

Case Report

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Histo-Pathological Assessment of Zinc Oxide Eugenol Canal Sealer on Periodontal Tissues: Three Case Reports

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Abstract

Purpose: Sealers used in endodontic dental treatment may leak into the periradicular tissues, increasing the risk of retained particles in oral mucosa, tissue irritation or discoloration, delayed healing or interfering on the erupting permanent teeth. Three clinical cases of primary teeth pulpectomy using Zinc Oxide Eugenol (ZOE) paste were observed, showing retained particles of filling cement in the oral mucosa during permanent dentition eruption. The aim of the study was to histologically analyze the possible causes of discoloration and to assess the effects of the sealer on cells by evaluating ultrastructure.

Materials and Methods: Three healthy children undergoing orthodontic therapy were enrolled in this study for the presence of gingival pigmentation. For all patients a biopsy of the discolored area was performed and histologically analyzed. Furthermore, sealer was added to the culture of SAOS-2 to evaluate cytotoxicity effects by Alamar blue tests and ultrastructure evaluation using Transmission Electrons Microscope (TEM).

Results: Black extracellular granules were appreciable in all Hematoxylin Eosin-stained sections. Deposition of pigment was observed particularly in the blood vessel walls, in connective tissue and in intercellular ground substance. In cell culture, sealers reduced viability about 43% after 24h, 17% after 48h and 12% after 72h compared with untreated cells. Furthermore, sealer affected morphology creating vacuoles inside the cytoplasmic area.

Conclusions: Sealer resulted to be mild toxic for the cells. Retention granules of bismuth in the oral mucosa after root canal treatment of deciduous teeth and subsequent mucosal discoloration analyzed in this study should alert pediatric dentists to use the filling materials very carefully and to check the radicular and filling paste resorption in order to prevent the prolonged retention of the overfilled material.

Keywords: Bismuth; Cytotoxicity; Deciduous Teeth; Gingival Discoloration; Overfilled Material; Ultrastructural Analysis

Introduction

Endodontic dental treatment creates a unique and complex biomaterial-tissue interface at the tooth root apex. The canal filling material and sealer are in intimate, long-term contact with multiple cell types in the periradicular tissues [1]. Sealers may

leak into the periradicular tissues, increasing the risk of tissue irritation or delayed healing. These risks increase if the sealers have inappropriate biological properties or do not effectively seal to prevent the ingress of bacteria [2-4]. In fact, although sealers should be confined within the root canal, their inadvertent extrusion into the periradicular tissues may occur [5], especially when a periapical lesion alters the anatomy of the apex. In many cases, the contact area between the sealer and the target cells, and

in turn, the concentration of the cytotoxic components on cells, may increase greatly [6]. Thus, toxic sealers can potentially cause tissue injury and may participate in the development of periapical inflammation or the continued existence of a preexisting periapical lesion, thereby delaying healing and adversely affecting the outcome of treatment. So, the biocompatibility of a sealer is of crucial importance [7-10].

It is determined by various parameters, such as composition and leachable components, setting characteristics, stability of the set sealer and the contact area between the sealer and the adjacent soft and hard tissues [7,9,10]. The sealers commonly used in endodontics are based on zinc oxide eugenol, calcium hydroxide, mineral trioxide aggregate, glass-ionomer or polymers, such as epoxy resins, polydimethylsiloxane and methacrylate [8]. Zinc Oxide-Eugenol (ZOE) paste is probably the most used root canal filling for primary teeth in the United States [11,12]. It is radiopaque, is very easy to fill and to remove and has a good antiseptic activity [13,14]. Although described as a resorbable material, long-term follow-up evaluations of pulpectomy on primary teeth treated with ZOE have revealed a high frequency of retention of the overfilled material in the periapical area, even after physiological root resorption [12, 15,16]. Another study revealed that 67% of all overfilled canals showed delayed resorption of the material when compared with physiological root resorption and retained particles of ZOE at 6 months follow-up [17]. Other studies showed that when ZOE extrusion occurs, there is a risk of deflecting the erupting permanent teeth due to its hardness [18,19]. In addition, it was demonstrated that ZOE components are a real obstacle for permanent successor eruption [20].

