

# Immunohistochemical changes and atrophy after chronic ethanol intoxication in rat salivary glands

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**Summary.** Alcoholism in humans is a chronic and progressive disease, characterized by loss of ethanol consumption control. Previous studies have reported that prolonged exposure to ethanol was responsible for alterations in glandular tissues of human and rodents. However, the interrelationship between ethanol and the glandular system is still the subject of numerous investigations, including the possible resistance of the submandibular gland (SG). In the present study, we investigated whether chronic ethanol exposure during adolescence may affect the parotid gland (PG) and SG in female rats. Female rats (n=16) were treated with distilled water or ethanol (dose of 6.5 g/kg/day, 22.5% w/v) through gavage for 55 days. Glands were collected, weighed and submitted to histological processing. Morphometric analysis was assessed by parenchymal and stromal area measurements. Smooth muscle actin ( $\alpha$ -SMA), cytokeratin-19 (CK19) and apoptotic caspase-3 (CAS) were measured using ImageJ<sup>®</sup> software. Chronic ethanol administration did not alter the body weight of rats after treatment, although it increased glandular weight (p<0.001), reduced the parenchyma area (p<0.001) and decreased CK19 and  $\alpha$ -SMA immunostaining in the PG. Besides, ethanol induced CK19 and CAS overexpression, and the occurrence of duct-like structures in SG. These results suggest that

ethanol induces histological and morphometric changes in salivary glands of female rats intoxicated with ethanol during adolescence. Furthermore, the mechanism underlying these alterations needs to be investigated but may be not related to the inflammatory process.

**Key words:** Ethanol, Salivary glands, Immunohistochemistry, Adolescence

## Introduction

Alcoholism is a chronic and progressive psychiatric disorder characterized by the loss of control of ethanol consumption (Friedlander et al., 2003). The World Health Organization (WHO) estimates that ethanol abuse is responsible for approximately 3 million premature deaths worldwide each year and approximately 4% of global morbidity (Schuckit, 2009). The use of the drug has increased in a heavy binge-drinking way (higher doses and longer period of time), among younger women, highlighting the vulnerability of this group and emerging as a public health emergency (Schuckit, 2009; INPAD, 2013).

The effects of chronic ethanol abuse are associated to damage in the tissues and organs in humans and animals, as a result of morphological and functional changes (Molina et al., 2002; Owczarek et al., 2005). Studies have pointed to several organs and systems affected by chronic alcohol exposure in rodents, such as upper digestive tract (Mascrec et al., 1984; Bagyánszki et al., 2010); liver, lungs, heart, and skeletal muscle (Molina et al., 2002; Owczarek et al., 2005); central

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nervous system (Oliveira et al., 2014; Teixeira et al., 2014); pancreas (Orabi et al., 2011); and salivary glands (Maier et al., 1986, 1988a,b; Banderas et al., 1992; Carrard et al., 2007; Nör et al., 2013). Studies in humans revealed damage to liver, lungs, heart, skeletal muscle and salivary glands (Scott et al., 1987, 1988; Ferraris et al., 1995, 1999; Bohl et al., 2008). The latter was one of the first structures affected by chronic ethanol consumption (Mandel and Baurmash, 1971).

Parotid (PG) and submandibular (SG) glands are classified as major salivary glands, and both are microscopically similar in architecture. The glandular parenchyma consists of continuous-secreting acini cells, which leads to a system of ducts that empty into the oral cavity (Actis et al., 2002). In rodents, the PG is located behind and below the ear, caudally bordering the SG. Microscopically, the PG consists of pure serous acini. On the other side, SG is a mixed gland that includes both serous and mucous acini (Amano et al., 2012). Both these glands contribute to 95% of the total salivary secretion produced and secreted into oral cavity, which the SG contributes 60-70% (Faustino and Stipp, 2003; Katchburian and Arana, 2004).

Ethanol rapidly diffuses into saliva and oral tissues (Gifford et al., 2008). Immediately after alcohol exposure, high concentrations of this drug are detected in the salivary glands (Waszkiewicz et al., 2013b). Thus, chronic alcohol exposure may promote effects on saliva, including a reduction in the salivary flow rate associated to gland atrophy, and reduced electrolytes, salivary proteins and glycoproteins (e.g. amylase, immunoglobulin A, lysozyme, lactoferrin) which modifies the morphology and physiology of the salivary glands (Lieber, 1991; Faustino and Stipp, 2003; Riedel et al., 2003; Waszkiewicz et al., 2013a,b).

However, there are insufficient studies in the literature in order to clarify these structural alterations, as well as in the morphometry and in the structure of its cellular components. Thus, our protocol aims to investigate the morphometric and immunohistochemical changes of the PG and SG in female rats exposed to heavy alcohol exposure during adolescence.

