Influence of Pregnancy on the Inflammatory Process Following Direct Pulp Capping: a Preliminary Study in Rats

Luiza Helena Silva Almeida1, Katerine Jahnecke Pilównic1, Sandra Beatriz Chaves Tarquínio1, Anelize Campello Felix2, Fernanda Geraldo Pappen1, Ana Regina Romano1

The purpose of this study was to evaluate the inflammatory process following direct pulp capping during pregnancy. This experimental study involved 48 maxillary first molars of female Wistar rats. The procedures were performed in pregnant and non-pregnant animals (n=20 each). Direct pulp capping with mineral trioxide aggregate (MTA) and restoration with a light-cured resin composite was performed in half of exposed pulp specimens. In the other half of specimens, light-cured composite was placed directly on the exposed pulp. In the control groups (n=4 each), no intervention was performed. Animals were euthanized at 3 and 7 days. All sections (three per slide) were viewed under an optical microscope. One previously calibrated pathologist performed descriptive analysis and assigned scores for inflammatory response and tissue organization adjacent to the pulp exposure. The Kappa value for intra-examiner variability was 0.91. At 3 days, in animals treated with MTA, inflammatory infiltrate was absent in non-pregnant animals while mild inflammatory infiltrate was observed in some pregnant animals. The inflammatory response ranged from mild to severe in both groups treated with composite alone. At 7 days, the inflammatory response was more intense in pregnant than in non-pregnant animals treated with MTA; while this difference were not evident in animals treated with composite alone. In conclusion, pregnancy may not influence the inflammatory process following direct pulp capping with light-cured resin composite, which was always harmful to the pulp; while the tissue response after the direct pulp with MTA were more favorable in non-pregnant animals.

Key Words: Dental pulp, direct pulp capping, inflammation, pregnancy.

Introduction

During pregnancy, physical and hormonal changes influence women's bodies and health significantly (1). Pregnancy increases estrogen and progesterone levels by 30 and 10 times, respectively (2). These high levels increase the permeability of the oral vasculature and reduce immunocompetence, increasing the tendency for and severity of periodontal tissue inflammation (3). Similar to periodontal tissue, the dental pulp is comprised of loose connective tissue; thus, it may also be affected by hormonal changes (4).

The increased susceptibility of tissues to inflammation may explain why emergency dental services due to acute dental pain are frequently necessary during pregnancy (5). Clinical survey data indicate that between 38.2% and 54.0% of pregnant women seek out to dental care during pregnancy having acute dental pain as the primary motivation (6–8), while the prevalence of dental pain is between 7.5–17.5% in the general population (9,10).

Additionally, women are at an increased risk for several clinical pain disorders, besides being more sensitive to experimentally induced pain in comparison to men (11,12). Among the possible reasons for the inequality in pain reaction between men and women, which include cognitive and sociocultural differences, there are also the role of the sex hormone estrogen in regulating nociception (11-13).

The presence of estrogen receptors in the pulp tissue has been previously described (14), and estrogens are known to influence the inflammatory process (15). Variations in estrogen levels throughout the lifespan may influence the physiological and pathological behavior of pulp tissue (16). In addition, Dezeletovic et al. (17) found a positive correlation between increased estrogen levels during the menstrual cycle and increased pulp blood flow. However, whether hormonal changes during pregnancy increase the intensity of this inflammatory response remains unknown. Thus, the aim of this preliminary study was to examine the inflammatory response of dental pulp after pulp exposure during pregnancy.

Material and Methods

Animals

The Research Ethics Institutional Committee for Animal Procedures approved this study (Protocol #3940). All procedures were carried out in accordance with institutional guidelines for animal care. Twelve pregnant and 12 non-
pregnant female Wistar rats (*Rattus norvegicus*; age, 3 months; weight, ~300-350 g) were used in this study. The animals' tails were marked for individual identification. The rats were housed in plastic cages (four per cage) placed in ventilated racks (Alesco, Monte Mor, SP, Brazil) at 22°C with a 12-h light/dark cycle (lights on at 7:00 am, off at 7:00 pm). During the experiments, the rats were provided with a standard diet of rat chow (Nuvilab, Colombo, PR, Brazil) and filtered water *ad libitum*.

