haversian canal system, the inflammatory mediators may spread to non-adjacent bone structures, thus, the bone involvement may contribute to the onset, dissemination, and persistence of the inflammatory status, in CRS. Browsing the literature, we found experimental models for osteitis associated with mechanical trauma, as well as infectious related osteitis. Although surgery has been shown to improve quality of life for patients with CRS, and improve long-term outcomes, the mechanism by which this is achieved is not well understood. There is not yet an established consensus on how potential perioperative therapeutic targets such as bacterial communities, biofilms, mucosal healing, and inflammatory changes should be managed (Jain et al 2017). Previous

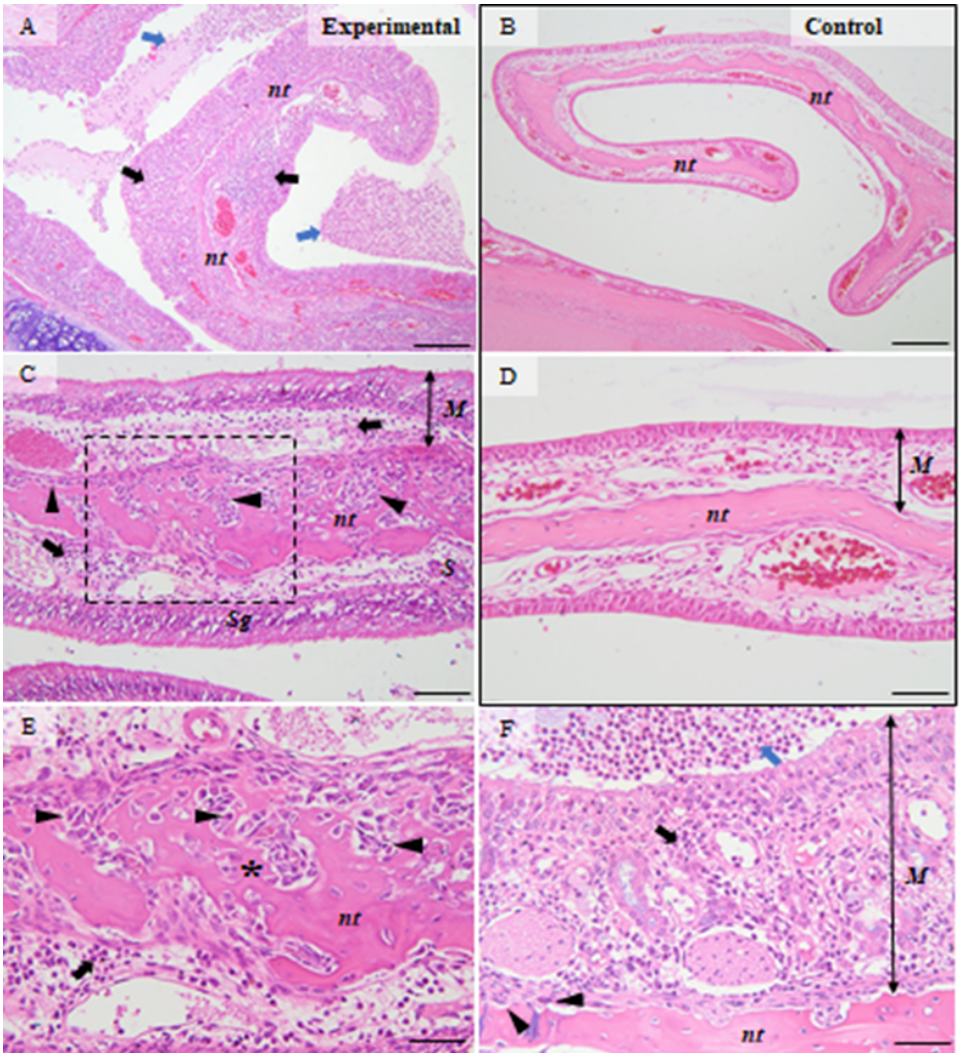


Fig. 6. Histological images presenting the nasal mucosa at the level of the turbinate (experimental group depicted in images A, C, E, and F; the control is presented in images B and D). For the experimental group, the respiratory meatus is partially occupied by purulent exudate (images A and F, indicated by the blue arrows) and the lamina propria of the respiratory mucosa (M) diffusely infiltrated by many lymphocytes admixed with plasma cells, neutrophils, and edema (image A, C, E and F, indicated by black arrows). The turbinate bones (nt) are irregularly expanded and extensively lined by a continuum border of hypertrophic, polygonal osteoblasts (images C and E, indicated by arrowheads) producing woven bone (image E, indicated by the asterisk) with variable degrees of mineralization. Occasionally, the turbinate bones are lined by active osteoclasts located in resorption lacunae (image F, indicated by arrowheads). HE stain, ob x 10 (images A and B; scale bar= 200 µm), x 20 (image C; scale bar= 100 µm) and x40 (images D, E and F; scale bar= 50 µm);