## Materials and methods

### Animals

A total of 16 adolescent female Wistar rats, 35 days old at the beginning of the experiments (50-60 g) were obtained from the Federal University of Pará (UFPA) and kept in collective cages (a maximum of five animals per cage). Animals were maintained in a climate-controlled room on a 12-h reverse light/dark cycle (7:00 AM lights on), with food and water *ad libitum*. All procedures were approved by the Ethics Committee on Experimental Animals of the Federal University of Pará under license number BIO-043-12 and followed the guidelines suggested by the NIH Guide for the Care and Use of Laboratory Animals.

### Experimental groups

Animals were initially allocated to the following groups: distilled water (n=8) or ethanol (6.5 g/kg/day, 22.5% w/v) (n=8). Every day, rats were administered through orogastric cannula over a period of 55 days (i.e., until the 90<sup>th</sup> day of life) according to a procedure previously described (Livy et al., 2001; Maier and West, 2001; Maia et al., 2009; Oliveira et al., 2014; Teixeira et al., 2014). The current ethanol administration protocol was based on previous studies from our group (Maia et al., 2009; Teixeira et al., 2014; Oliveira et al., 2014) showing that ethanol intoxication (6.5 g/kg/day) in the developing central nervous system may induce long-lasting neurobehavioral impairments in rats. In order to investigate putative effects of ethanol exposure on overall poor nutrition levels, which may directly affect the quality of the tissues, were noted the animals' body weight, behavioral dysfunctions, loss or changes in hair coat, diarrhea, and edema during the entire period of ethanol administration (Campana et al., 1975).

### Collection and preparation of tissue

After 7.5 hours of the last administration of alcohol, which is the period related to non-detectable amounts of ethanol in the blood (Livy et al., 2003), animals were anaesthetised with a mixture of ketamine hydrochloride (72 mg/kg, i.p.) and xylazine (9 mg/kg, i.p.) and then perfused with heparinized saline followed by paraformaldehyde 4%. The PG and SG were removed and weighed by analytical balance (FA 2104 N, Eletronic Balance Bioprecisa, Shanghai, China). The relative glandular weight (RGW) was calculated by the formula:  $RGW = \text{gland weight} \times 100 / \text{final body weight}$ .

After weighing, glands were post-fixed in formalin solution (10%), and submitted to histological process by dehydration in increasing alcohol battery and xylene, and finally embedded in Paraplast<sup>®</sup> resin.

### Histomorphometry

For histochemistry procedures, 7- $\mu\text{m}$  samples were cut from the region corresponding to the largest cross-sectional area of the organ with a microtome (RM2255, Leica Microsystems, Nussloch, Germany), deparaffinized in xylene, rehydrated in decreasing concentrations of alcohol and water, and finally stained with hematoxylin/eosin (HE). Thereafter, for morphometric analysis, the mean percentage equivalent to the glandular parenchyma and stroma region was evaluated.

The area of the samples was evaluated by counting point planimetry method. The sections were stained by HE and illustrative images from all experimental groups were obtained with a digital camera (Cyber-shot DSC-W230 4X optical zoom, Sony, Tokyo, Japan) attached to a stereo microscope (1.5x; Eclipse E200, Nikon, Tokyo,

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Japan). Subsequently, we used five random sections per animal of both PG and SG computerized images, which were plotted in the computer monitor followed by overlapping of the grid of planimetry, which measures 21x16 cm and contains 2,852 points equidistant of 0.5 cm. Coincident points with parenchymal elements and stromal glandular were counted, and the percentage obtained of each region was calculated by the formulas: Morphometric Parenchymal Area (%MPA) = number of coincident points in the parenchymal x 100/ number total of points on the grid; and, Morphometric Stromal Area (%MSA) = number of coincident points in the stroma x 100/ number total of points on the grid (Borgers et al., 1993).

### Immunohistochemistry

Immunohistochemical studies were performed on paraffin-embedded tissues using streptavidin (Spring bioscience, Pleasanton, USA) and 3,3-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, USA) methods. Briefly, 3- $\mu$ m sections were deparaffinized and rehydrated in decreasing concentration of alcohol battery. After antigen retrieval in chamber Pascal (S2800, Dako, Carpinteria, USA) and blocking of endogenous peroxidase activity, sections were incubated with the primary antibody anti- $\alpha$  smooth muscle actin ( $\alpha$ -SMA) (1:50 dilution, Dako, Carpinteria, USA), a cytoplasmic myofilaments marker (Takahashi et al. 2001); anti-cytokeratin 19 (CK19) (1:100 dilution, Bioss, Boston, USA), a marker of intermediate filaments in the cytoplasm cells ductal (Fradette et al., 1998); and anti-caspase 3 (CAS) (1:250 dilution, Promega, Madison, USA), which reacts with the active form of caspase 3 and is associated with induction of apoptosis (Takahashi et al., 2004), in 0.1 M phosphate buffer saline (PBS). After, sections were submitted to streptavidin and revealed by DAB and counter-stained by Mayer hematoxylin methods.