The rats were divided into four experimental groups according to the restorative material used and pregnancy status (Fig. 1). As the full-term gestational period in rats is 21 days, interventions were performed on the 15th gestational day. The same procedures were performed in pregnant and non-pregnant rats. Rats were allocated randomly to the experimental and control groups.

**Intervention**

The animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg by weight) combined with xylazine (4 mg/kg by weight; both from SDC E-Commerce for Agricultural Products LTDA, Marilia, SP, Brazil). A surgical table adapted to maintain the oral cavity open during dental interventions was used. The buccal mucosa was retracted, and oral antisepsis was performed using iodinated alcohol and sterile distilled water.

All procedures were performed following the method described by Simon et al. (18) Cavities were drilled with a carbide bur (FG1011; KG Sorensen, Cotia, SP, Brazil) on the occlusal aspect of the mesiobuccal cusps of the maxillary right and left first molars, until the pulp was visible through the transparent dentin floors of the cavities. Pulp exposure was then performed mechanically using an endodontic hand K-file with a diameter of 0.40 mm (Dentsply Maillefer, Ballaigues, JN, Switzerland); this approach enabled control of pulp exposure size. Following hemostasis, pulp capping was performed using grey mineral trioxide aggregate (grey-MTA, Angelus, Londrina, PR, Brazil) or light-cured composite resin (Z 250; 3M ESPE®, Campinas, SP, Brazil). For teeth in the MTA groups (*n* = 20), the pulp capping material was placed in contact with the pulp tissue using the tip of a probe, and condensed gently with a sterile paper point (Dentsply-Maillefer, Curitiba, PR, Brazil). The cavity was then sealed with light-cured composite resin (Z 250; 3M ESPE®) using a one-step adhesive system (Adper Single Bond; 3M ESPE®, Campinas, SP, Brazil). Teeth in the remaining experimental groups (*n* = 20) were restored directly with the same composite resin and adhesive system. Eight teeth in pregnant and non-pregnant rats served as controls, with no intervention.

![Experimental design](image-url)

Figure 1. Experimental design.
The animals were placed in individual cages until they recovered from anesthesia. To aid recovery, paracetamol (0.06 mg g⁻¹ day⁻¹) was added to their drinking water for 72 h.

**Euthanasia**

The animals were euthanized at 3 and 7 days after the dental intervention (n = 5/group at each time point). They were anesthetized with an intraperitoneal injection of chloral hydrate (350 mg/kg) (Sigma Aldrich, St. Louis, MO, USA), and physiological saline (Sigma Aldrich) followed by 10% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) (Sigma Aldrich) was perfused transcardially. The maxillae were removed en bloc and immersed in the same fixative for 24 h.

**Histological Processing and Analysis**

After fixation, the samples were rinsed in phosphate-buffered saline for 20 min and then decalcified with 20% formic acid (Sigma Aldrich), which was stirred at room temperature for 3-4 weeks. The samples were then dehydrated in increasing concentrations of ethanol, cleaned, and embedded in paraffin. The paraffin-embedded teeth were sagittally sectioned at a thickness of about 3 μm with a microtome (RM2235; Leica, São Paulo, SP, Brazil). The paraffin sections were stained with hematoxylin and eosin (HE) and mounted with Fisher Chemical™ Permount™ Mounting Medium (Fischer Scientific, Ireland Ltd., Dublin, LE, Ireland).

A previously calibrated experienced pathologist who was blinded to sample group assignment performed histological evaluation of the HE-stained sections based on the ISO 7405-2008 (19, 20).

