ORIGINAL ARTICLE

Lorazepam induces acinar cells apoptosis of rat parotid glands

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1. Introduction

The prevalence of insomnia, depression and anxiety have recently been increasing (Kessler et al., 2005) with approximately 18.4% of the Brazilian population having experienced depression at least once during their lifetimes. These data characterize Brazil as the developing country with the largest number of people suffering from this disease (WHO, 2018).

Depression is commonly accompanied by anxiety disorders and chronic psychiatric conditions with an estimated prevalence of approximately 29% (Kessler et al., 2005), which results in functional impairment that is associated with significant social costs. Treatment of these conditions includes psychological therapy and antidepressants. These drugs, such as benzodiazepines (BZDs), have been widely studied since the 1960s (Llorente et al., 2000) (WHO, 2018).

BZDs are used by 9–25% of the elderly population aged 65 years of age and over, mainly among women (15–21.7% in Brazil, 9.9% in the United States, 24% in Canada and 25% in Sweden) (Jorm et al., 2000; Alvarenga et al., 2008; Johnell and Fastbom, 2009; Leggettet al., 2015). BZDs can

be divided into two categories, anxiolytics and hypnotics, and can be used as anticonvulsants due to their muscle relaxant effects (De Almeida et al., 2008).

As with all central-acting drugs, BZDs have side effects, such as decreased psychomotor activity, memory loss, physical dependence on long-term treatments, (Longo and Johnson,2000, Uzun et al., 2010) and hyposalivation and/or xerostomia, which is one of the main adverse effects described in the literature (Guggenheimer and Moore, 2003; Lambrecht et al., 2013; Raghavan et al., 2014). BZDs are among the drugs with the highest rates of complaints by patients in dental offices (Smith and Burtner, 1994) where these drugs are prescribed to reduce anxiety related to dental treatments of non-cooperative patients (Zaclikevis et al., 2009). Among the licensed BZDs, Lorazepam is one of the most widely used drugs for both insomnia and anxiety (Amato et al., 2010), particularly among elderly individuals with anxiety disorders and related conditions (Pomara et al., 2015).

Previous studies, by our research group, demonstrated the damage Lorazepam and midazolam, another BDZ commonly prescribed for anxiety, caused to the acinar cells of parotid glands (Rinaldi et al., 2015; Mattioli et al., 2016; Rinaldi et al., 2018). We observed that the chronic use of BZDs significantly decreased the number and proliferation of acinar cells, reduced salivary flow and increased cell volume. In light of this, we hypothesize that the reduction in the number of acinar cells caused by Lorazepam in the salivary glands is possibly due to an apoptotic effect on these cells of the parotid glands of rats caused by the chronic use of this drug.

Apoptosis plays a fundamental role in the elimination of unwanted, damaged or infected cells in multicellular organisms and serves as an important biological process in cellular development, differentiation and proliferation (So et al., 2016). The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end-labeling (TUNEL) (Tilly, 1996) technique is the gold standard test employed to detect apoptotic cells (Burattiniet al., 2009; Heisler-Taylor et al., 2018). Because of the its wide-spread use of TUNEL, there is no need to use another technique to detect apoptotic cells.

The literature lacks studies demonstrating Lorazepam-induced apoptosis in acinar cells. The aim of this study was to evaluate the apoptotic action of a BZD on the acinar cells of the parotid glands of rats by quantification of the number of apoptotic cell nuclei (NCA), the number of acinar cells (N) and the immunoexpression of nuclear proliferation cell proliferation (PCNA) protein.

2. Material and methods

Apoptosis was examined in this study using the paraffin-embedded salivary gland tissue collected from rats studied in a previous report by this research group, Rinaldi et al., (2015). Previously published data from our lab have been used in the current study to correlate with our new data regarding apoptosis.

All animal and project experiments were conducted after approval by the Committee of Ethics in the Use of Animals of the Pontifical Catholic University of Paraná (PUCPR) (CEUA/PUCPR n 01084/2016-2nd version) in accordance with the Guide to the Care and Use of Laboratory Animals. Forty male Wistar rats (250 g of body weight) were obtained from the Central Vivarium of the Pontifical Catholic University of Paraná/PUCPR. The animals were housed in plastic cages and fed with Nuvilab® CR-1 (Nuvital Nutrientes S.A.) pellets and water ad libitum. The animals were housed in rooms with a 12/12 light-dark cycle and a constant temperature of 25 ± 1 °C.