### Digital image analysis

Evaluation of immunostaining was performed by the percentage of labelled area of six regions randomly selected of each gland by the operator. Briefly, images selected were obtained by microscope (Axio Scope, Carl Zeiss, Jena, Germany) attached to the camera (AxioCam HRC, Carl Zeiss, Jena, Germany) with a magnification of 400x.

Digital images were prepared for analyses using ImageJ<sup>®</sup> software [Bethesda, Maryland, USA, (<http://rsb.info.nih.gov/ij/download.html>)]. According to Ruifrok and Johnston (2001), each color images were selected and separated from the rest of the field using image segmentation tool. CK19, CAS and  $\alpha$ -SMA positive area (brown stain) and total gland area in the analysed field were automatically measured using color deconvolution plugin by calculating the contribution of each stain based on the stain-specific RGB absorption. In

our protocol, color deconvolution was used to separate DAB stain (positive areas) from hematoxylin (total area of the glands). Each image was changed to black/white color image. The measurements button was calibrated to calculate the area of the field (total gland area) and the area fraction (black color representing DAB stain). Finally, scores for CK19, CAS and  $\alpha$ -SMA expression in the analysed field were calculated by the positively stained area over the total gland area.

### Statistical analysis

All values are expressed as means + S.E.M. (n=8 animals per group). Statistical comparison was performed using Mann-Whitney test. The accepted level of significance was  $p \leq 0.05$ . All tests were performed using the GraphPad Prism software version 5.0 (San Diego, USA).

## Results

### *Body weight of female rats in adulthood was not affected by chronic ethanol exposure during adolescence*

As illustrated in Fig. 1, the chronic ethanol (6.5 g/kg/day) administration over a period of 55 days (i.e., from the 35<sup>th</sup> day until the 90<sup>th</sup> day of life) did not alter the final body weight of ethanol-treated rats.

### *Ethanol exposure increases PG weight, but does not change SG weight*

The chronic ethanol (6.5 g/kg/day) administration over a period of 55 days (i.e., from the 35<sup>th</sup> day until the 90<sup>th</sup> day of life) induced a significant increase in the RGW of PG of female rats from the 35<sup>th</sup> to 90<sup>th</sup> days of life ( $p < 0.001$ ). However, the total SG weight between control- and ethanol-treated rats after the period of 55 days of treatment did not differ statistically (Fig. 2).

### *Chronic ethanol exposure induces reduction and atrophy of PG glandular parenchyma and increases SG glandular stroma area*

The histomorphometric results evaluated in the salivary glands are summarised in Fig. 3. Mann-Whitney test revealed that chronic ethanol exposure reduced the percentage of the parenchymal area of the PG ( $p < 0.001$ ) that was not observed in the SG (Fig. 3A).

In addition, chronic ethanol exposure increased stromal area of SG ( $p < 0.001$ ) that was not noticed in the PG (Fig. 3B).

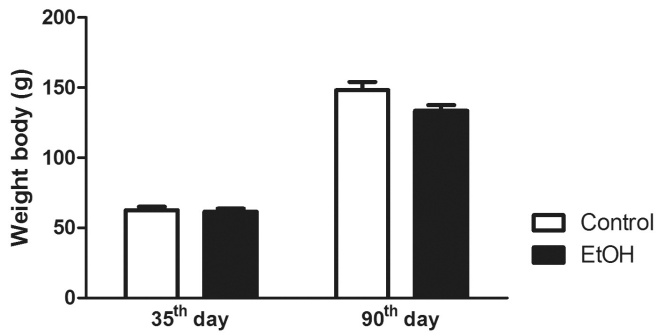
### *Chronic ethanol intoxication during adolescence does not induce inflammatory infiltration in PG and SG*

The HE method did not reveal inflammatory processes in the salivary glands of female rats after

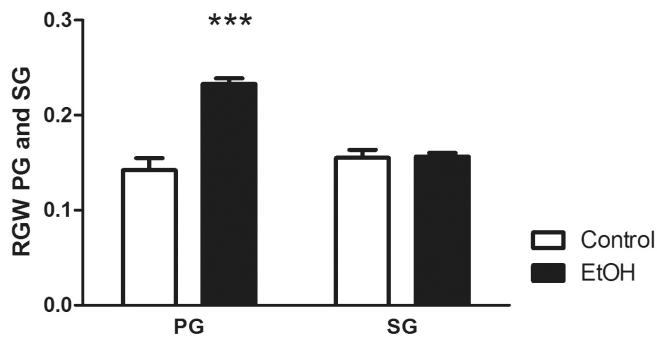
chronic ethanol exposure during adolescence, from the 35th to 90th days of life (Fig. 4). In the PG and SG glands, control (Fig. 4A,C) and ethanol-treated groups (Fig. 4B,D), did not present periductal lymphocyte infiltration. Moreover, as demonstrated in the photomicrograph in Fig. 4D, rats chronically treated with ethanol during adolescence showed alterations in the SG, consisting of ductal-like structures (indicated by arrows).

