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Effect of red wine or its polyphenols on induced apical periodontitis in rats

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ABSTRACT

Aim: To evaluate the effect of red wine consumption or its polyphenols on the inflammation/resorption processes associated with apical periodontitis in rats.

Methodology: Thirty-two 3-month-old Wistar rats had the apical periodontitis induced in the four first molars, arranged into four groups: control (C) - rats with apical periodontitis; wine (W) - rats with apical periodontitis receiving 4.28 mL/kg of red wine; resveratrol+quercetin (R+Q) - rats with apical periodontitis receiving 4.28 mL/kg of solution containing 1.00 mg/L of quercetin and 0.86 mg/L of resveratrol; and alcohol (ALC) - rats with apical periodontitis receiving the alcoholic dose contained in the wine. The oral gavage treatments were administered daily, from day 0 to day 45th. On the 15th day apical periodontitis was induced, and on the 45th day the animals were euthanized; histological, immunohistochemical (RANKL, OPG, TRAP, IL-10, TNF-α and IL-1β), and micro-computed tomography for bone resorption analysis were performed in the jaws. The Kruskal-Wallis with Dunn's test was performed for nonparametric data, and the ANOVA with Tukey's test for parametric data, $p<0.05$.

Results: The median score of the inflammatory process was significantly lower in the R+Q group (1) compared to the C (2) ($p=0.0305$) and ALC (3) ($p=0.0003$) groups, and not different from the W (1.5) group. The immunolabelling for OPG was higher in the R+Q group ($p=0.0054$) compared to all groups; the same observed for IL-10 ($p=0.0185$), different from groups C and ALC. The R+Q group had the lowest TRAP cell count ($p<0.0001$), followed by the W group, both inferior to C and ALC groups. The lowest bone resorption value was in the R+Q group ($0.50mm^3 \pm 0.21mm^3$), significantly lower ($p=0.0292$) than the C group ($0.88mm^3 \pm 0.10mm^3$). The W group ($0.60\ mm^3 \pm 0.25\ mm^3$) and R+Q group had less bone resorption compared to the ALC group ($0.97mm^3 \pm 0.22mm^3$), $p=0.0297$ and $p=0.0042$ respectively.

Conclusion: Red wine administration to rats for 15 days before induction of apical periodontitis decreased inflammation, TRAP marking, and periapical bone resorption compared to Alcohol. Resveratrol-quercetin administration reduced the inflammatory process in apical periodontitis, periapical bone resorption, and altered the OPG, IL-10, and TRAP expression compared to Control and Alcohol groups.

INTRODUCTION

Red wine is a popular worldwide drink and is reported to be beneficial for the body when ingested in appropriate amounts (Artero *et al.* 2015). The wine is composed mainly of water, ethanol, glycerol, polysaccharides, various acids, and phenolic compounds (Snopek *et al.* 2018). The beneficial properties of red wine such as the cardioprotective potential, the inhibition of the low-density lipoprotein (LDL) oxidation, and prevention of endothelial dysfunction occur at the expense of the ingestion of wine

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polyphenols, especially resveratrol, anthocyanins, and catechins, which are the most effective wine antioxidants (Haseeb *et al.* 2017). The phenolic compounds of wine can be divided into flavonoids and non-flavonoids, and the precise content of each one is dependent on elements, such as the grape variety, and manufacturing technique; but it is known that red wine contains 10-fold more phenolic compounds than white wine (Markoski *et al.* 2016). In addition to the polyphenols, the low alcohol content is reported to be responsible for the beneficial effect that red wine can exert (Golan *et al.* 2019). In general, the beneficial effect of regular and moderate wine consumption is obtained with approximately 150 mL/day for women and 300 mL/day for men, as defined by previous studies and by the Dietary Guidelines for Americans, 2020-2025 (Rotondo *et al.* 2001, Pavlidou *et al.* 2018, U.S. Department of Agriculture and U.S. Department of Health and Human Services. 2020).

Resveratrol (3,5,4'-trihydroxystilbene) is a nonflavonoid polyphenol present in red wine and in foods that are ingested commonly in the human diet, such as strawberries, blueberries, mulberries, grapes, grape juice, peanuts, and dark chocolate (Galiniak *et al.* 2019). The compound started to gain importance in 1992 when it was postulated to explain some of the cardioprotective effects of red wine consumption, referred to as the "French Paradox", which described the inverse relationship between mortality from coronary heart disease and the red wine consumption predominantly seen in France (Renaud & de Lorgeril 1992). Since then, the substance has been extensively investigated in the health field, with benefits reported to the cancer prevention, neuroprotection, cardiovascular disease, ischaemic injuries, anti-aging, enhance stress resistance and extend the lifespans of various organisms (Rauf *et al.* 2018, Galiniak *et al.* 2019, Li *et al.* 2019). Specifically in bone tissue, the non-flavonoid resveratrol acts by inducing SIRT1 and regulating RUNX2, to induce osteoblasts differentiation. It also suppresses the NF-κB activation, leading to reduced differentiation and osteoclastic activity (Shakibaei *et al.* 2011, Pandey *et al.* 2018).

In addition to resveratrol, the flavonoid quercetin (3, 3', 4', 5, 7-pentahydroxyflavone) is also found in red wine, as well as in vegetables, fruits, and teas, being one of the most prominent dietary antioxidants in health research (Li *et al.* 2016). Its properties have been gaining notoriety due to antioxidant, anti-inflammatory, anti-tumor, metabolic regulation, and neuroprotective activities (Li *et al.* 2016, Khan *et al.* 2019, Reyes-Farias & Carrasco-Pozo 2019, Wong *et al.* 2020). In bone tissue, quercetin has been reported to decrease osteoclastogenesis via inhibition of the activating receptor for nuclear factor-kB ligand (RANKL), involved in osteoclastic differentiation (Wong *et al.* 2020), in addition to its ability to directly induce apoptosis of mature osteoclasts (Wong *et al.* 2020). Quercetin is also believed to activate osteoblasts, either by activating the TGF beta signaling pathway, p38 mitogen-activated protein kinases, and WNT/β-Catenin pathways, thus regulating bone metabolism (Yamaguchi & Weitzmann

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2011, Casado-Diaz *et al.* 2016, Pandey *et al.* 2018). These studies demonstrate that quercetin has great potential to be used as a bone health supplement.