Various daily used endodontic sealers contain heavy metal; in 2002 Greenberg demonstrated that the presence of heavy metal could cause mucocutaneous discoloration [21]. If the combination of ZOE and heavy metal may increase the risk of mucocutaneous discoloration and may damage periodontal cells are questions still to be answered. This report presents three clinical cases of primary teeth pulpectomy using ZOE paste showing retained particles of filling cement in the oral mucosa during permanent dentition eruption. The retained particles of sealers migrated from the periapical area to the gingival vestibular area, in a way like a pus drainage, causing a poor aesthetics appearance. In order to remove the ZOE retained particles that created a poor aesthetic appearance, the periodontal surgery was necessary. The removed tissue was histologically analyzed in order to contribute to the understanding of the genesis of discoloration. At the same time, ZOE sealer was put in culture with cells with the aim to evaluate morphologically possible adverse effects on cells in contact.

Material and Methods

Clinical Procedures

During a periodical orthodontic evaluation, an altered intraoral gingival pigmentation was observed in three healthy children. The first patient was a 7-years-old girl (Figure 1,2). The upper lateral incisors eruption was hindered by lack of space. After a rapid maxillary expansion protocol, the upper left lateral incisor did not erupt yet, thus root canal therapy and mesial slicing of the deciduous upper left canine were executed (Figure 1a,b), allowing a correct eruption of the lateral incisor. When the patient was 10 years old, she noticed a discoloration on the free gingival margin of the deciduous upper left canine. An orthopantomography showed root filling paste extrusion in the periradicular area (Figure 1d). The darkening seemed to be referred to the bulge of the permanent maxillary canine (Figure 1c), but the discoloration also remained after the canine eruption (Figure 1e,f). The removal of the discoloration, a gingival graft and a histological assessment of the removed tissue were planned at the end of permanent dentition, when the patient was 11 years old (Figure 2). The graft has been stabilized to the periosteum with 6-0 Prolene Ethicon sutures (Figure 2). The second patient was a 13-years-old girl (Figure 3). She had all permanent teeth, except for the lower right second premolar, affected by agenesis, and the left first molar that was extracted after an unsuccessful root canal treatment. Moreover, a darkening of the gingiva of the upper left second premolar was present (Figure 3). Patient referred an endodontic therapy on the upper left second primary molar at age of 5 years. A gingival graft was planned to remove the discolored area and histological study was executed.

The third patient was a 10-years-old boy (Figure 4). His medical history included two endodontic therapies of both upper left and right first primary molars. After the removal of residual roots, the corresponding free gingival area seemed dark. The discoloration also remained after the premolar eruption (Figure 4). The removal of the discoloration and a histological assessment were executed to evaluate its nature. For all patients, a biopsy of the discolored area of buccal mucosa was performed using a surgical blade (Figure 5, 7). For practical reasons for the first two patients the biopsy was made during periodontal surgery. The pigmented tissue has been removed surgically by means of a layered partial thickness flap, starting from the superficial layer and moving deeper when necessary. A free gingival graft was harvested from the tuberosity, trimmed to fit the wound area and the epithelial layer was removed, so the graft was a free connective tissue graft. This technique leads to a better esthetic outcome in terms of color and tissue thickness when compared to a free gingival graft [22].

Specimens Processing

Immediately after harvesting, soft tissue biopsies were immersion fixed in 10% formalin 0.1M phosphate buffer saline (PBS) (pH 7.4) 24 hours at room temperature, then routinely dehydrated in increasing concentrations of ethanol (from 50 to 100%), xylol for 12 hours and then paraffin embedded. Serial 4-5 μm buccal-lingual sections were obtained, mounted on 3-Amino-propyl-trietoxi- silane coated slides, and then hydrated in decreasing concentrations of xylol and ethanol (from 100 to 70%) and then immersed in distilled water. To evaluate the tissue morphology four sections for site were stained with Mayer's Hematoxylin (Bio-Optica, Milan, Italy) and Eosin (Bio-Optica, Milan, Italy) according to the standard protocol [23]. All the histological sections were observed and photographed in a Nikon light microscope (Eclipse E600) equipped with a calibrated digital camera (DXM1200, Nikon, Tokyo, Japan) and morphological assessments were performed.