2.1. Experimental treatment

The treatment of the animals was performed as described by Mattioli et al. (2016). The rats were randomly assigned to four groups of ten animals/group, according to Fig. 1.

2.1.1. Death of animals and excisions of the parotid glands

The animals of the control and experimental groups were euthanized by overdose of thiopental sodium 120 mg/kg (Thionembutal®, Abbott Laboratórios do Brasil Ltda., São Paulo, Brazil), by intraperitoneal and intracardiac administration after thirty or sixty days. This dose was found to be safe and effective.

The right and left parotid glands were removed using a number 12 scalpel blade and placed in previously identified universal collectors with 10% buffered formalin for 48 h. The choice to collect the parotid gland was made because the parotid glands are the major salivary gland in many animals. In humans, the parotid gland is the largest of the salivary glands, weighs 20–30 g but produces only approximately 30% of the total saliva.

2.1.2. TUNEL processing and technique

The formalin-fixed parotid glands were embedded in paraffin (donor block). From this donor block, a 3 mm diameter cylinder with trephine drill (Neodent®, Curitiba, Brazil) was coupled to a 130 Watt suspension motor from Bethil (Prometal Ind. Met. LTDA®, Marília, Brazil). Ten of these cylinders were organized into rows and columns and embedded in paraffin (receptor block), and this constituted the tissues microarray (TMA) (Sawaki et al., 1995; Kosuge et al., 2009). In the sequence, microscopic sections with a thickness of 4 μm were cut and mounted on slides to be submitted to the TUNEL technique.

The TUNEL technique was used to detect apoptosis in acinar cell, based on the staining of DNA fragments resulting from the breakage of genomic DNA in situ. The Cell Death Detection Kit, POD-Roche® (Roche Diagnostics GmbH, 11684817910, Mannheim, Germany) was used according to the manufacturer's instructions. This technique has been the gold standard for the evaluation of cellular apoptosis (Burattini et al., 2009).

After dewaxing the sections, endogenous peroxidase activity was blocked in 5% hydrogen peroxide solution and methanol for 15 min at room temperature. Sections were then treated with 0.1 citrate buffer, pH 6.0 in a water bath, for 25 min at 99° C for antigenic recovery.

After washing in TBS-tris (pH 7.0), the slides were covered with 50 μL of the solution enzyme reaction mixture with the label solution and then incubated for 1 h at 37° C in a humidified atmosphere in the dark. After being washed in PBS, the slides were covered with 50 μL convert POD, and incubated for 30 min at 37 °C in a humid chamber.

Groups	Drug	Daily dose	Administration	Treatment period
G_{S30} (Control)	injectable 0.9% saline solution	0,1 mL	intraperitoneally, (once a day, at the same time)	30 days
G_{S60} (Control)	injectable 0.9% saline solution	0,1 mL	intraperitoneally, (once a day, at the same time)	60 days
G_{L30} (Experimental)	injectable solution of Lorazepam 1%*	0,5mg/Kg	Intramuscularly (once a day, at the same time)	30 days
$G_{L30 + S30}$ (Experimental)	injectable solution of Lorazepam 1%*	0,5 mg/Kg	Intramuscularly (once a day, at the same time)	30 days
	injectable 0.9% saline solution	0,1mL	intraperitoneally, (once a day, at the same time)	31 – 60 days

Fig. 1 Study design of control and experimental groups according to drug used, daily dose, administration and treatment period. *Cosmética Farmácia de Manipulação Ltda, Curitiba, Parana, Brazil.

After washing in PBS, the slides were incubated in diaminobenzidine solution (DAB, K3468 DAKO DAB + chromogen substrate liquid system, Carpinteria, CA, USA) for 15 s at room temperature. The slides were contrasted with Harris hematoxylin. In the negative controls, the enzyme solution was omitted.