Studies specifically evaluating the influences of red wine on the inflammatory response and bone tissue are important, but scarce. These investigations are included in epidemiological studies on alcohol consumption, and only few specify the type of alcoholic beverage. Red wine is reported to be more beneficially than other beverages, due to the rich phenolic components allied to a low alcohol content, which would facilitate the absorption of these phenolic compounds (Kutlesa & Budimir Mrsic 2016). To date, only one study has evaluated the effect of red wine and its major components on periodontitis and revealed that animals exposed to red wine had a lower occurrence of spontaneous marginal periodontitis, and lower levels of TNF-α and C-reactive protein (Wagner *et al.* 2019).

Apical periodontitis results from persistent bacterial contamination of the root canal system, characterized by an inflammation of periradicular tissues (Kakehashi *et al.* 1965). When the dental pulp becomes infected, bacteria and their byproducts evoke nonspecific inflammatory responses, as well as specific immunological reactions, leading to the destruction of bone by osteoclasts and resorption cementum and dentine by multinucleated cells designated as odontoclasts (Galler *et al.* 2021). Red wine and the isolated polyphenols (resveratrol and quercetin) have been reported to alter the functioning of bone tissue and the immune system, both crucial elements for the development of apical periodontitis (Das & Das 2007, Wong & Rabie 2008a, Wong & Rabie 2008b, Pervaiz & Holme 2009, Li *et al.* 2016, Esteban-Fernández *et al.* 2017, Wong *et al.* 2020).

This study aimed to evaluate the effect of red wine or a polyphenols solution consumption on apical periodontitis induced in rats. Thus, the null hypothesis tested in this study was that exposure to wine or resveratrol associated with quercetin does not alter the lesion volume and severity of apical periodontitis.

MATERIAL AND METHODS

Animals

Thirty-two three-month-old adults male Wistar rats *(Rattus norvegicus albinus)*, weighing between 250-300g, were used. The animals were maintained in a temperature-controlled environment (22 °C ± 1 °C, 70% humidity) with a 12h light-dark cycle and ad libitum access to water and food. All experimental protocols were approved by the Institutional Ethics Committee on Animal Use (00154-2019) of Universidade Estadual Paulista, São Paulo, Brazil. The general health condition was evaluated weekly. The animals were arranged randomly into four groups (n=8): Control (C) - rats with apical periodontitis; Wine (W) - rats with apical periodontitis receiving wine; Resveratrol + Quercetin (R+Q) - rats with apical periodontitis receiving a solution with resveratrol and quercetin; and Alcohol (ALC) - rats

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with apical periodontitis receiving an alcoholic solution. All the treatments were conducted through oral gavage for forty-five days, starting fifteen days before induction of apical periodontitis, and extending for thirty more days after induction of the disease (Figure 1).

Sample size calculation

The sample size was estimated based on the parameters used in previous studies (Cintra *et al.* 2014). Using an alpha error of 0.05% and 95% power to recognize a significant difference, a minimum of seven animals per group was considered necessary. Taking into consideration possible complications that could occur during the study, one more animal was added in each group, resulting in eight rats per group, giving a total of thirty-two animals.

Quantification of the phenolic compounds present in the red wine

The total phenolic compounds present in the red wine Miolo Seleção ® (Miolo Wine Group Vitivinicultura S.A, Campanha, RS, Brazi) were determined using a high-performance liquid chromatographic/diode array detector (HPLC/DAD) coupled with electrospray ionization/mass spectrometry (ESI-MS). For quantification, the wine sample was diluted three times with a solution of water acidified with 0.2% formic acid and filtered using a nylon filter with 0.45 μm porosity. The quantification of phenolic compounds was performed using a high-performance liquid chromatograph (Shimadzu LC-20T; Shimadzu Corporation, Tokyo, Japan), equipped with a diode array detector, degassing system, autosampler, and oven column. Chromatographic separation was performed on a reverse-phase column (C18, 150 mm x 4.6 mm, particle size 3.5 μm (Agilent Technology, Santa Clara, CA, United States). The mobile phase consisted of water acidified with 0.2% formic acid (v/v) (mobile phase A) and acetonitrile (mobile phase B). For chromatographic separation, the gradient elution used was composed of 0 min 100% A, 10 min 88% A, 15 min 45% A, 17 min 65% A, 23 min 100% A, 30 min 100% A. The temperature of the column was maintained at 40 ° C, the sample injection volume was 10 μL, with a flow rate of 0.5 mL/min. The wavelengths used were 306 nm for t-resveratrol and 360 nm for quercetin. Quantification was performed by external calibration, with seven concentrations equidistant from the analytical standards (Nixdorf & Hermosin-Gutierrez 2010, Lago-Vanzela *et al.* 2011a, 2011b). The concentrations of resveratrol (0.86 ± 0.02 mg / L) and quercetin (1.00 ± 0.02 mg / L) found in the red wine were used to prepare the solution administered to the R+Q group.

Oral gavage treatments

The animals from the Control group (C) received water at 4.28 mL/kg body weight through gavage to mimic the same procedure endured by the other animals. The animals from the Wine group (W) received 4.28 mL/kg body weight of red wine (Miolo Seleção ®, Wine Group Vitivinicultura S.A) (Schmatz *et al.* 2013). For the animals from Resveratrol + Quercetin group (R+Q) a solution with 1.00 mg/L of quercetin and 0.86 mg/L of resveratrol was prepared and administered in the same volume to red

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wine (4.28 mL/kg body weight). The animals from the Alcohol group (ALC) received 4.28 mL/kg body weight of an alcoholic solution containing 12.5% alcohol by volume (the same amount of alcohol present in the wine administered to group W), prepared by diluting absolute ethyl alcohol in water (Dal-Fabbro *et al.* 2019b).

