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Gross, organoleptic and histologic assessment of cadaveric equine heads preserved using chemical methods for veterinary surgical teaching

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ABSTRACT

Background: Preservation of biological tissues has been used since ancient times. Regardless of the method employed, tissue preservation is thought to be a vital step in veterinary surgery teaching and learning.
Objectives: This study was designed to determine the usability of chemically preserved cadaveric equine heads for surgical teaching in veterinary medicine.
Methods: Six cadaveric equine heads were collected immediately after death or euthanasia and frozen until fixation. Fixation was achieved by using a hypertonic solution consisting of sodium chloride, sodium nitrite and sodium nitrate, and an alcoholic solution containing ethanol and glycerin. Chemically preserved specimens were stored at low temperatures (2°C to 6°C) in a conventional refrigerator. The specimens were submitted to gross and organoleptic assessment right after fixative solution injection (D0) and within 10, 20, and 30 days of fixation (D10, D20, and D30, respectively). Samples of tissue from skin, tongue, oral vestibule, and masseter muscle were collected for histological evaluation at the same time points.
Results: Physical and organoleptic assessments revealed excellent specimen quality (mean scores higher than 4 on a 5-point scale) in most cases. In some specimens, lower scores (3) were assigned to the range of mouth opening, particularly on D0 and D10. A reduced the range of mouth opening may be a limiting factor in teaching activities involving structures located in the oral cavity.
Conclusions: The excellent physical, histologic, and organoleptic characteristics of the specimens in this sample support their usability in teaching within the time frame considered. Appropriate physical and organoleptic characteristics (color, texture, odor, and flexibility) of the specimens in this study support the use of the method described for preparation of reusable anatomical specimens.

Keywords: Educational techniques; anatomy; biological preservation; horses

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Conflict of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support this study are available from the corresponding author (Mendes RP) upon reasonable request.

INTRODUCTION

Use of synthetic body parts and cadavers as anatomical models is a common practice in veterinary surgery teaching worldwide [1]. In the last few years, researchers, animal welfare organizations, and animal model manufacturers have tried to develop alternative methods to replace natural anatomical specimens [2,3]. However, the preservation of biological materials is still considered essential for surgical training [1]. The use of preserved specimens allows procedures to be repeated, optimizing learning and increasing students' confidence at a lower cost and with lower levels of stress compared to use of live animals [3-5].

Video-based sessions, anatomical models, software, and use of cadaveric specimens preserved in glycerin or Laskowski solution are some examples of alternative non-invasive methods [1,6-9] for teaching and learning. However, these methods have limitations and may not faithfully reproduce procedures performed on live animals, which in turn may interfere with appropriate teaching and learning [10].

Formaldehyde is still widely used to preserve tissues. However, tissue hardening, foul odor, mucosal irritation, and carcinogenic potential are some of the disadvantages associated with this fixative [11,12].

Novel alternative preservation techniques include chemical preservatives such as 30% sodium chloride [3], an appropriate, inexpensive and widely available product [4]. Ethanol (96° GL), another inexpensive, and widely available chemical with strong affinity for biological tissues and high penetrating capacity can also be used as a fixative agent in small animals and small anatomical specimens [3,11,12].

The combination of alcoholic fixation and preservation using hypertonic salt solution (30% sodium chloride) provides an effective method for the preparation of surgical training models [13]. Saturated salt solutions inhibit bacterial growth while producing specimens with flexible joints and excellent quality for surgical skill training [9,14,15].

Fixation of canine viscera using absolute alcohol and sodium chloride yielded promising results [3,9,13]. Saturated salt solutions were also successfully used in other species [16]. Curing Salt Solution (CSS, 20% sodium chloride, and 1% sodium nitrate and nitrite) provided appropriate preservation of feline cadavers [17,18], however, the use of this solution has not been reported in horses.

This study was designed to examine the physical and organoleptic characteristics of cadaveric equine heads prepared for veterinary surgical teaching. Specimens were preserved using a CSS (20% sodium chloride and 1% sodium nitrate and nitrite) followed by an alcoholic solution containing ethyl alcohol (EA, 99.9°GL) and 98% glycerin (95% and 5%, respectively) and examined within 10, 20 and 30 days of fixation. Correlations between physical and organoleptic characteristics and structural changes detected in histological evaluation were also investigated.