Ten images for each cylinder (totaling 400 images) were captured by a micro Dino-Lite® (AM 423 × AmMo Electronics Corporation, New Taipei City, Taiwan) coupled to an Olympus BX50® microscope (Olympus Corporation, Ishikawa, Japan) at a magnification of 400×, connected to a Dell Inspiron 15,459 notebook (Round Rock, TX, USA). The images were analyzed by a single observer using morphometry Image Pro-Plus™ 4.5 software (Media Cybernetics, Silver Spring, MD), in which a virtual grid was applied to images in order to count the apoptotic nuclei (dark brown coloration) and non-apoptotic nuclei (purple coloration) (Muhvic-urek et al., 2006).

The examiner counted one image of each cylinder and repeated the counts of the same image after twenty-one days to evaluate the reproducibility and absence of systematic error. The entire sample was counted after this verification. A Dahl-

berg error of 5.10%, indicated that the examiner reliably reproduced the apoptotic cell counts (Midtgard et al., 1974; Houston, 1983). Student's t-test showed that there was no systematic error in the count ($p = 0.16$).

From the total number of nuclei (sum of apoptotic + non-apoptotic nuclei), the percentages of apoptotic cell nuclei (NCA) were calculated. Final values were determined from the means of the percentages of the ten fields.

The data of number of N and PCNA were also extracted from the works of Rinaldi et al. (2015) and Mattioli et al. (2016), respectively.

2.2. Statistical analysis

SPSS® 23.0 (SPSS Inc., Chicago, IL) was used with a significance level of 5% ($p < 0.05$).

Data were submitted to Shapiro-Wilk testing for normality. Since the data presented a normal distribution, the comparison between the groups was performed using ANOVA, followed by the Tukey HSD test, since Levene's test for homogeneity of variances suggested homogeneous variances.

Table 1 Average values and standard deviation (SD) of the variables according to the groups treated for 30 days with saline (G $_{S30}$) and Lorazepam (G $_{L30}$).

Groups variables	G $_{S30}$ Mean ± SD	G $_{L30}$ Mean ± SD
NCA%	37.94 ± 5.94[a]	33.14 ± 8.02[a]
PCNA	55.80 ± 14.38[a]	43.90 ± 22.46[a]
N (×10^6)	44.73 ± 7.29[a]	27.26 ± 7.48[b]

Anova test p < 0.0000; Observed Power: 10,000.
Different letters mean statistically significant differences.
NCA – Apoptotic cell nuclei.
PCNA – Immunoexpression nuclear antigen of cell proliferation.
N – Number of acinar cell nuclei.

3. Results

3.1. Thirty days of treatment

There was no statistically significant difference between the animals that received the psychotropic drug for 30 days, (the G S30 and G L30 groups) in terms of the NCA and PCNA (Table 1) (Fig. 2 A and B). There was no statistically significant difference in N between G L30 group and the G S30 group (Table 1).