ESS was associated with high osteitis prevalence, greater endoscopic score severity, worse computed tomography grading and poor outcome (Lee et al 2006). Thus, the need for a proper murine experimental model of osteitis after ESS, it becomes a real challenge. As well, there are evidence of osteitis typically associated with nasal polyps, eosinophilia, and recurrent CRS, however its roles and pathogenesis remain unclear due to the lack of animal models (Lee et al 2006; Khalmuratrova et al 2020).

Therefore, the initial purpose of our experimental model was to study the specific role of sino- nasal inflammation in bone remodeling in CRS. We started our experimental activity from the definition of osteitis in CRS, which is likely a development of new bone and bone remodeling. We conducted the experimental model following the previous model described by (Joo et al 2019) to demonstrate the relationship between mucosal denudation and subsequent subjacent osteitis. Because the microscopic neo-osteogenesis changes were observed mainly at the bone level away from the targeted site, we concluded that this model could provide new insights into the pathophysiology of neo-osteogenesis after ESS and develop a basis for new effective therapies. Despite the prior existing literature experience with rabbits and mice (Snidvongs et al 2014; Khamuratrova et al 2020; de Campos et al 2015), we developed a murine model on rats, because of the easy manipulation, higher resistance, lower costs, and availability of the breed.

At present time, evidence on mucosal changes such as edema, goblet cell hyperplasia, decreased number of ciliate cells that occur in CRS, has been well described. However, the changes in the underlying bone have only recently been investigated (Giacchi et al 2001). True "osteitis" with inflammatory infiltrate of the bone was not observed. Osteitis appears to be a process of neo-osteogenesis and bone remodeling, rather than bony inflammation (Snidvongs et al 2019). On the other hand, (Joo et

al 2019) demonstrated the presence of exudate, inflammation, neo-osteogenesis and bony synechia.

In our study we have reported results in accordance with this current state of knowledge. We identified criteria for neo- osteogenesis in 35.3 % of the specimens in the experimental group. The bone thickening and irregular extension at the level of the nasal turbinate of these individuals was observed. Also bone neo- formation, with variable degrees of mineralization, was highlighted by the hypertrophic osteoblasts producing woven bone. Bony resorption and remodeling were described by the active osteoclasts located in resorption lacunae, lining the turbinate bones.

Regarding the inflammatory mucosal changes (edema, decreased number of ciliated cells, and goblet cell hyperplasia), we identified the specific features in 11 of the 17 rats (64.7 %), for the experimental group. The respiratory meatus was partially occupied by purulent exudate, the lamina propria of the respiratory mucosa was diffusely infiltrated by many lymphocytes admixed with plasma cells, neutrophils, and edema.

What we consider important to emphasize is that our experimental model followed the previous described nasal septum lesion model (Joo et al 2019). Nevertheless, instead of the bony synechia described by the author, we identified neo-osteogenesis under the mechanically damaged mucosa of the nasal turbinate, not at the septum area. Our histopathological findings were conclusive for inflammatory catthar, inflammation of the nasal mucosa, with oedema in lamina propria, mononuclear cells and osteoid reaction in the bone, activated osteoblasts in different stages of activity, from osteoblast strings that start to produce bone tissue, to osteoblasts surrounded by osteoid matrix. Also, we observed bone remodeling, with osteoclasts in resorption lacunae, neo- osteogenesis with lamellar bone deposition.

As to the incidence of osteitis, the exact prevalence varies depending on the evaluation criteria in human patients with CRS. According to different investigators, osteitis was identified in 39,6% of CRS patients who underwent endoscopic sinus surgery (Sacks et al 2013), in around 51% of CRS patients, with higher prevalence in those who had prior sinus surgery (76%) (Snidvongs et al 2012) comparing to 33% in primary CRS patients or 36% in patients with primary surgery (Georgalas et al 2010). In our pilot study we validated an experimental model that mimics the lesion of the mucosa induced by ESS and we observed inclusion criteria for osteitis (neo- osteogenesis, bone resorption, bone thickening), in accordance with the existing data, in a percentage of 35.3 %.