Cell Culture

SAOS-2 cells, a permanent line of osteosarcoma cells (Invitrogen, Germany), were cultured with L-glutamine, 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin (GIBCO, USA) in 5% CO_2 at 37°C in 12-well culture plates (Corning, USA).

Viability Assessment

Three mother solutions were prepared adding 10%, 5%, 1% of ZOE past to medium of culture. After 24h in culture the plate was observed under contrast phase microscope, images were acquired to perform semi-quantitative analysis of viability cells [24]. Cells in adhesion were counted and the ratio between treated cells and untreated cells were expressed in percentage. Then the variation between treated cells with 10%, 5%, 1% of ZOE was calculated compared with untreated control in order to report the reduction of the viability in presence of the sealer. The same cells were pelleted and used for ultrastructural analysis by Transmission Electron Microscope (TEM).

Ultrastructure Evaluation

For the assessment by TEM, cellular pellets were fixed overnight in a solution containing 2% of paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) and then post - fixed in 1% osmium tetroxide in cacodylate buffer, washed, dehydrated and embedded in Epon - Azaldite resin. Semi-thin sections to evaluate pellets were obtained using microtome, stained with toluidine blue in heater and acquired under light microscope (Eclipse E600) [25]. Furthermore, ultra- thin sections were cut by a Leica Supernova ultramicrotome (Reichert Ultracut E) and stained with lead citrate for morphological observation under TE (Zeiss EM10).

Results

Study Population

In three subjects that had a previous history of dental treatment, a blackish blue oral pigmentation due to ZOE paste was found and collected for histological examination. In two patients, the biopsy was carried out for aesthetic reasons, while in one case the study was conducted at the request of parents. All biopsies were obtained by oral surgeon. Pigmentation areas were measured between 0.2 and 0.7 cm (with a mean diameter of 0.45 cm).

Histological assessment

At histological evaluation, no signs of necrosis, fibro-encapsulation, adipose tissue fatty infiltration or severe inflammatory infiltrations were detected. Only in the third patient some areas of a mild inflammatory infiltration were observed. All samples presented a well-organized connective tissue underneath the oral epithelium. The method used to harvest the biopsies did not assure the integrity of the junctional epithelium. Fine dark brown or black extracellular granules were appreciable in all Hematoxylin Eosin-stained sections (Figures 5-7). Deposition of pigment was observed particularly in the blood vessel walls, in connective tissue and in intercellular ground substance of the lamina propria. In basal membrane we found light brown micro-granules uniformly distributed all over its extension. In contrast, the granules found in the connective tissue assumed variable sizes, mostly larger than those observed in basal membrane; these were preferentially distributed in extra cellular matrix and showed an almost black coloration. In some cases, fibroblasts and endothelial cells were also marked. Macro-granules observed in the connective tissue presented jagged and irregular margins. At higher magnification, the granules in the vessel walls and in basal membrane appeared similar, both size and color. In all the samples epithelial layers did not show any deposit of pigment.

Viability Assessment and Ultrastructural Analysis of Cells in Contact With ZOE

The viability was evaluated using semi-quantitative methodology with the aim to compare treated cells (Figure 8a-c) to untreated cells (Figure 8d) behavior. SAOS-2 are little cells of elongated shape in adhesion (Figure 8d). All concentrations reduced the viability of the cells when the presence of sealer was increased (10%) (Figure 8e). Observing cellular pellets on semi-thin sections obtained by sample with medium containing sealer at 10%, the adverse effects on cells were confirmed by structural analysis that showed well stained and round cells in suspension in no treated group (Figure 9a) and cellular debridement in treated group (Figure 9c). Furthermore, ultrastructural analysis conducted on ultra-thin sections allowed to visualize numerous cytoplasmatic vacuoles in treated cells treated (Figure 9d) compared with no treated cells (Figure 9b). The presence of numerous vacuoles seem

to indicate a cellular foam degeneration with probably consequent cellular death (Figure 9d).