Induction of apical periodontitis

Fifteen days after the oral gavage has been started the pulp tissue of rats were exposured (Cosme-Silva *et al.* 2019). Briefly, rats were anaesthetized with 87 $mg.kg^{-1}$ ketamine (Francotar, Virbac do Brazil Ind e Com Ltda, Roseira, São Paulo, Brazil) and 13 $mg.kg^{-1}$ xylazine (Rompum, Bayer AS, São Paulo, Brazil) by intramuscular injection and placed on a jaw-retraction board. All four first molars of each rat had the dental pulps surgically exposed using a no. 1/4 dental round bur (Jet Carbide, Kavo Kerr Group, Orange, CA, USA) using an electric handpiece. The size of the exposure was approximately equivalent to the diameter of the bur. The access cavity was left open to the oral cavity after removal of the pulp tissue using an endodontic file, allowing contamination of root canals with oral commensal microorganisms. Rats were killed on day 30 after pulp exposure.

Sample collection

The animals were killed with an overdose of anaesthetic solution (Thiopentax, Cristalia Produtos Quimicos Farmaceuticos Ltda., São Paulo, Brazil). After, the right and left sides of the jaws were removed, stored in a 4% buffered formaldehyde solution, and subsequently sent for microtomographic and histological/immunohistological analysis respectively.

Histological/Immunohistochemical analysis

Fixed left side semi-jaws were decalcified in 10% EDTA solution, embedded in paraffin, and sectioned at 6 μm thickness following a general histology protocol. Haematoxylin and Eosin (H&E) staining were used for the analysis of the inflammatory profile and condition of the periapical region of the first mandibular molar. Histopathological analysis was conducted following the guidelines of quality of inflammation and the cellularity pattern of dental and periodontal tissues to score the inflammatory infiltrate as follows: low (0 to few inflammatory cells, score = 0), mild (<25 cells, score = 1), moderate (25– 125 cells, score = 2) and severe (>125 cells, score = 3) (Cintra *et al.* 2016). The immunohistochemical analysis was performed using the immunoperoxidase technique as previously described (Dal-Fabbro *et al.* 2021). Histological sections were arranged into six batches, incubated with one of the following primary antibodies 1:100 diluted: Receptor Activator of Nuclear factor Kappa-B Ligand (RANKL) (Goat anti-RANKL - SC7627; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Osteoprotegerin (OPG) (Rabbit anti-OPG - SC11383; Santa Cruz Biotechnology), Tumor Necrosis Factor – Alpha (TNF-α) (Goat anti-TNF-α SC1348, Santa Cruz Biotechnology), Interleukin 1-beta (IL-1β) (Rabbit - orb101745 Biorbyt, San Francisco, CA, USA), Interleukin 10 (IL-10) (Rabbit - orb221323,

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Biorbyt), and Tartrate-Resistant Acid Phosphatase (TRAP) (Goat anti-TRAP - SC30832; Santa Cruz Biotechnology). In sequence, biotinylated secondary universal antibodies were applied to the slices for 2h followed by a streptavidin–horseradish peroxidase conjugate for 2h (Universal Dako-Labeled Streptavidin-Biotin Kit; Dako Laboratories, Carpinteria, CA, USA). Finally, the slices received the chromogen 3,3'-di-aminobenzidine tetrahydrochloride (DAB chromogen Kit - Dako Laboratories). The negative controls contained sections following the same protocol described above, excluding the application of the primary antibody. Semiquantitative immunolabeling analyses of RANKL, OPG, TNF-α, IL-1β, and IL-10 through the designation of the ensuing score system: 0 = absence of immunoreactive (IR) cells; 1 = low immunolabeling of both IR cells and extracellular matrix; approximately one-quarter of the field; 2 = moderate immunolabeling of both IR cells and extracellular matrix; approximately one half of the field and 3 = high immunolabeling of both IR cells and extracellular matrix; approximately three-quarters of the field (Azuma *et al.* 2017). The TRAP analysis was performed as follows, first surrounding the entire perimeter of bone resorption resulting from apical periodontitis, followed by counting the positive multinucleated TRAP cells. Lastly, the ratio between TRAP cells and perimeter size was obtained (Gomes-Filho *et al.* 2015). All the analyses were performed by a single-certified histologist using a light microscope (DM 4000 B; Leica) and a colour camera (DFC 500; Leica, Wetzlar, Germany) blinded regarding the groups.

Micro-computed tomography

Fixed right side semi-jaws were scanned as previously described using a cone beam-type tomograph (SkyScan 1272; Bruker, Aartselaar, Belgium) using the following parameters: 50 kV, 800 µA, 1º rotation step, 7000 ms exposure, 3 frame averages, 11 a pixel image and 13 a pixel camera size (Cosme-Silva *et al.* 2020). In brief, the images were reconstructed using the software NRecon (SkyScan) and the alveolar bone volume loss were calculated using the CTAn software (SkyScan) and expressed in cubic millimetres. The region of interest (ROI) comprised the empty space of periapical resorption area, containing the distal root canal and apical foramen, starting from the first transversal cut at the beginning of the resorption of the periapical bone, continuing through the apex of the root, ending at the last slice where the bone resorption was seen.

Statistical analysis

The data were analyzed using Prism 8 software (GraphPad; La Jolla, CA, USA). After the test of normality, the Kruskal-Wallis with Dunn's multiple comparisons test was performed for nonparametric data, and the one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was performed for parametric data. The level of significance was 5%.

RESULTS

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Histological analysis

The inflammatory scores and the representative haematoxylin–eosin-stained sections are shown in Figures 2 and 3. All the animals submitted to pulp exposure had pulp necrosis with an inflammatory infiltrate in the periapical region consisting mainly of neutrophils and mononuclear cells, concomitantly with bone destruction, confirming the effectiveness of the thirty days pulp exposure method. The C group had moderated levels of inflammation, attributed a median of score 2, significantly greater than that presented by the R+Q group, which had a mild inflammatory reaction in the periapical region, with a median score of 1 ($p=0.0305$). There was no significant difference when the W group (median of scores 1.5) was compared with Control and neither with R+Q. Moreover, the ALC group had the highest median score for inflammation (3), significantly higher than the inflammatory process found in groups W ($p=0.0037$) and R+Q ($p=0.0003$).