According to (Lindsay et al 2006), in murine model, the inflammation was evaluated by the severity or grade of inflammation and the focal or diffuse nature of the inflammation. The severity or grade was scored on a scale of none (0), minimal (1)- rare individual inflammatory cells within the mucosa and submucosa, mild (2)- light infiltrate of individual and occasional clusters of inflammatory cells, moderate (3)- dense infiltrate of inflammatory cells, and severe (4)- inflammatory infiltrate so dense as to obscure the normal architecture of the mucosa or submucosa. The secretory hyperplasia was scored on a scale of none (0)- no secretory cells, minimal (1)- rare secretory cells, mild (2)- scattered areas of secretory hyperplasia, moderate (3)- diffuse areas of hyperplasia, and severe (4)- diffuse areas of secretory cells so dense to obscure normal architecture and coupled with secretory metaplasia of subepithelial glands such as septal glands. De Campos et al (2015) described the semi- quantitative parameters used in mucosal inflammation and osteitis gradations, as follows: grade (0)- absence of inflammation; grade (1)- mild inflammation, with slight mucosal inflammatory cells; grade (2) moderate inflammation with diffuse inflammatory cells in the mucosa; grade (3)- intense inflammation, with diffuse inflammatory infiltrate, associated with epithelial injury, abnormal mucosal and submucosal architecture. In regard with the bone changes, the same author considered the following: grade (0)- absence of inflammation; grade (1)- mild inflammation, mild periosteal thickening; grade (2) moderate inflammation with moderate periosteal thickening and osteoblastic rimming along the new bone; grade (3)- intense inflammation, with pronounced periosteal thickening, osteoblastic rimming, and presence of non- mineralized osteoid. Given the above, we followed the mentioned features criteria in our histopathological evaluation.

Our murine model has several advantages comparing other existing similar experiments. First, we demonstrated the feasibility of reproducibly chronic inflammation and subsequent neo-osteogenesis, after endoscopic sinus surgery, by a simple brushing mechanical injury. Regarding the protocol complexity and technical difficulty, comparing to other previous literature experience, such as Rosen needle injury (Tansavatdi et al 2010) or the exposure to different biological agents as Aspergillus Fumigatus (Tansavatdi et al 2010), Pseudomonas Aeruginosa (Perloff et al 2000), staphylococcal and streptococcal toxoid (de Campos et al 2015), our study protocol had a low degree of difficulty, and high safety profile, in inducing the similar ESS lesion, with minimal stress on animals. Regarding the follow- up period, comparing to many other investigators (Joo et al 2019; de Campos et al 2015) who studied the evolution of inflammatory cells, neo- osteo- formation and bony cell maturation, we have a generous follow-up of 80 days after injury, vs 28-30 days.

We identified certain limitations of our study, such as having a small sample size (n=20) that may not be reliable to validate the osteitis threshold. However, most of the experimental animal model studies also used similar, even fewer animal number (Snidvongs et al 2014; de Campos et al 2015). Additionally, like in other experimental models (Snidvongs et al 2014), no correlation was made between lesion- inflammation, lesion- osteitis and inflammation- osteitis, new scales should be implemented. Another limitation that could be addressed in our further study is the impossibility to quantify the degree of bone mineralization due to the embedded procedures, if necessary, different, and more expensive embedded procedures may be used. To better asses the mineral deposition, for the main study protocol, we may take into consideration beside the standard HE and the optic microscopy used in the actual experiment, the Alizarin red S staining (Khalmuratova et al 2019) and the Polarized light microscopy, to evaluate the histomorphometry of bone structure, consistent with bone remodeling, and to assess the orientation of collagen fibers (Giacchi et al 2001).

Conclusions

Based on the results of this experimental study, we demonstrated that simple brushing lesions of nasal mucosa may be sufficient to induce inflammation and osteitis, in an experimental rat model. Moreover, the results of the study demonstrated that we

accurately mimicked the injury created by endoscopic sinus surgery (ESS) and its effect on inflammation, thus we can conclude that reducing inflammation may reduce the risk to develop osteitis and to prevent recalcitrant CRS to patients that underwent ESS. This will represent our main objective in the consequently experiment. Our analysis revealed histopathological features consistent with mucosal inflammation in 64.7 % and varying grades of bone remodeling, in 35.3 % of the studied specimens. Simple light microscopy demonstrated bone resorption and neo-osteogenesis in the extracellular matrix. Due to the paucity of studies that describe how ESS changes the sinus environment, this animal model could provide new insights into the induced osteitis and a better understanding of these changes that may be crucial for the development of more effective surgery and post-operative care, particularly for the recalcitrant cases.

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