Discussion

An ideal endodontic sealer must be biocompatible, to have adequate physicochemical properties, bioactivity and antimicrobial activity. Although many endodontic sealers are available on the market, none of them meets all these requirements [26]. ZOE has been found to be potentially irritating to periapical tissues; it may even produce necrosis of bone and cementum, and extruded particles may develop a fibrous capsule that prevents resorption of the paste [27]. Moreover, it was already reported that the overfilled ZOE induced inflammatory reactions, chronic or subacute, on the dental follicle of permanent successor [20]. Frequently osteoclastic activity was too slow to eliminate the retained ZOE and suggests the possibility of not- resorption [28]. Pulp canal sealer (Sybron dental specialties orange, CA) is an endodontic filling material commonly used in contemporary endodontic treatment also for deciduous teeth [29,30]. It is composed of liquid and powder containing eugenol and zinc oxide, staybelite resin, bismuth sub carbonate, barium sulfate, sodium borate anhydrous with significantly less toxic effects in connective tissue in rats [29-31].

In literature, relations between heavy metal deposition and the following mucocutaneous discoloration have been demonstrated [21]. The bismuth subsalicylate seems to be a pigment-inducing agent; it determines the formation of a characteristic linear black rib at free gingival margin level. The tissues inflammation, caused by extrusion of endodontic sealer, lead to an increase of capillary permeability thus allowing the deposition of the metal sulfides in the connective structure, resulting in a mucocutaneous discoloration [32]. The three cases of oral pigmentation evaluated in the current study show both clinical and histological features that are typical for heavy oral metal deposition. This is the first histological report that analyzed the presence of granules in the oral mucosa due to the bismuth subsalicylate deposition. The aspect of intraoral discoloration and the localization of micro and macro-granules at histological analysis, seem to be similar to those already observed in tissues exposed to amalgam [33]. Amalgam pigmentation is harmless [34] and relatively frequent, mainly affecting the mandibular gingival mucosa [35], followed by the buccal mucosa, floor of mouth, tongue, retromolar mandibular

area, lips, and palate. Amalgam may produce local adverse effects [33], including mucosal pigmentation due to its metal components (silver, mercury, and tin). This is the most prevalent exogenous oral pigmentation [35,36] and can be confused with melanin pigmentation, in which case biopsy studies are indicated. Also, bismuth deposition can be added with melanin pigmentation. In our cases, granules were vastly different from melanin deposits that are typically localized in the cytoplasm of melanocytes. Therefore, while melanocytes are usually scattered within the basal layer of the epidermis, the black granules were placed also in the connective tissue.

Oral pigmentation caused by amalgam is caused by several mechanisms, including mechanical penetration into soft tissues, corrosion phenomena, and release of metallic components [37]. The mechanism of bismuth penetration in oral tissue is still unknown; in future studies it could be interesting to determine if this heavy metal works in a similar way. An in vitro model was applied to investigate ZOE sealer behavior. Cytotoxicity assays are used to verify the level of biocompatibility although may be influenced by many factors such as cells type used in the experiments [26,38]. The literature, in fact, reports diffused cytotoxicity effects of sealers towards cells in culture [26,38,39]. The cytotoxicity of zinc oxide-eugenol had been previously observed reporting similar data to this study where the viability was affected by the presence of the sealer and that cytotoxic effects were reducing over time [39]. Furthermore, Huang et al 2002 reported that zinc oxide-eugenol-based sealer caused moderate to severe cytotoxicity probably attributable to free eugenol liberated from the set material [39]. Vacuolization of the cells reported in the results confirmed the negative effects. Nowadays, the process is not completely clarified in all passages, but it was observed in mammalian cells after exposure to bacterial or viral pathogens as well as to various natural and artificial compounds [40]. Cytoplasmic vacuolization is a known process, morphologically recognizable, and associated with cell death.

Conclusion

As a result of these considerations, the pediatric dentist should carefully evaluate the primary treated teeth, and periodically check the radicular and filling paste resorption in order to prevent the prolonged retention of the overfilled material, especially during permanent dentition eruption.



Figure 1: Clinical case from patient 1. A: Clinical intraoral images shows patient 1 after a rapid maxillary expansion; B: Detail of orthopantomography shows the root canal therapy of the deciduous upper left canine; C: Photo reports the bulge of the permanent maxillary canine; D: Detail of orthopantomography shows root filling paste extrusion in the periradicular area; E: Photos reporting the erupted permanent upper left canine. F: Yellow arrow indicates pigmented gingiva.