*Ethanol intoxication during adolescence induces reduction of CK19 expression in the PG and overexpression in the SG*

The effects of chronic ethanol intoxication during



**Fig. 1.** Effects of chronic ethanol (EtOH) (6.5 g/kg/day) administration from adolescence (35 days old) to adulthood (90 days old) on body weight of female Wistar rats. The results are expressed as mean  $\pm$  S.E.M. of initial body weight and final body weight (g) (n=8 animals per group), (Mann-Whitney test).

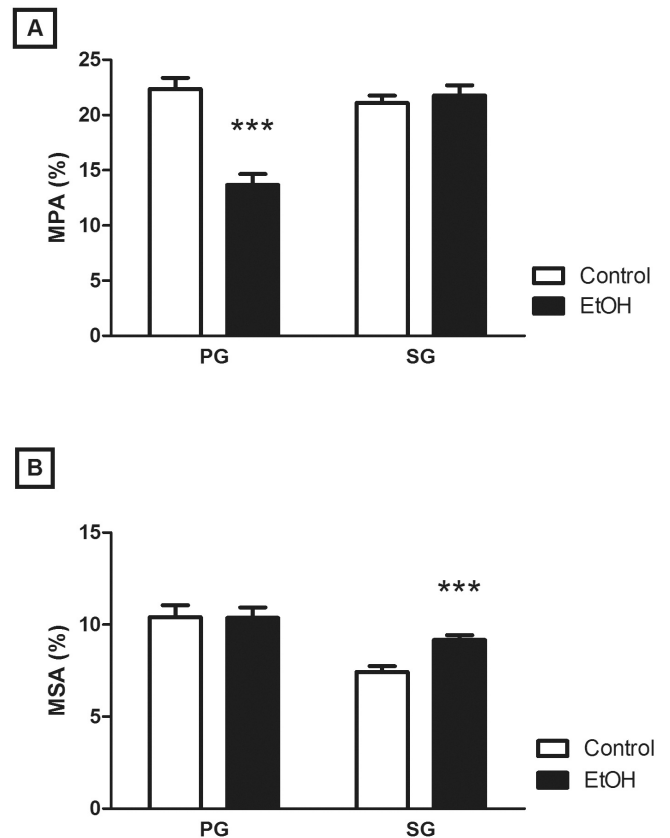


**Fig. 2.** Effects of chronic ethanol (EtOH) (6.5 g/kg/day) administration from adolescence (35 days old) to adulthood (90 days old) on relative glandular weight (RGW) of Parotid (PG) and Submandibular (SG) glands of female Wistar rats. The results are expressed as mean  $\pm$  S.E.M. of RGW (RGW=gland weight  $\times$  100/final body weight) of PG and SG (n=8 animals per group). \*\*\* $p \leq 0.001$  compared to the control group (Mann-Whitney test).

adolescence on the labelling of three different antibodies in both PG and SG are illustrated in Figure 5. Mann-Whitney test revealed reduced  $\alpha$ -SMA positive cells on the PG ( $p < 0.05$ ) that was not noticed in SG (Fig. 5A). Of high importance, as detected in the sample images, rats chronically treated with ethanol during adolescence developed alterations in the tissue glands, in which both acinar and ductal cells were more dispersed than control group in SG and PG.

Moreover, CK19 positive cells were reduced in PG ( $p < 0.001$ ) associated to contradictory increase in the SG ( $p < 0.01$ ), detected through immunostaining method with CK19 antibody that labelled intercalated, striated and excretory ductal cells of female rats chronically treated with ethanol from adolescence till adulthood (Fig. 5B,E,H,K,N).

CAS-positive cells were observed in all groups analysed (Fig. 5F,I,L,O). Besides, chronic ethanol intoxication in rats increased CAS-positive cells in SG



**Fig. 3.** Effects of chronic ethanol (EtOH) (6.5 g/kg/day) administration from adolescence (35 days old) to adulthood (90 days old) on morphometric evaluation of Parotid (PG) and Submandibular (SG) glands of female Wistar rats. The results are expressed as mean  $\pm$  S.E.M. Panel A represents the MPA- Morphometric Parenchymal Area (%); Panel B represents the MSA- Morphometric Stromal Area (%) (n=8 animals per group). \*\*\*  $p \leq 0.001$  compared to the control group (Mann-Whitney test).