All sections (three per slide) were viewed under an optical microscope (DM1000, Leica, São Paulo, SP, Brazil). The pathologist performed descriptive analysis and assigned scores for inflammatory response and tissue organization adjacent to the pulp exposure (Table 1).

In order to test the intra-examiner variability, Kappa coefficient was obtained using a sample of 10 sections. The Kappa value for intra-examiner variability was 0.91, showing excellent agreement.

**Results**

Table 2 summarizes histological responses according to group and time point. Inflammatory infiltrate was

<table>
<thead>
<tr>
<th>Histological criterion</th>
<th>Score</th>
<th>3 days</th>
<th>7 days</th>
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<tr>
<td></td>
<td>MTA</td>
<td>Composite resin</td>
<td>MTA</td>
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<td></td>
<td>Non-pregnant</td>
<td>Pregnant</td>
<td>Non-pregnant</td>
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<tr>
<td>Inflammatory response</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>None</td>
<td>5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>0</td>
<td>2</td>
<td>3</td>
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<tr>
<td>Moderate</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Severe</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Normal</td>
<td>5</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Soft-tissue organization</td>
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<tr>
<td>Mild</td>
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<td>5</td>
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<tr>
<td>Extensive</td>
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MTA, mineral trioxide aggregate.
predominantly absent in samples from the MTA groups. The remaining pulp tissue retained its integrity, or superficial cell death was observed at the site of injury. In samples treated with composite resin, the inflammatory response ranged from mild to severe and loss of tissue was observed consistently in focal areas or involving a significant amount of pulp tissue. A tendency for the exacerbation of pulp tissue inflammation was observed in pregnant animals.

No injury, inflammatory response, or disruption or loss of cell survival occurred in the control groups. No morphological change in the pulp tissue was observed in these groups (Fig. 2).

3 days

The inflammatory response was more intense in pregnant than in non-pregnant animals, especially in the MTA group (Fig. 3). In non-pregnant animals treated with MTA, inflammatory infiltrate was absent. In two pregnant animals treated with MTA, mild inflammatory infiltrate involving the pulp exposure area was observed. In both groups treated with composite alone, the coronal pulp exhibited predominantly polymorphonuclear inflammatory infiltrate, ranging from mild to severe. Focal tissue necrosis and congested and enlarged vasculature were observed in the exposure area.

In the MTA groups, superficial tissue loss was observed in five pregnant animals, whereas no tissue disruption was observed in non-pregnant animals. Tissue organization was similar in pregnant and non-pregnant animals treated with composite alone; focal to extensive tissue loss was observed.

7 days

No inflammatory response was observed in most non-pregnant animals treated with MTA; in two cases, a moderate inflammatory response was observed. The pulp tissue of most pregnant animals treated with MTA showed a moderate inflammatory response. Tissue organization was similar in pregnant and non-pregnant animals in the MTA groups. In non-pregnant animals treated with composite alone, the inflammatory response ranged from absent to

Figure 2. No injury, inflammatory response, or disruption or loss of cell survival occurred in the control groups. No morphological change in the pulp tissue of non-pregnant (A) (HE, 100x) and (B) (HE, 400x), or pregnant animals (C) (HE, 100x) and (D) (HE, 400x).
Figure 3. Pulp response at 3 days after pulp exposure and direct pulp capping with MTA (A - D) and composite (E - H). (A) no tissue disruption in a non pregnant animal treated with MTA (HE, 100x); (B) high magnification of (A), in the area of pulp exposure and in contact with the material (HE, 400x); (C) intense reaction to the material, characterized by tissue necrosis, cell-rich zone and adjacent inflammation involving the pulp exposure area in pregnant animal treated with MTA (HE, 100x); (D) high magnification of (C), presenting intense polymorphonuclear inflammatory infiltrate and congested vasculature (HE, 400x); (E) Superficial necrosis and polymorphonuclear inflammatory infiltrate in the exposure area of non-pregnant animal upper molar, in which the pulp capping was performed with composite (HE, 200x); (F) high magnification of (E) (HE, 400x); (G) polymorphonuclear inflammatory infiltrate and extensive areas of necrotic tissue in pulp tissue of pregnant animal treated with composite-only; (H) high magnification of (G), with emphasis in the inflammatory infiltrate adjacent to the exposure area (HE, 400x).
severe; the greatest number of animals showed moderate inflammatory response. In pregnant animals treated with composite alone, the inflammatory response ranged from mild to severe. Tissue organization findings ranged from normal to extensive loss in non-pregnant animals and from mild to extensive loss, in pregnant animals (Fig. 4).