Immunohistochemical analysis

The immunoreactivity pattern for IL-10, RANKL, OPG, TNF-α, IL-1β, and TRAP are described below and in Figures 2 and 4. The R+Q group had moderate immunoreaction for IL-10 expression (median of score 2), which was significantly greater ($p=0.0185$) than groups C and ALC (median of score 1); although the W group had the same median as the R+Q group, the statistical analysis revealed no differences when compared to the other groups. With regard to OPG, all groups had mild immunoreaction (score 1), except for the R+Q group, which had a significantly greater ($p=0.0054$) expression of this osteoclastogenesis inhibitory factor (score 2), when compared with the other three groups. Although the groups W and R+Q had fewer immunoreactive cells for RANKL, TNF-α and IL-1β, these differences were not significant ($p>0.05$). The TRAP immunolabelling technique applied was specific to identify osteoclasts. The W and R+Q groups had a significantly lower ($p<0.0001$) load of TRAP-positive multinucleated cells per mm of the perimeter in the periapical region, 2.44 ± 0.53 and 1.88 ± 0.36, respectively, when compared to groups C (3.07 ± 0.35) and ALC ($3.95 \pm .048$). The ALC was significantly ($p<0.05$) more TRAP inductive than the C group. No difference was found when comparing W and R+Q groups.

Micro-computed tomography analysis

The micro-CT slices and data are shown in Figures 2 and 3. All animals subjected to pulp exposure had increased periapical hypodense areas on microtomography by day 30, proving the development of apical periodontitis. The R+Q group had a bone resorption mean volume of $0.50\ mm^3 \pm 0.21\ mm^3$, significantly lower ($p=0.0292$) than the bone resorption volume in groups C ($0.88\ mm^3 \pm 0.10\ mm^3$) and ALC ($0.97\ mm^3 \pm 0.22\ mm^3$) ($p=0.0042$). The W group had a volume of bone resorption ($0.60\ mm^3 \pm 0.25\ mm^3$), that was significantly lower ($p=0.0297$) than that resorbed in the ALC group, however, without significant differences when compared to groups C and R+Q.

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DISCUSSION

Red wine or its polyphenols compounds are consumed throughout the world, regardless of the source. The present study is the first to evaluate the effect of red wine consumption or the association between resveratrol and quercetin through oral gavage on the development of apical periodontitis in rats. The results revealed a beneficial effect of the consumption of the associated polyphenols on the magnitude of the inflammatory process and bone resorption associated with apical periodontitis. Red wine consumption also led to less osteoclastic marking than the control, rejecting the null hypothesis. Although light to moderate wine intake seems to have several beneficial effects, definitive recommendations on wine intake cannot be made at this time (Minzer *et al.* 2020). Furthermore, excessive alcohol consumption is well known as a major public health concern (Ventura-Cots *et al.* 2019).

The administration of wine and its constituents via oral gavage in rats is widely used, since it allows the precise control of the amount to be ingested by the animal, which is not possible in the freely available water source (Venturini *et al.* 2010, Correa *et al.* 2018). Also, in human studies, the polyphenols investigated may come from countless types of food. It is difficult to determine wine consumption throughout an experiment on humans, since they are based on the collaboration of the patient regarding the protocol imposed for prospective studies and the accuracy of the answers provided in cases of retrospective studies. The 4.28 mL/kg of body weight dosage of red wine given to each animal in the present study was used based on the general recommendation of consumption of 300 mL/day of red wine for humans weighing 70 kg (Rotondo *et al.* 2001, Pavlidou *et al.* 2018). In order to have a homogeneous comparison, the wine used in the present investigation was first subjected to the quantification of the polyphenols of interest, through an established methodology (HPLC / DAD / ESI-MS) (Nixdorf & Hermosin-Gutierrez 2010, Lago-Vanzela *et al.* 2011a, Lago-Vanzela *et al.* 2011b). This quantification allowed the group receiving the two associated polyphenols to receive the same amount that would be present in the wine administered to animals in group W.

The model used to induce apical periodontitis through the exposure of the pulp molars was chosen due to the similarity of periapical response to pulp exposure as seen in humans. That is, the bacterial infection from the oral environment infects the pulp tissue leading to pulp necrosis, resulting in apical periodontitis (Yamasaki *et al.* 1994). The 30th induction period is considered an adequate time to assess the extent of the inflammatory process and bone resorption resulting from the condition, since the plateau stage of development has been reached (Yamasaki *et al.* 1994).

The histological analysis revealed an inflammatory process associated with apical periodontitis of lesser extent and intensity in the R+Q group when compared to the other groups. This finding agrees with those already published in the literature demonstrating the anti-inflammatory activity of the two

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polyphenols. Resveratrol, for instance, protects from inflammation by acting at different phases of inflammation (Das & Das 2007); this anti-inflammatory activity may occur through the inhibition of both cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) mediated pro-inflammatory signaling, suppression of pro-inflammatory mediator production, acting in nuclear transcription factor-kB (NFkB) in macrophage inhibition, suppressing interleukin-6 release and interleukin-8, which plays an important role in inflammation as it recruits leucocytes to the lesion area (Das & Das 2007). Quercetin, downregulates nitric oxide synthase (NOS) expression, inhibit matrix metalloproteinases, inhibits the production of inflammation-producing enzymes (COX) and lipoxygenase (LOX), blocks TNF-α, and prevents it from directly activating extracellular signal-related kinase (ERK) and (NF-kB), which are potent inducers of inflammatory gene expression and protein secretion (Li *et al.* 2016).

The alcoholic solution in the 12.5% concentration administered alone was associated with greater inflammation, thus excluding the hypothesis that low alcoholic levels could be one of the factors responsible for the beneficial effects of red wine consumption. This result agrees with those already published in the literature, which revealed a deleterious effect of 15% and 20% alcohol concentration on apical periodontitis (Dal-Fabbro *et al.* 2019a, 2019b). Although the red wine given in the present study has the same alcohol concentration, the inflammatory infiltrate was significantly lower when comparing these two groups. A possible explanation is that the nonalcoholic compounds present in the red wine, such as polyphenols, may have reversed the harmful effect evoked by the alcohol concentration present in the red wine (Esteban-Fernández *et al.* 2017).