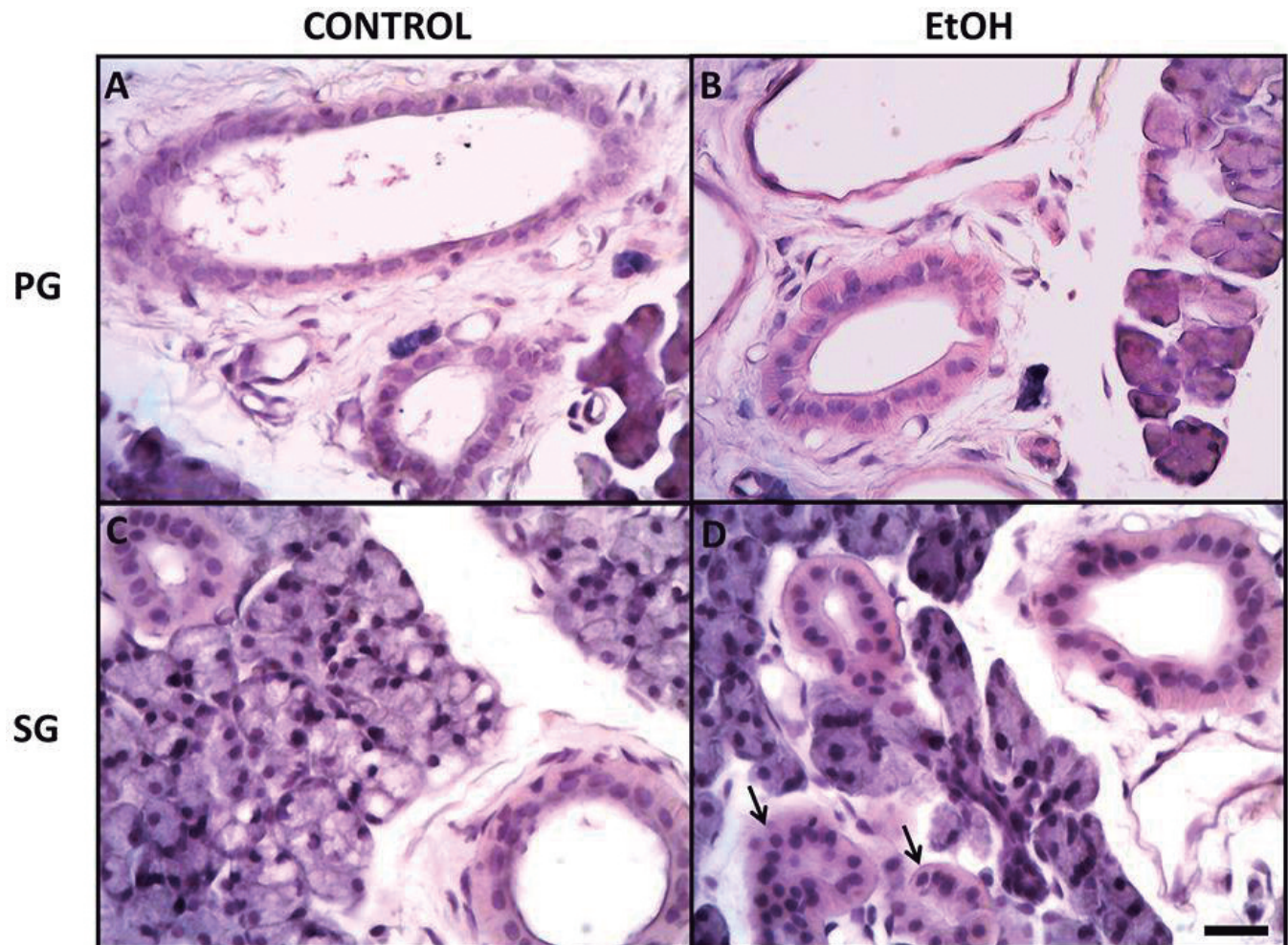
( $p < 0.05$ ) but not in PG (Fig. 5C).