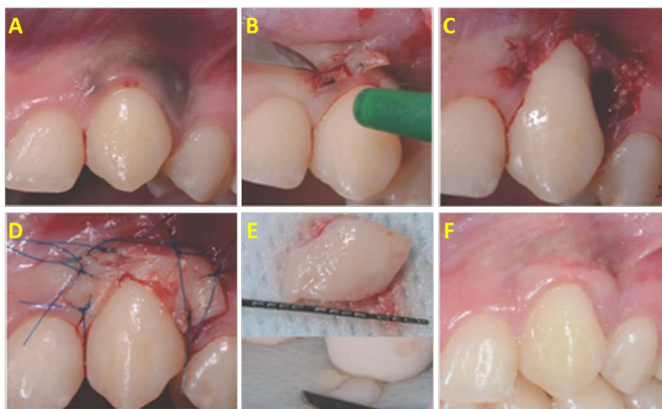


Figure 2: Surgery on patients 1. Intraoral images shows passages of periodontal surgery for patient 1. A: Area of gingival discoloration; B, C: Removal of pigmented tissue by means of a layered partial thickness flap; D: 6-0 Prolene sutures; E: Measurement of free connective tissue graft from the tuberosity; F: Healed site after surgery.



Figure 3: Clinical case from patient 2. Clinical intraoral image shows darkening of the gingiva (yellow arrow) of the upper left second premolar in the second patient.



Figure 4: Clinical case from patient 3. Clinical intraoral image shows darkening of the gingiva (yellow arrows) on: A right and B left upper jaw in the third patient.

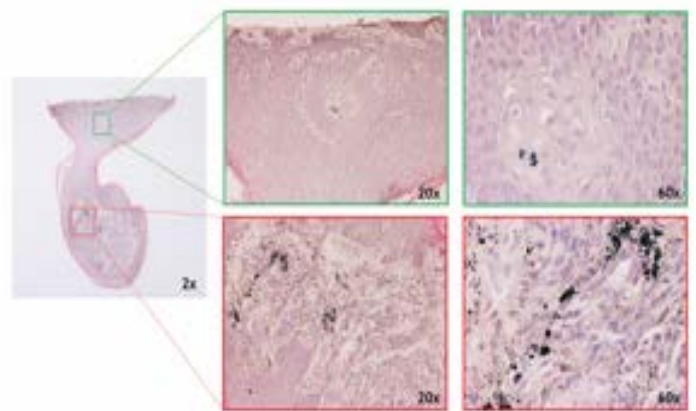


Figure 5: Histological sample from patient 1. Histological image at different total magnification (20x, 200x, 600x) that shows black extracellular granules distribution and in intercellular ground substance of the proprial layer (green box) and in connective tissue (red box), Hematoxylin and Eosin staining.

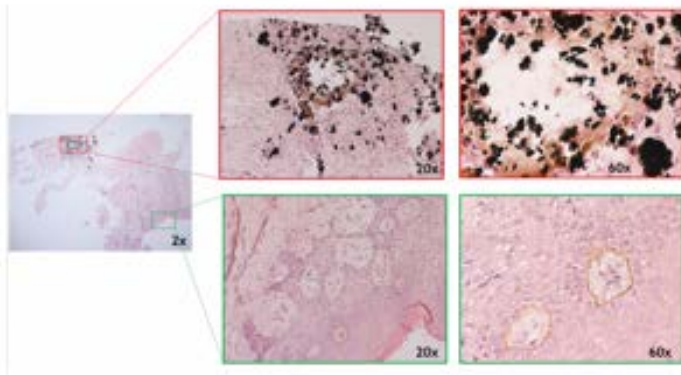


Figure 6: Histological sample from patient 2. Histological image at different total magnification (20x, 200x, 600x) that shows black extracellular granules distribution in connective tissue (red box) and in intercellular ground substance of the proprial layer (green box), Hematoxylin and Eosin staining.