Discussion

Increased levels of estrogen and progesterone are related to increased permeability of oral vasculatures and decreased host immunocompetence, which increase the tendency for and severity of oral inflammation in reaction to bacterial, physical, and chemical irritants (3). Although the role of hormones in the periodontal inflammation is well documented, there is a lack related to the of inflammatory exacerbation or greater predisposition of pulp tissue to inflammation as a result of hormonal changes during pregnancy (3). The exacerbation of inflammation in the pulp tissue could explain the increased prevalence of dental pain among pregnant women (6-8).

This is a preliminary study, the first one investigating changes in pulp tissue after pulp exposure and direct pulp capping in Wistar rats. Wistar rats are suitable animal models and have been widely used for such evaluation (21,22). Pulp exposure and restorative procedures were performed in first upper molars because these teeth have three roots and three cusps, with innervation and vascularization similar to those in the human dentition (21,22). Larger number of animals would give results that are more robust. However, similar to the present study, recent articles also have been describing pulp reaction to different materials using a reduced number of animals (21,23). Moreover, the use of animals in scientific procedures should always follow the principles of the replacement, reduction and refinement), which provides a framework for performing more humane animal research. In this case, reduction refers to methods that minimize the number of animals used per experiment or study consistent with the scientific aims.

As in humans, estrogen and progesterone levels are increased in Wistar rats during pregnancy to develop and maintain the fetus (24). Although gestational cycles differ between humans and rats in terms of duration and the timing of hormonal concentration peaks (24), differences do not contraindicate the use of this animal model. Rats have been commonly used in studies of fertilization, breast cancer and preeclampsia (25, 26). The gestational period of the Wistar rat is approximately 21 days, and the progesterone concentration peak occurs around gestational day 15; hormone levels then remain constant until the end of pregnancy, and decrease around the time of birth (24). For this reason, dental interventions in pregnant rats were conducted on approximately the 15th day of gestation in this study. Euthanasia was performed 3 and 7 days after the interventions, with the aim of evaluating the inflammatory response to pulp dental capping. In fact, the pulp repair is only complete in these cases, when the deposition of mineralized tissue occurs. However, it would only be possible to observe approximately 30 days after intervention, when the animal tissues would not have any influence of gestational hormones anymore.

MTA was used to evaluate the response of dental pulp to direct pulp capping due to its well-established biocompatibility (27). MTA is the gold standard material for dental pulp capping, and it was expected a favorable response when this material was used. On the other hand, the cytotoxicity of composite resin is well established; and when used directly on the pulp, it maintains the inflammatory process and prevents pulp tissue repair (18). So, in this study, this material was used in order to simulate an aggression to pulp tissue, in which higher inflammation levels were predictable. In the MTA groups in the present study, the inflammatory process was absent or less severe than in the composite groups, which can be explained by the properties of these materials.

Due to the injury caused by pulp exposure, even with the use of a biocompatible material, the development of an inflammatory process in dental pulp tissue is expected after 3 days (28). Non-pregnant animals treated with MTA showed no inflammatory response, and pregnant animals in which direct pulp capping was performed with MTA showed mild inflammatory infiltrate. Overall, treatment with composite resin alone showed no differences in tissue response related to pregnancy status. Composites induced inflammation ranging from moderate to severe, in pregnant and non-pregnant animals.