### Discussion

In the present study, we investigated whether chronic ethanol exposure during adolescence affects the salivary glands in female rats. The current findings demonstrate, for the first time, that chronic ethanol exposure during adolescence induces parenchyma atrophy related to gland weight increases and reduction in ductal and myoepithelial cell population in the PG as evaluated in the histomorphometry and immunohistochemical analysis. Moreover, histological evaluation revealed that our alcohol exposure altered cell population in the SG, appearing ductal-type structures and inducing apoptosis. Heavy binge-drinking (i.e., high doses and long period of time) has increased worldwide mainly among

adolescents (Schuckit, 2009; INPAD, 2013). Therefore, our group has studied the effects of chronic ethanol exposure in body tissues in adolescent rats. Heavy chronic ethanol intoxication (6.5 g/kg/day) during adolescence induces long-lasting neurobehavioral impairments as well as brain histochemical damage (Oliveira et al., 2014; Teixeira et al., 2014). In this sense, our group also observed that chronic ethanol consumption from adolescence till adulthood alters the homeostasis of the oral cavity promoting alveolar bone loss in rats (Bannach et al., 2015).

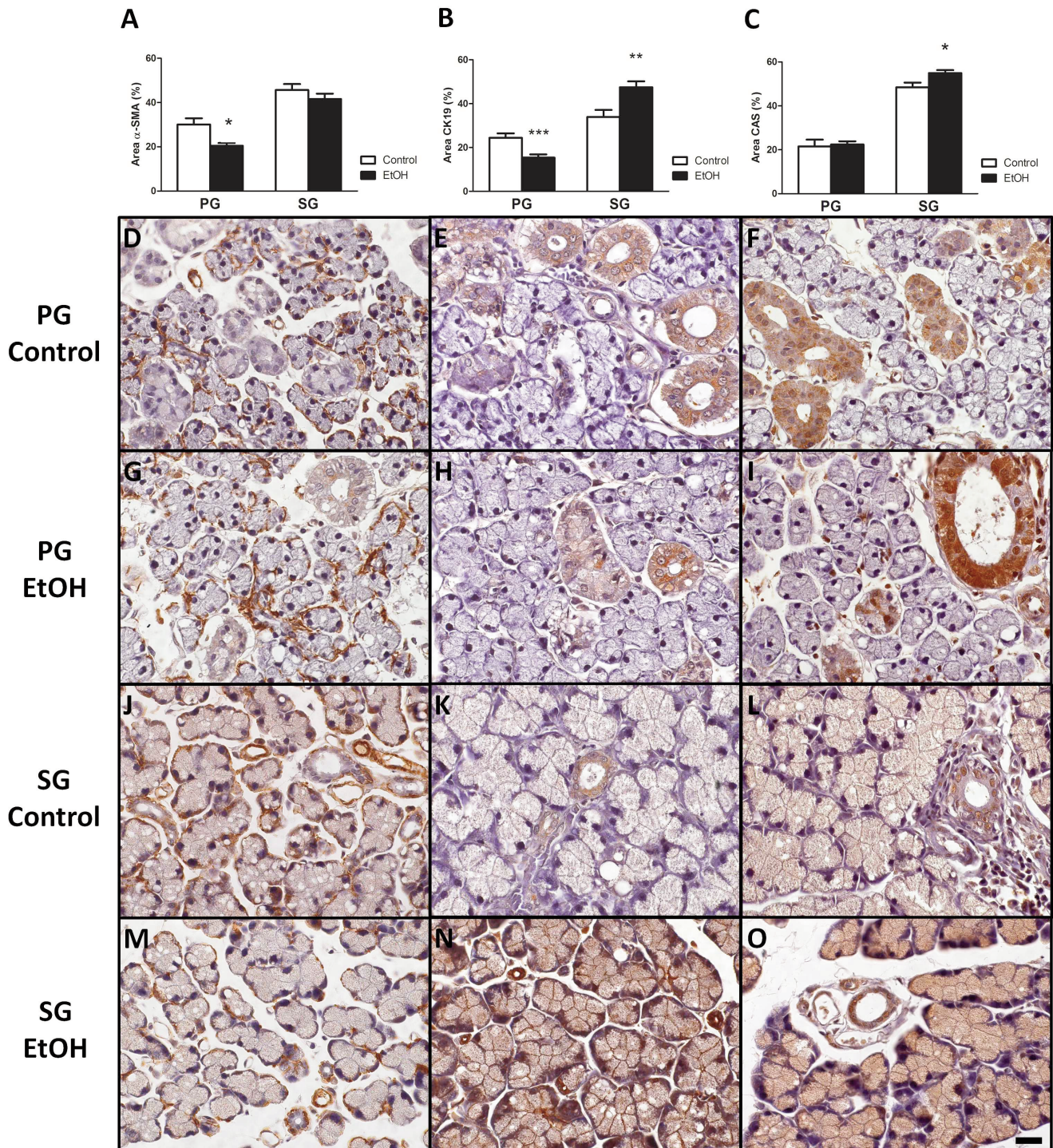
It has been shown that chronic ethanol consumption induces glandular atrophy and reduces salivary flow rate, salivary protein and glycoprotein levels and electrolytes promoting, among alcoholics, worse dental state and significantly worse periodontal state (Waszkiewicz et al., 2013a,b). Whereas saliva ensures the homeostasis of the