3.2. Sixty days of treatment

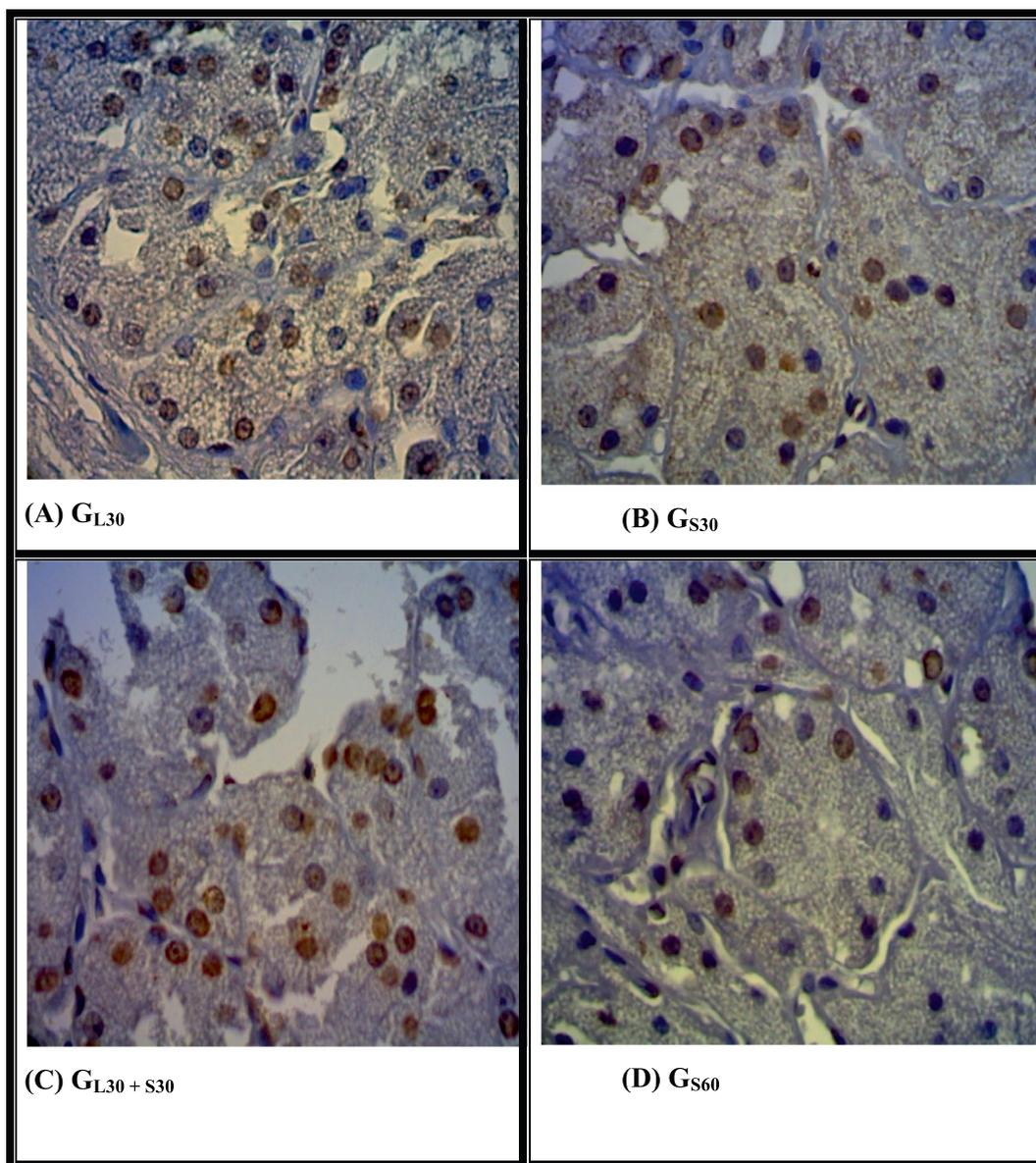Regarding the animals that received BZD for 60 days, the percentage of NCA in the G L30 + S30 group was significantly

Fig. 2 Parotid gland photomicrograph of rat subjected to (A): thirty days with Lorazepam G $_{L30;}$ (B): Thirty days with saline G $_{S30,}$ (C): Thirty days with Lorazepam and from the thirty-first day with saline G $_{130 + S30,}$ (D): Sixty days with saline G$_{S60}$. Apoptotic cells nuclei were stained brown at 400× magnification.

Table 2 Average values and standard deviation (SD) of the variables according to the groups treated for 60 days with saline (G $_{S60}$) and Lorazepam and saline (G $_{L30\ +\ S30}$).

Groups variables	G $_{S60}$ Mean ± SD	G $_{L30\ +\ S30}$ Mean ± SD
NCA%	31.85 ± 9.18[a]*	54.05 ± 8.00[b]*
PCNA	50.00 ± 17.53[a]	41.50 ± 18.06[a]
N (× 10^6)	40.55 ± 4.64[a]*	30.62 ± 8.13[b]*

Anova test p < 0.0000; Observed Power: 10,000.
NCA – Apoptotic cell nuclei.
PCNA – Immunoexpression Nuclear Antigen of Cellular Proliferation (PCNA).
N – Number of acinar cell nuclei.
* Different letters mean statistically significant differences.

higher when compared to the control G S60 (Table 2) (Fig. 2 C and D). The value of N (Table 2) was significantly lower. No statistically significant differences in PCNA were found between the G L30 + S30 S60 groups and G (Table 2).