Even though in the present study no significant reduction in IL-1β and TNF-α levels were associated with red wine consumption or polyphenols therapy, a propensity to a higher inhibition of these cytokines was observed in these two groups. However, numerous studies have confirmed that resveratrol and quercetin can suppresses the release of TNF-α (Takada *et al.* 2004, Lee & Moon 2005, Yuan *et al.* 2018, Lee *et al.* 2019, Chen *et al.* 2020, Wang *et al.* 2020). TNF-α is a proinflammatory cytokine released by macrophages and has a vital role in periodontitis mediated bone loss by inducing the expression of mediators that amplify or sustain the inflammatory response such as the production of prostaglandins and matrix metalloproteinases (Algate *et al.* 2016). Also, IL-1 and TNF-α acts synergistically increasing bone resorption, and TNF plays a critical role in preparing the innate host response to defend against bacteria, except when overstimulated, which can cause significant collateral damage (Graves & Cochran 2003). Moreover, the pro-inflammatory cytokine IL-1β, primarily secreted by macrophages, is a key regulator of host responses to microbial infection and is found frequently in elevated levels in persistent apical periodontitis (Yang *et al.* 2018). IL-1β is a potent stimulator of periodontal tissue breakdown, and its properties include the promotion of bone resorption and the production of tissue-degrading proteinases (Cheng *et al.* 2020). A possible explanation for the lack of significance in the reduction found in the

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present study may be due to the dosage of resveratrol and quercetin administered, since they were lower than those applied by other studies that reported the effectiveness in reducing this interleukin (Napimoga *et al.* 2013, Ribeiro *et al.* 2017).

The supplementation with the mixture of resveratrol and quercetin caused an elevated and significantly greater release of IL-10. Red wine, in turn, also increased the production of IL-10, but without significance, probably due to the presence of alcohol in the drink, hindering the beneficial effects of polyphenols (Gavala *et al.* 2015). IL-10 is a pleiotropic cytokine with potent anti-inflammatory ability that suppresses both immunoproliferative and inflammatory responses, regulates B-cell proliferation and differentiation, and downregulates a number of processes such as the release of proinflammatory cytokines and chemokines, such as IL-1, IL-6, and TNF-α, the production of nitric oxide, and collagenase (Akdis *et al.* 2016, Sun *et al.* 2019). Moreover, IL-10 affects osteoclast precursors, and inhibits osteoclast activation and has been regarded as a key regulator of bone homeostasis, in homeostatic and inflammatory conditions, since the lack of IL-10 in animals leads to increased femur and alveolar bone loss (Cheng *et al.* 2020). Besides, Il-10 is recognized as an important suppressor for periodontal disease and apical periodontitis development *in vivo* (Sasaki *et al.* 2000, Sasaki *et al.* 2004). The molecular mechanism of bone loss prevention evoked by the IL-10 is based on the upregulation of OPG expression and downregulation expression of the RANKL (Cheng *et al.* 2020).

Bone is a dynamic complex tissue that undergoes renovation and repair constantly (or remodeling). The cells responsible for this process are osteoblasts, which secrete new bone, and osteoclasts that remove the existing tissue. Normally, the fine balance between these two cells is in harmony, with the result there is no change in bone mass. The control of bone metabolism is a key factor in reducing bone resorption in inflammatory diseases (Xiao *et al.* 2016). Therefore, the use of substances that are capable of interfering positively in inflammatory processes that lead to bone resorption should be considered.

Several signaling pathways maintain the activities of osteoblasts and osteoclasts; one of the most important and frequently targeted as a new treatment strategy in bone related-disease conditions is the RANK/RANKL/OPG system (Silva & Branco 2011). The OPG/RANKL ratio is considered an important information to assess the cellular state of bone tissue, since the OPG is an osteoprotective protein, acting by binding to RANKL preventing it from binding to RANK and sequencing osteoclastic formation (Silva & Branco 2011). In the present study, as the OPG-RANKL pathway was shifted towards OPG in group R+Q, due to a higher expression of this protein and no differences in RANKL, less formation of bone resorptive cells occurred, as previously reported (Ribeiro *et al.* 2017, Ge *et al.* 2020). This data is confirmed by the decreased number of positive TRAP multinucleated cells (osteoclasts) per millimetre of the perimeter of the apical lesion in the same group. Reduced periapical bone destruction was evidenced

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by the μCT analysis in the R+Q group reinforcing the correlation between reduced TRAP-positive cells found on immunohistochemistry, displaying attenuated bone loss due to the lower osteoclast activating in the apical lesion when compared to the control. In addition to this pathway, others not evaluated in the present study, but already published in other areas, may be related to the diminished bone resorption volume by the phenolic group (Wattel *et al.* 2004, He *et al.* 2010).

Red wine consumption also led to fewer TRAP multinucleated cells when compared to the control, however, no significant difference was found in the lesion volume between these two groups. Nevertheless, a strong tendency towards a reduction in bone resorption analyzed by μCT was observed in the red wine group. A longer observation period (6 weeks, for example) might have resulted in a significant reduction in bone resorption since small changes in periapical bone remodeling still occur at this timepoint (Xu *et al.* 2019). The group receiving alcohol alone had the greatest number of TRAP-positive cells per millimetre of the lesion perimeter, confirming the deleterious effect of alcohol consumption on this marker in the rats experimental model (Dal-Fabbro *et al.* 2019a, Dal-Fabbro *et al.* 2019b). Interestingly, when comparing this group with wine, the latter one had a significantly lower cell count, leading to the hypothesis that the polyphenols present in the drink counterbalanced the deleterious effect of alcohol.