**Fig. 4.** Effects of chronic ethanol (EtOH) (6.5 g/kg/day) administration from adolescence (35 days old) to adulthood (90 days old) on histological sections of Parotid (PG) and Submandibular (SG) glands stained by hematoxylin/eosin (HE) of female Wistar rats. **Panel A** represents the PG of the control group; **Panel B** represents the PG of ethanol-treated group; **Panel C** represents the SG of the control group; **Panel D** represents the SG of ethanol-treated group. Arrows indicate ductal-like structures. Scale bar: 20  $\mu\text{m}$ .



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**Fig. 5.** Effects of chronic ethanol (EtOH) (6.5 g/kg/day) administration from adolescence (35 days old) to adulthood (90 days old) on Parotid (PG) and Submandibular (SG) glands immune expression of anti- $\alpha$  smooth muscle actin ( $\alpha$ -SMA), anti-cytokeratin 19 (CK19), and anti-caspase 3 (CAS) positive cells of female Wistar rats. The results are expressed as mean  $\pm$  S.E.M. **Panel A** represents the labelled fraction area (%) of  $\alpha$ -SMA positive cells; **Panel B** represents the labelled fraction area (%) of CK19 positive cells; **Panel C** represents the labelled fraction area (%) of CAS positive cells. Labelled fraction area (%) was calculated by positively stained area over the total glandular area. Photomicrographs illustrate immunohistochemical staining of antibodies  $\alpha$ -SMA (**panels D, G, J, M**), CK19 (**panels E, H, K, N**) and CAS (**panels F, I, L, O**) in PG and SG (n=8 animals per group). \* $p \leq 0.05$  compared to the control group, \*\*  $p \leq 0.01$  compared to the control group, \*\*\*  $p \leq 0.001$  compared to the control group (Mann-Whitney test). Scale bar: 20  $\mu$ m.



### *Ethanol intoxication in salivary glands*

oral environment, defence of the oral cavity, oropharynx and upper region of the gastrointestinal tract, and protection of hard and soft tissues of the oral cavity as well as of the teeth and periodont (Pronko et al., 2002; Prestifilippo et al., 2009), saliva flow rate reduced may induce oral cavity vulnerability. Thereby, investigations are necessary about alterations that chronic ethanol consumption induce on salivary gland tissues.

Studies have demonstrated that alcohol exposure affects salivary glands, inducing fat accumulation, acini cell swelling and reduction of protein content in the PG. Besides, salivary flow rate was reduced related to gland atrophy of the secretory parenchyma (Scott et al., 1988; Maier et al., 1990; Banderas et al., 1992; Riedel et al., 2003; Carranza et al., 2005). However, the effects on the gland size after ethanol exposure remain unclear (Mandel and Baumash, 1971; Maier et al., 1986; Scott et al., 1988; Campos et al., 2005; Mandel et al., 2005).

Ethanol intoxication protocol used in our study revealed weight increase of PG (but not SG). Similar results were found in Campos and colleagues study (Campos et al., 2005). On the contrary, Maier et al. (1986) related that chronic ethanol exposure reduced the PG weight, not affecting SG.

The effects of heavy alcohol consumption on nutrition are well recognized and may contribute to poor development of organs (Campos et al., 2005). The malnutrition status may alter the physiology and morphology of salivary glands (Nör et al., 2013) which in rodents is characterized by behavioral dysfunction, loss or changes in hair coat, diarrhea, and edema, apart from remarkable weight loss (Campana et al., 1975). In fact, in the present study was not reveal any of malnutrition characteristics in the female rats in the adulthood, as previously observed in Oliveira et al. (2014) study. However, our data demonstrated PG atrophy (observed in the parenchyma area reduced) associated to weight increased displayed by alcohol exposure as previously reported by Campos and colleagues (2005) that noticed a weight gain and salivary gland atrophy in PG (Riedel et al., 2003; Maier et al., 1986). Moreover, glandular atrophy depends on the dose and duration of ethanol consumption (Scott et al., 1988), but accurate data related to the minimum period required for atrophy is poorly understood.

Besides, our results also reveal that the stroma area of SG was increased which was not followed by inflammatory process, since there was not noticed inflammatory infiltration in the glandular stroma. Our results are contradictory to other studies that claim that chronic ethanol intoxication increased periductal lymphocyte infiltration (Dale, 2001; Carda et al., 2005). However, it is well reported that chronic alcoholism is one of the primary causes of sialadenosis, a non-inflammatory pathology that induces glandular enlargement and alters gland weight (Mandiy and Suraffanon, 2002), which could partially explain our findings.