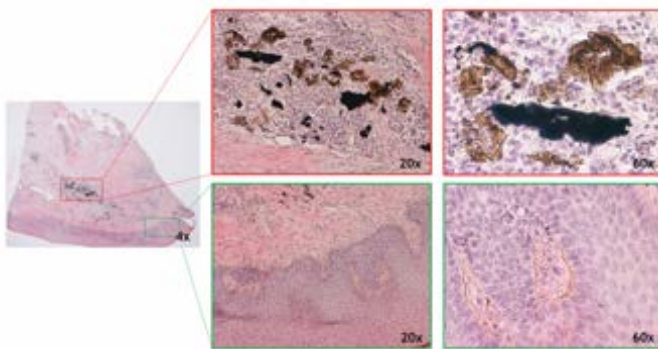


Figure 7: Histological sample from patient 3. Histological image at different total magnification (40x, 200x, 600x) that highlight black extracellular granules distribution in connective tissue (red box) and in intercellular ground substance of the proprial layer (green box), Hematoxylin and Eosin staining.

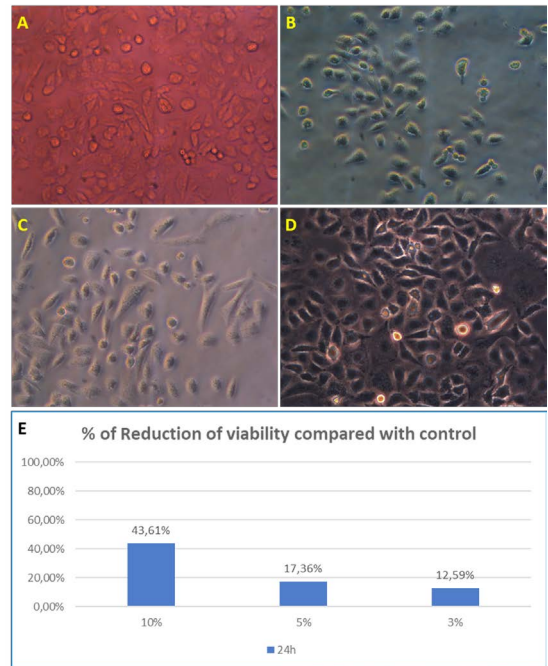


Figure 8: Viability evaluation in contact with sealer. **A:** cells treated with 10% of sealer are partially detached, and show a rounded shape indicating a suffering condition. **B:** The cells treated with 5% of sealer present a major adhesion if compared to cells treated with 5% of medium. It is notable that cellular density is slightly lower than in control. **C:** The cells treated with 1% of sealed for density and morphology are resembling to control. **D:** untreated cells are well attached to the plate, in close contact among them with high density. Morphologically they present elongated shape characterized by cytoplasmic processes that allow adhesion on plate and intracellular contact maintaining stimuli to promote cellular proliferation. (contrast phase microscope, total magnification 200x). **E:** Semi- quantitative analysis reported in graphic shows reduction of the viability in presence of sealer at all concentrations.

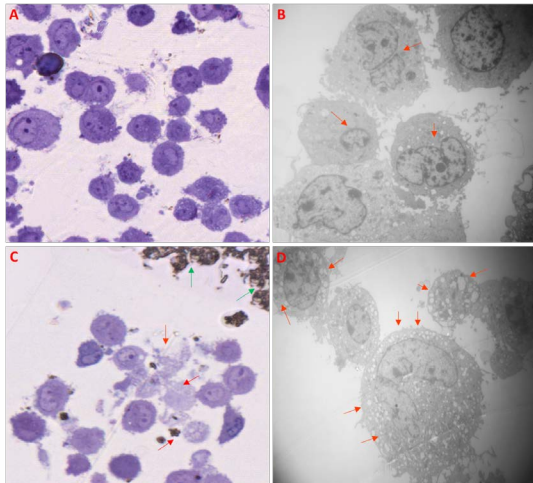


Figure 9: Ultrastructural analysis of SAOS-2. A: Untreated SAOS-2 cells appeared well stained with toluidine blue confirming the integrity of the cellular membrane, nucleus, nucleoli (total magnification 500x, light microscope, semi-thin sections). **B:** Image of a group of untreated cells. Some cells are observed in division phase (red arrows). Arrows indicate nucleus in division filled with chromatin and nucleoli. **C:** Red arrows indicate some cellular debris in treated cells. SAOS-2 did not show marked color confirming that cells did not result well preserved in contact with sealer in brown indicated by green arrows (total magnification 500x, light microscope, toluidine blue, semi-thin sections). **D:** Treated cells resulted not preserved and characterized by numerous bubbles/vacuoles inside (red arrows) which indicate the non-integrity of the nucleus and organelles in the cytoplasm (400x).

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