MATERIALS AND METHODS

First phase

Animals

This project was developed at the University of São Paulo (FMVZ-USP) Fernando Costa campus, Equine Dentistry Center (COE) of the School of Veterinary Medicine and Animal Science in Pirassununga, SP, Brazil following approval by the Ethics Committee (CEUA) of the School of Animal Science and Food Engineering (FZEA-USP), protocol No. 7010030220 (ID 001436). Six cadaveric equine heads were used. Specimens were obtained from female and male horses weighing between 400 kg and 600 kg, who died at the FMVZ-USP Veterinary Hospital.

Preservation method

Cadaveric heads (6) were collected from adult female and male horses aged 8 to 20 years with intact head structures who died or were euthanized for reasons unrelated to this study. Heads were separated at the level of the atlanto-occipital joint and washed in running water immediately after death or euthanasia. The oral cavity was also washed for food material removal. Specimens were placed in plastic bags and stored in a cold chamber at –18°C. Cadaveric heads were collected over the course of 60 days. Frozen specimens were placed into thermal insulation boxes, covered with crushed ice, transported to COE/FMVZ-USP, and stored in a horizontal freezer.

Specimens were left to thaw in a temperature-controlled (25°C) room for 14 h, then immersed in water for 6 h. Water was changed every 2 h during the thawing process. Thawed specimens (including the oral cavity) were washed in running water and kept in the vertical position (i.e., lying on the vertical mandibular rami) for 30 min for dripping. Specimens were then numbered 1 to 6 and weighed. Specimen weight (12 kg, 13 kg, 16 kg, 24 kg, 17 kg and 25 kg; specimens No. 1, 2, 3, 4, 5, and 6, respectively) was used to calculate fixative solution volume.

The left and right common and internal carotid arteries were dissected and cannulated just proximal to the origin of the occipital artery using Levin catheters (FR 12 and FR 8, respectively). Catheters were fixed with 2-0 silk suture (**Fig. 1**). Arterial accesses were used for infusion of a CSS (150 mL/kg, 75 mL/kg per side). The CSS was prepared with 200 g/L of sodium chloride, 10 g/L of sodium nitrite, and 10 g/L of sodium nitrate per liter of water. After 30 min, 150 mL/kg of an alcoholic solution consisting of 99.8% ethyl alcohol and 98% glycerin (95% and 5%, respectively; **Supplementary Table 1**) were injected into the specimens. The infusion was carried out via the common and internal carotid arteries (90% and 10% of the total volume, respectively) using a 20 L knapsack manual sprayer pump.

Following infusion completion, specimens were placed in duly identified plastic bags and stored in a conventional refrigerator at 2°C to 6°C. Three to six days later, specimens were washed in running water to remove any blood remaining from the injection procedure and then kept refrigerated until use.

Second phase

Specimens were submitted to physical and organoleptic assessment immediately after fixative solution injection (D0) and within 10, 20 and 30 days of fixation (D10, D20, and D30, respectively). The following characteristics were evaluated: appearance, texture, flexibility, odor, color, and range of mouth opening. Assessments were carried out individually by three blinded examiners (surgeons familiar with the use of anatomical specimens for teaching and