Furthermore, the glandular atrophy produced by

alcohol exposure is associated with increased proliferative activity of the oral mucosa epithelium that may be caused by ethanol cytotoxic effect (Riedel et al., 2003). Recent study in rats (Nör et al., 2013) demonstrated that chronic ethanol exposure during adulthood associated to SG partially removed induced cell proliferative profile and enhanced convoluted ducts that are a source of growth factors and are present in the SG but not PG (Amano et al., 2012), at the 7<sup>th</sup> post-surgical day. In the present study, chronic ethanol intoxication during 55 days showed increased expression of CK19 and appearing ductal-type structures in the SG, however, reduction in CK19 expression in PG after a harmful stimulus.

Cytokeratins are epithelium-specific intermediate filament proteins that support cellular integrity, contributing to cell attachments (Fradette et al., 1998). However, previous studies affirm that CK19 overexpression by suprabasal cells of the oral mucosa indicates cell dysfunction and probable premalignant changes (Michel et al., 1996; Zhong et al., 2007). On the other hand, long-term glandular atrophy leads to a reduction in ductal cells in PG (Walker and Gobé, 1987). Considering that ductal cells in the total SG volume is about 20% compared to PG (5%) and these cells exhibit characteristics of resistance, we suggest that SG may be less susceptible to stressor factors (i.e., alcohol exposure) than PG, since the SG presents resistance factors in higher proportion than PG (Dehaye and Turner, 1991; Burgess and Dardick, 1998). However, the present results cannot provide direct evidence to account for these changes or the mechanisms responsible for gland alteration.

The immunohistochemistry performed to identify myoepithelial cells showed reduced labelling intensity in the samples from the ethanol group in PG but not SG. According to studies with experimental induction of ductal atrophy by other mechanisms (i.e., ligation), the persistence of experimental induction of ductal atrophy promotes reduced population of myoepithelial cells (Burgess et al., 1996; Burgess and Dardick, 1998). In this sense, our study showed that myoepithelial cell population of the glandular parenchyma in PG was reduced by chronic stress caused by ethanol intake during adolescence.

The caspase 3-positive cells found in the glands were presented in ductal and acini cells. It has also been reported that apoptosis participates in progressive and regressive processes which occurs both in development and regeneration process of salivary gland (Takahashi et al., 2004). Apoptosis contributes to the deletion of terminal tubule cells (Hecht et al., 2000) and lumen formation of ducts (Jaskoll and Melnick, 1999). In our study, after alcohol intoxication during adolescence, apoptotic cell immunostaining in the treated group was increased in SG but not in PG. However, the mechanisms that induce caspase-3 levels in the SG, but not PG are not understood. Campos et al. (2005) reported that the biochemical mechanisms of both glands

are diverse, PG have a predominantly aerobic biochemical mechanism, while SG have a predominantly anaerobic mechanism. Such differences may indicate that the events of cell death in PG (more susceptible to oxidative damage) differ from occurring in SG, which this could be more susceptible to apoptotic events

For the first time, our study performed immunohistochemical analyses by three different antibodies in order to investigate the cell-type and mechanisms involved in the damage of chronic ethanol intoxication during adolescence. The anti CK19 e anti  $\alpha$ -SMA are specific markers of glandular salivary cells that label ductal and myoepithelial cells respectively, which are very important to salivary gland morphology and physiology studies. Indeed,  $\alpha$ -SMA is the marker of myoepithelial cells more indicated to studies in glandular tissue and was performed in this study (Takahashi et al., 1999). Therefore, we investigated whether ethanol exposure alters specific proteins and/or induces apoptosis in the parenchyma glandular cells. It is well noticed that previous immunohistochemical studies that assess atrophy in salivary glands are related to identification the diagnosis, or in experimental conditions, are caused by ductal ligation as noxious stimulus (Burgess et al., 1996; Burgess and Dardick, 1998; Takahashi et al., 1998; Safadi et al., 2010; Laco et al., 2012). Thereby, our study emerges as a relevant work that highlight the immunohistochemical evaluation in salivary glands after damage produced by chronic chemical stimulus.

In conclusion, our results provide new evidence that ethanol exposure during adolescence induces PG atrophy, but not SG. Paradoxically, alcohol exposure induced the occurrence of increased duct-like cells related to caspase-3 overexpression in SG. Of significance are the current findings indicating that differences between both PG and SG have been demonstrated. The exact mechanisms involved in the observed harmful alcohol effects in PG, as well as proliferating capacity of duct cells in SG should be investigated in future research, but the present data provide evidence that alcohol during adolescence damages salivary glands.

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