In the periodontology field, a prospective cohort study reported that intake of wine was inversely associated with clinical periodontal attachment loss in men (Kongstad *et al.* 2008). Another study in adults in southern Brazil reported evidence of a beneficial effect of wine on periodontal status (Susin *et al.* 2015). Previous investigations in ligature-induced periodontitis in animals demonstrated good properties regarding the use of the polyphenols present in the red wine. Firstly, continuous administration of resveratrol was reported to decrease periodontal breakdown induced experimentally in rats (Casati *et al.* 2013). Resveratrol administered via oral gavage to rats caused a significant reduction in inflammation-mediated destruction of periodontal soft tissues and bone (Correa *et al.* 2017). When given by subcutaneous injection in the same experimental model, it protected rats from periodontal tissue damage by inhibiting inflammatory responses and by stimulating antioxidant defense systems (Bhattarai *et al.* 2016). The same results were observed when administered freely in drinking water (Tamaki *et al.* 2014). Moreover, resveratrol had a positive influence in decreasing periodontal breakdown during smoking in rats (Ribeiro *et al.* 2017). Similar to resveratrol, quercetin exhibited protective effects in bacterial-induced periodontitis, reducing alveolar bone loss by mechanisms involving the reduction of pro-inflammatory cytokine production and down-regulation of the osteoclastogenic cytokine RANKL (Napimoga *et al.* 2013). In addition, it reduced alveolar bone loss in ligature-induced periodontitis by increasing osteoblastic activity, decreasing osteoclastic activity, apoptosis, and inflammation (Taskan & Gevrek

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2020). These data, concomitantly with the findings in the present study, highlight a promising approach to inhibit the development of bone loss during apical periodontitis development.

Considering the high prevalence of apical periodontitis throughout life, combined with frequent intake of red wine and the polyphenols through other sources, the present study offers some insights regarding the mechanisms of how these compounds may affect the apical periodontitis. However, due to some limitations such as the use of an animal model, the resveratrol and quercetin dosage, the ingestion frequency, and time of administration treatment before the periapical injury induction, the results cannot be extrapolated to humans, and therefore, more studies are encouraged considering these relevant parameters.

CONCLUSION

Red wine administration decreased apical periodontitis inflammation, TRAP marking, and periapical bone resorption compared to Alcohol. Resveratrol-quercetin administration reduced the inflammatory process in apical periodontitis, periapical bone resorption, and altered the OPG, IL-10, and TRAP expression compared to Control and Alcohol groups.

CONFLICT OF INTEREST STATEMENT

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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FIGURE LEGENDS

FIGURE 1 - Flowchart showing the experimental stages and their order fulfillment.

FIGURE 2 - A) Chart showing scores for the intensity of inflammatory process; red line indicates the median of each group. B) Chart showing scores for the IL-10 immunolabeling; red line indicates the median of each group. C) Chart showing scores for the RANKL immunolabeling; red line indicates the median of each group. No significant differences were present between the groups. D) Chart showing scores for the OPG immunolabeling; red line indicates the median of each group. E) Chart showing scores for the TNF-α immunolabeling; red line indicates the median of each group. No significant differences were present between the groups. F) Chart showing scores for the IL-1β immunolabeling; red line indicates the median of each group. No significant differences were present between the groups. G) Bar graph for positive TRAP cells per millimetre perimeter of apical periodontitis showing mean and standard deviation for each group. H) Bar graph for lesion volume showing mean and standard deviation for each group. Symbols: *: $p<0.05$ vs. Control; ‡: $p<0.05$ vs. Wine; #: $p<0.05$ vs. Alcohol.

FIGURE 3 - Photomicrographs showing histological aspects of apical regions around the foraminal opening of the distal root (R) of the lower first molar (A-L). Moderate inflammatory process can be observed in the Control group (A, B, and C at 50x, 100x, and 400x magnification, respectively). Mild to moderate inflammatory process can be observed in the Wine group (D, E, and F at 50x, 100x, and 400x magnification, respectively). Mild inflammatory process can be observed in the Resveratrol+Quercetin group (G, H, and I at 50x, 100x, and 400x magnification, respectively). And severe inflammatory infiltrate is observed in the Alcohol group (J, K, and L at 50x, 100x, and 400x magnification, respectively). Haematoxylin and eosin staining. Rectangles indicate the enlarged area in the next magnification. (A, D, G, J) Scale bars: 800 μm, (B, E, H, K) Scale bars: 400 μm, and (C, F, I, L) Scale bars: 100 μm. Micro-computed tomography images (μCT) of apical periodontitis in distal root (R) sections of the mandibular first molars (M, N, O, P). Note a diminished bone resorption on microtomography in the resveratrol+quercetin group. White arrowheads points to the area of the apical periodontitis surrounded by radiopaque alveolar bone (AB).

FIGURE 4 - Photomicrographs showing the immunostaining for OPG (A, D, G, J), IL-10 (B, E, H, K), TRAP (C, F, I, L) in apical periodontitis around the foraminal opening of the distal root of the lower first molar in Control group (A, B, C), Wine group (D, E, F), Resveratrol+Quercetin group (G, H, I), and

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Alcohol group (J, K, L). Counterstaining: Harris haematoxylin. Original magnification for OPG and IL-10: 400X with scale bars: 100 µm, and 100X for TRAP with scale bars: 400 µm. Regarding OPG and IL-10 note a large amount of immunoexpression of these proteins in the R+Q group. For TRAP, black arrowheads points to osteoclastic cells reabsorbing the alveolar bone (AB) located below the opening of the apical foramen. Note a reduced TRAP immunolabeled cells in the R+Q group.