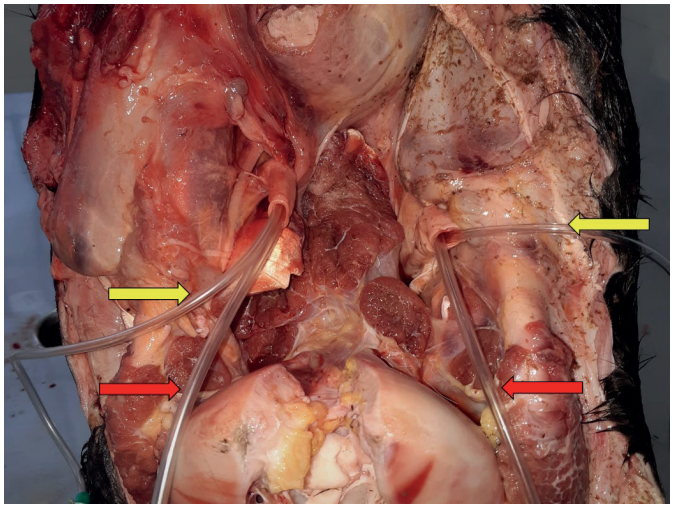

Fig. 1. Identification and cannulation of arteries used for fixative solution infusion (specimen No. 6). Levin catheters FR 12 and FR 8 were placed in the right and left common carotid arteries (red arrows) and in the right and left internal carotid arteries (yellow arrows) respectively.

Specimen identification: _____ Date:__/__/

Examiner: _____

Sensory assessment				
	D0	D10	D20	D30
Appearance				
Texture				
Flexibility				
Smell				
Color				
Range of mouth opening				

Score: 1 to 5, as follows:
1 Very bad: severe deterioration of physical characteristics; not suitable for use.
2 Bad: severe deterioration of physical characteristics; partially suitable for use.
3 Regular: severe deterioration of physical characteristics; suitable for use.
4 Good: mild deterioration of physical characteristics; suitable for use.
5 Very good: intact physical characteristics.

Fig. 2. Specimen assessment form filled out by examiners on D0, D10, D20 and D30.

research purposes) using a standardized form. Characteristics were scored 1 to 5, and mean scores were used in the analysis (**Fig. 2**).

Collection and processing of samples for histologic evaluation

Tissue samples (skin, tongue, oral vestibule, and masseter muscle) were collected (**Fig. 3**), immersed in 10% phosphate-buffered formaldehyde for 48 h and stored in 70% alcohol until use. Samples were processed using conventional paraffin embedding methods, stained with hematoxylin and eosin, and examined under a light microscope (Axiolab 2.0, Carl Zeiss,

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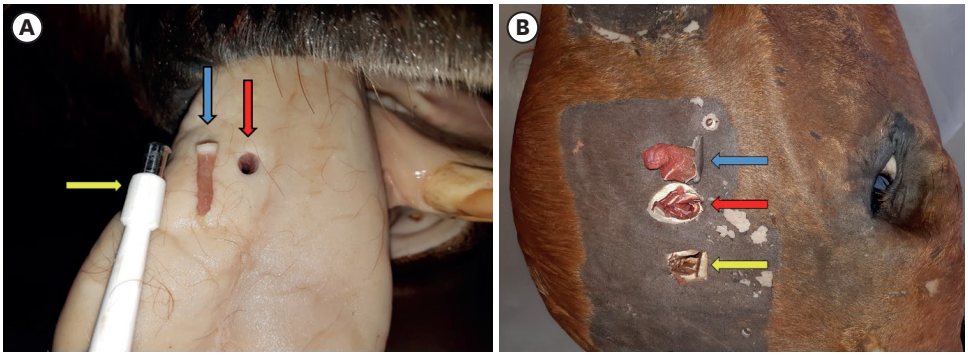

Fig. 3. Collection of tongue tissue sample for histologic evaluation (specimen No. 1, D10). (A) Punch (yellow arrow), fragment (blue arrow) and sampling site (red arrow). Collection of masseter muscle sample for histologic evaluation (specimen No. 5, D20). (B) Muscle tissue fragment (blue arrow), sampling site (red arrow). The yellow arrow indicates the site of prior (D10) sample collection.

Germany). Histologic evaluations were performed at the Veterinary Pathology Department of FZEA-USP.

Samples were collected immediately after physical and organoleptic assessment on D10, D20 and D30. Samples were collected after specimen fixation to prevent fixative solution extravasation.

RESULTS

Preservation method

The fixation method proposed in this study enabled satisfactory preservation of anatomical specimens throughout the experimental period. Hence, specimens preserved in this manner can be reused in teaching activities aimed at expanding anatomical knowledge.