Less inflammation in pulp tissues was expected after 7 days, especially with the use of MTA, which has the ability to maintain the integrity of the pulp and stimulate the formation of a dentinal barrier (28). However, most pregnant animals treated with MTA showed moderate inflammation. These findings demonstrate the tendency toward increased inflammation in pregnant animals, similarly to reported outcomes in periodontal tissues (3,20).

After 7 days of dental pulp capping, two specimens of MTA group, even in non-pregnant animals, presented moderate inflammatory response. It is important to discuss that MTA itself does not have an anti-inflammatory effect, and other authors had reported similar results with those from the present study. Louwakul et al. (29) reported that more than 60% of cavities capped with MTA, presented moderate acute inflammation after 8 days period. Dianat et al. (23) reported that the inflammation levels induced by MTA was similar in both 3 and 7 days. It indicates that
Figure 4. Pulp response at 7 days after pulp exposure and direct pulp capping with MTA (A - D) and composite (E - H). (A) no inflammatory response in the pulp tissue of a non-pregnant animal treated with MTA (HE, 100×); (B) high magnification of (A), showing the exposure area (HE, 400×); (C) pulp tissue of pregnant animal in which the pulp capping was performed with MTA, demonstrating focal necrosis and mild inflammatory infiltrate restricted to the exposure area (HE, 100×); (D) high magnification of (C) (HE, 200×); (E) moderate inflammatory response, and mild tissue loss in non-pregnant animal treated with composite-only (HE, 100×); (F) high magnification of (E) (HE, 400×); (G) extensive area of severe inflammatory response in the adjacent area of pulp tissue treated with composite-only in pregnant animal (HE, 100×); (H) focal area of (G), showing inflammatory infiltrate, edema and congesting vasculature (HE, 200×).
MTA promotes a mild to moderate chronic inflammatory response, which was characterized by organized connective tissue with the presence of macrophages and inflammatory multinuclear cells. Moreover, according to Long et al. (30) moderate inflammatory response induced by MTA can still be noticed after 30 days of intervention.

Bowles et al. (31) reported that that sex differences exist and can affect the role of NGF in the modulation of inflammation through the regulation of neuropeptide release and content. Furthermore, hormone levels fluctuate widely in women across the lifespan, including changes during the menstrual cycle, pregnancy, and following menopause. These variations in estrogen levels may influence the physiological and pathological response of pulp tissue (16). In addition, Dezeletovic et al. (17) found that plasma estrogen levels affect blood flow, then, greater vasodilatation may occur not only during the menstrual cycle but also during pregnancy (17,32).

Other systemic conditions also affect the pulp response. An increased and exacerbated expression of proinflammatory mediators in diabetic patient has been observed (33). Elevated blood levels of glucose might be associated with decrease or increase of proinflammatory cytokines such as interleukin (IL)-6, tumor necrosis factor (TNF)-α (34), and IL-17 (35). Furthermore, Cintra et al. (36) demonstrated that pulp tissue of diabetic rats had accentuated inflammation when compared to normoglycaemic rats after dental bleaching, resulting in accelerated deposition of reactionary dentine and alteration in collagen fibers, featuring pulp aging. These data support our finding of an increasing inflammatory response in the teeth of pregnant rats, probably due to greater hormone concentrations and increased blood supply in the pulp tissue.

The results of this preliminary experimental study in rats suggest that the tissue response after the direct pulp with MTA were more favorable in non-pregnant animals, while pregnancy may not influence the inflammatory process following direct pulp capping with light-cured resin composite, which was unsatisfactory regardless of pregnancy. These findings confirm the importance of additional histological studies to clarify the role of hormonal changes in the inflammatory response of the pulp tissue.

References
17. Dzeletovic B, Grga D, Krsiljak E, Strativimirovic D, Brkovic B, Stojic D.


Received June 8, 2018
Accepted August 2, 2018