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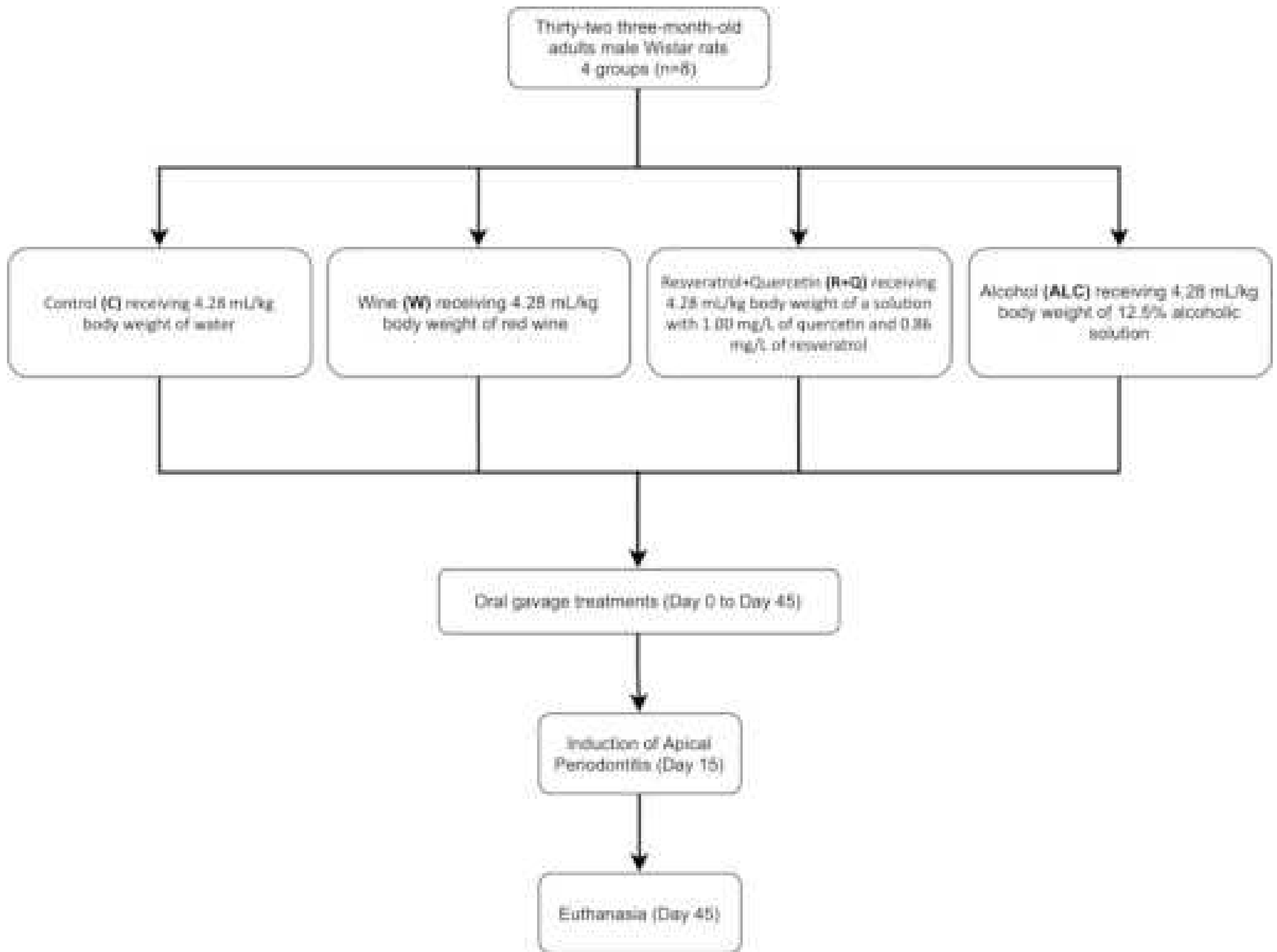

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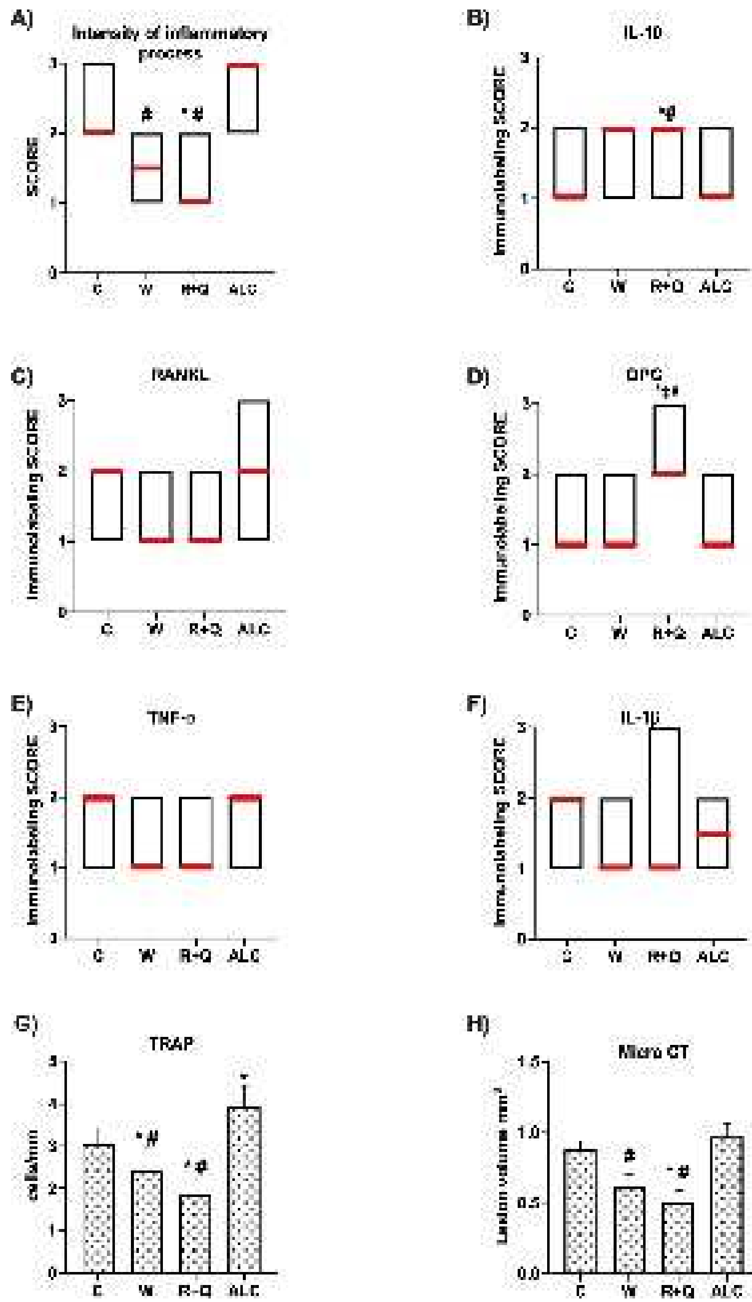