4. Discussion

The use of BZDs is increasing, and Lorazepam is one of the most frequently prescribed drugs, but studies on its effects on salivary glands are limited. This is the first study to evaluate apoptosis in the salivary glands of rats treated with Lorazepam, where the following results were verified after the use of this BZD compared with the control: a) at thirty days, a statistically significant difference was observed in the number of acinar cell nuclei (N) in the G L30 group compared with the G S30, but no statistically significant difference was observed in the number of apoptotic cell nuclei (NCA) or cell proliferation (PCNA); b) at sixty days, the percentage of apoptotic cells in the G L30 + S30 group proved to be significantly higher than in the control G S60. A significant reduction in the number of acinar cell nuclei was found in the lorazepam-treated group compared to the control.

These unprecedented findings demonstrate the deleterious effects of Lorazepam on the parotid glands of rats. In accordance with our previous work, these effects are not limited to a reduction in saliva volume but extend to the induction of apoptosis (Zaclikevis et al., 2009; Rinaldi et al., 2015; Mattioli et al., 2016).

The studies of Rinaldi et al. (2015) and Mattioli et al. (2016), who evaluated N and PCNA, respectively, using the same samples from this study, showed that the least number of acinar cells found in the G L30 group may have been the result of a lower PCNA, which was not statistically significant compared to the G S30; however, that observation may have led to fewer acinar cells due to the balance of proliferation/apoptosis within the group. At the thirty days timepoint, Lorazepam had not yet increased apoptosis in the acinar cells compared to the control group, probably because of a pharmacokinetic effect, since it is a very liposoluble drug and has a long-lasting plasma half-life (De Almeida et al., 2012).

The later effects of Lorazepam, evaluated at sixty days of use in the G L30 + S30 group showed fewer acinar cells, resulting in an increased apoptosis rate compared to the treatment at thirty days in the G L30 group, which confirms the hypothesis considered in the study by Rinaldi et al. (2015). It should be noted that in the groups treated for sixty days, BZD was suspended on the thirtieth day and saline was administered from the thirty-first day forward. Lorazepam does not have any active metabolites and is conjugated directly with a glycoside radical with half-life of 12 to 18 h (De Almeida et al., 2012). This increased half-life could explain the late apoptotic effects of lorazepam seen in this study.

The study by Zaclikevis et al. (2009) on the action of BZDs on parotid glands of rats revealed a reduction in salivary flow and cell volume. In light of this, it was postulated that this finding could be due to an alteration in the number of acinar cells. Rinaldi et al. (2015) have shown that the use of this drug contributed to a decrease in the number of acinar cells, hypothesizing a possible apoptotic action of Lorazepam on these cells. In accordance with the previous studies, Lorazepam has been found here to cause an apoptotic effect on the cells of the parotid glands of rats. This finding is in line with previous work of Mattioli et al. (2016) who showed a reduction of acinar cell proliferation due to the chronic use of lorazepam.

Similar to the present study, Pavlovic et al. (2012) evaluated the apoptotic power of BZDs on rat thymocytes, since they incubated cells for a twenty-four-hour period with a BZD. After this period, the authors found that exposure to the drug resulted in an increase in cell death by apoptosis. There are no previous studies evaluating the apoptotic action of Lorazepam on acinar cells. Further studies should be carried out on this topic.

Fafalios et al. (2009) demonstrated that Lorazepam has antiproliferative and pro-apoptotic actions. Lorazepam inhibited the growth and viability of cancerous cells in the prostate in vitro for eighteen hours. In addition, in vivo, when introduced in mice that were grafted with prostate cancer cells for tumor development, the BZD reduced tumor size and volume. In spite of being aware of all the genetic and molecular changes that occur in cancer cells, and in the absence of other studies to compare Lorazepam-induced apoptosis, this study is in line with the present research, since it has demonstrated that Lorazepam has a pro-apoptotic effect on acinar cells of parotid glands of rats at sixty days, as observed in the acinar cancer cells of the prostate.

5. Conclusion

Chronic Lorazepam use has been shown to decrease the number of acinar cell nuclei and increase the percentage of apoptotic cell nuclei in rat parotid glands. According to these results, lorazepam is not recommended for use over long periods of time to avoid the development of these apoptotic effects, especially in elderly patients.

Declaration of Competing Interest

None.

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Further reading

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