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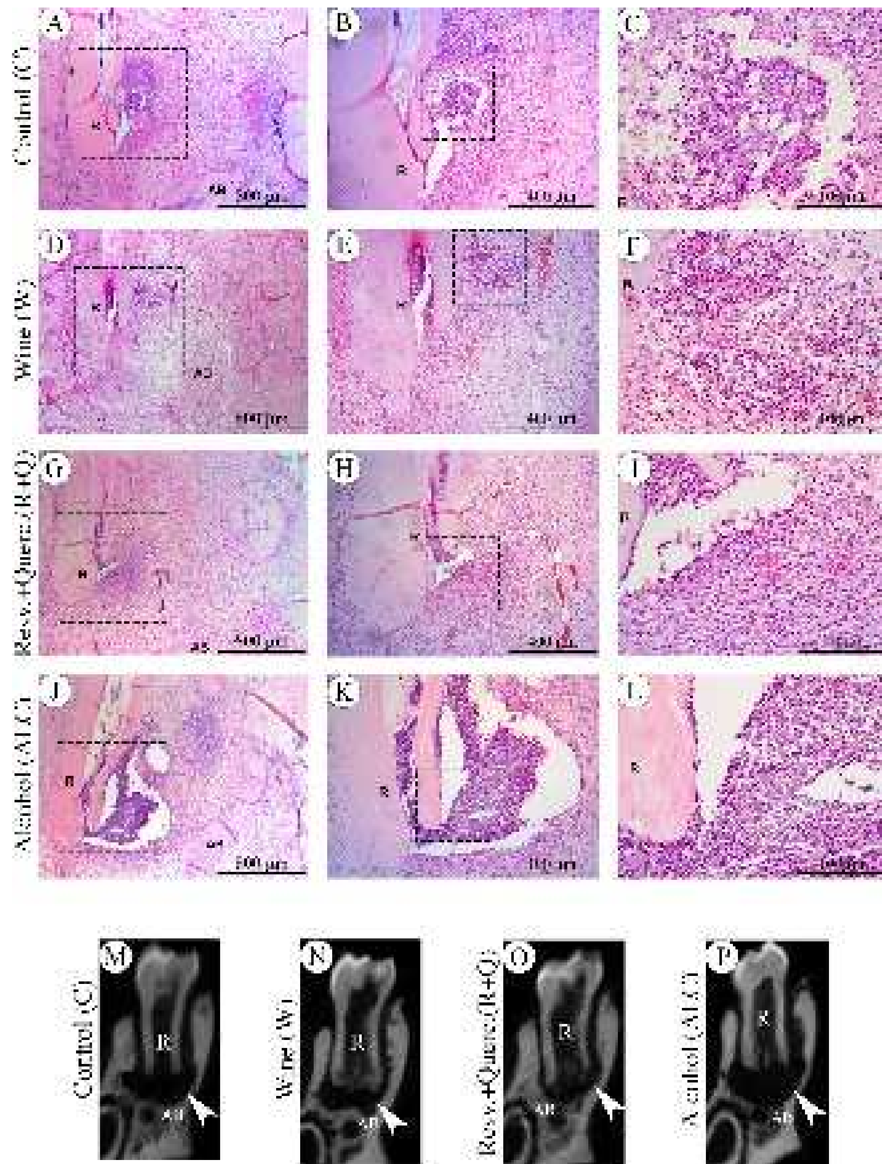

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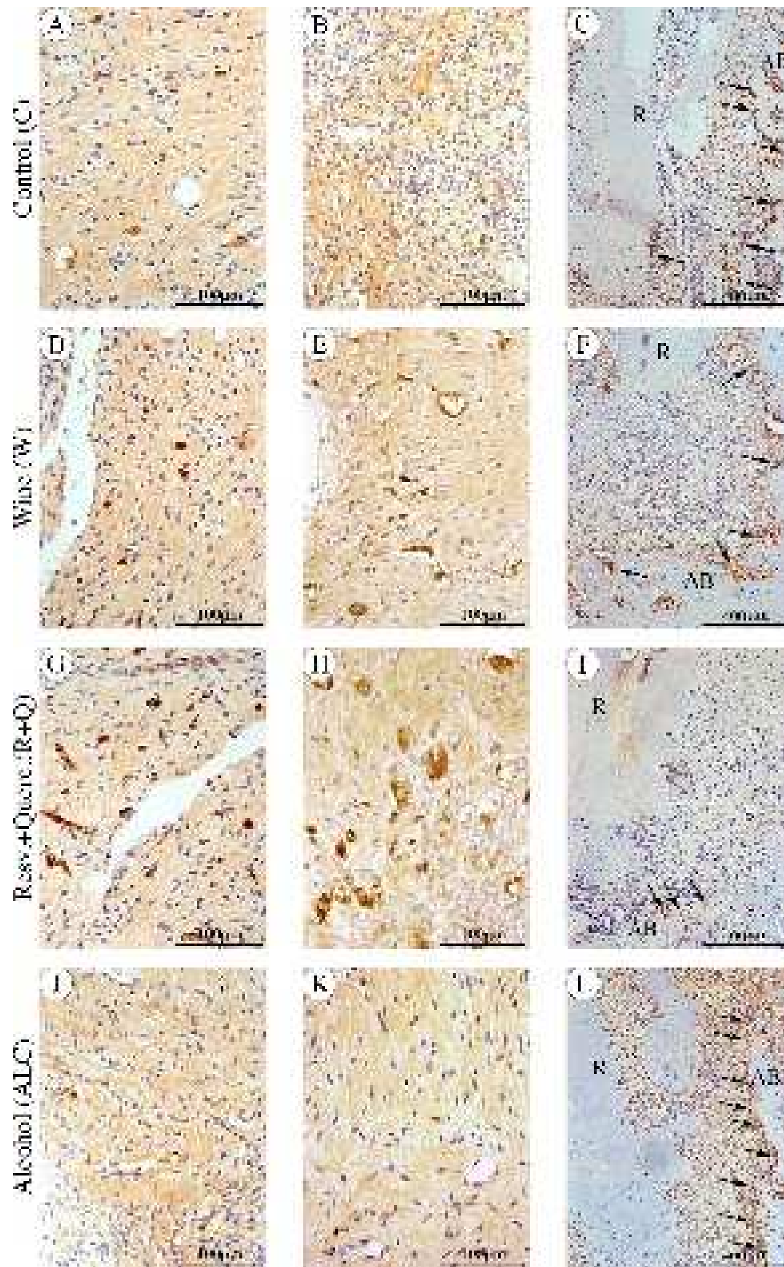

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