The use of a CSS injection was more time consuming and higher pressure was required to overcome tissue resistance. Still, the procedure was completed as planned. The replacement of the blood remaining in tissues and vessels was achieved closer to the end of the injection procedure when the CSS began to leak through major venous trunks, particularly the external jugular and the linguofacial veins. Alcoholic solution injection was faster and easier.

The following changes were noted after completion of injection procedures: swelling of the subcutaneous and submucosal tissues and eyelids, enlargement of the distal aspect of the tongue, oral vestibule and masseter muscle, and ingurgitation of facial vessels (**Supplementary Fig. 1**). Fluid also escaped through the gums and oral mucosa. These changes were less evident between D10 and D30. Tissues and anatomical structures regained their baseline characteristics (D0) over time. Swelling and enlargement did not interfere with the use of specimens in surgical training. Specimens had to be washed on the third and sixth days after fixation to remove the blood accumulated in storage bags. An unpleasant, mildly putrid odor was noted on the third day after fixation. In spite of the large amount of blood in the storage bags, the foul odor was no longer perceived on the sixth day. At this time, the alcoholic odor prevailed.

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Physical and organoleptic evaluation

Specimens retained organoleptic characteristics deemed essential for surgical training, supporting the effectiveness of the preservation method proposed in this study. In order to facilitate the interpretation and comparative analysis of findings, scores were assigned to different characteristics by different examiners on D0, D10, D20, and D30 were presented as means and medians (**Table 1**).

Specimens were scored 4 to 5 (good to very good) regarding appearance, texture, flexibility, and odor at all assessment time points (**Table 1**). Mean scores decreased progressively from D0 to D30. Color was scored lower than 4 on D30.

Mouth opening scores ranged from 3.17 to 4.02 (D0 and D30, respectively) and increased progressively over time. The lower range of mouth opening scores can be explained by low scores (2, poor) assigned to specimens 4 and 6 on D0, and to specimen 5 at all assessment time points (**Supplementary Tables 2-4**, respectively). Specimens 1, 2, and 3 were scored 4 to 5 (good to very good) (**Supplementary Tables 5-7**, respectively).

Aside from the characteristics described, other relevant changes were detected. Soft tissues in the oral vestibule, lips and tongue became extremely swollen and increased in size after the injection of fixative solutions. As of D10, the oral and lingual mucosa started to detach and could be easily peeled off. However, head specimens had good visual appearance and smell 30 days after fixation (**Supplementary Fig. 2**).

Histologic evaluation

Histologic evaluation revealed appropriate tissue preservation (skin, masseter muscle, tongue, and oral vestibule) and mild structural changes following impregnation with CSS.

The skin was well preserved, particularly the epidermis, hair follicles, sebaceous glands, and collagen fibers in the dermal connective tissue. Skin characteristics did not differ over time (D10 to D30) (**Fig. 4A, E, and I**).

The masseter muscle was also well preserved. Muscle fibers and myofibrils could be identified and individualized in cross-sectional and longitudinal sections. Nuclei were satisfactorily preserved and could be identified and examined. Poor definition of muscle bands at all time points precluded comparisons with muscle tissues preserved using traditional methods (**Fig. 4B, F, and J**).

The tongue was partially preserved. In spite of the good histologic appearance, mild cell retraction and myofibrillar compression noted at different time points, possibly the partial preservation observed was due to the dehydration induced by the preservation method (**Fig. 4C, G, and K**).

Table 1. Average and median value of organoleptic characteristics at different time points

Parameter	Day-0		Day-10		Day-20		Day-30	
	Mean	Median	Mean	Median	Mean	Median	Mean	Median
Appearance	5.00	5.00	4.85	4.85	4.60	4.65	4.12	4.00
Texture	4.85	4.85	4.73	4.70	4.45	4.50	4.15	4.15
Flexibility	4.35	4.35	4.23	4.35	4.23	4.00	4.50	4.50
Odor	4.48	4.30	4.50	4.50	4.43	4.40	4.50	4.50
Color	4.58	4.70	4.38	4.30	4.27	4.30	3.98	4.00
Range of mouth opening	3.17	3.00	3.57	3.65	3.77	3.50	4.02	4.35

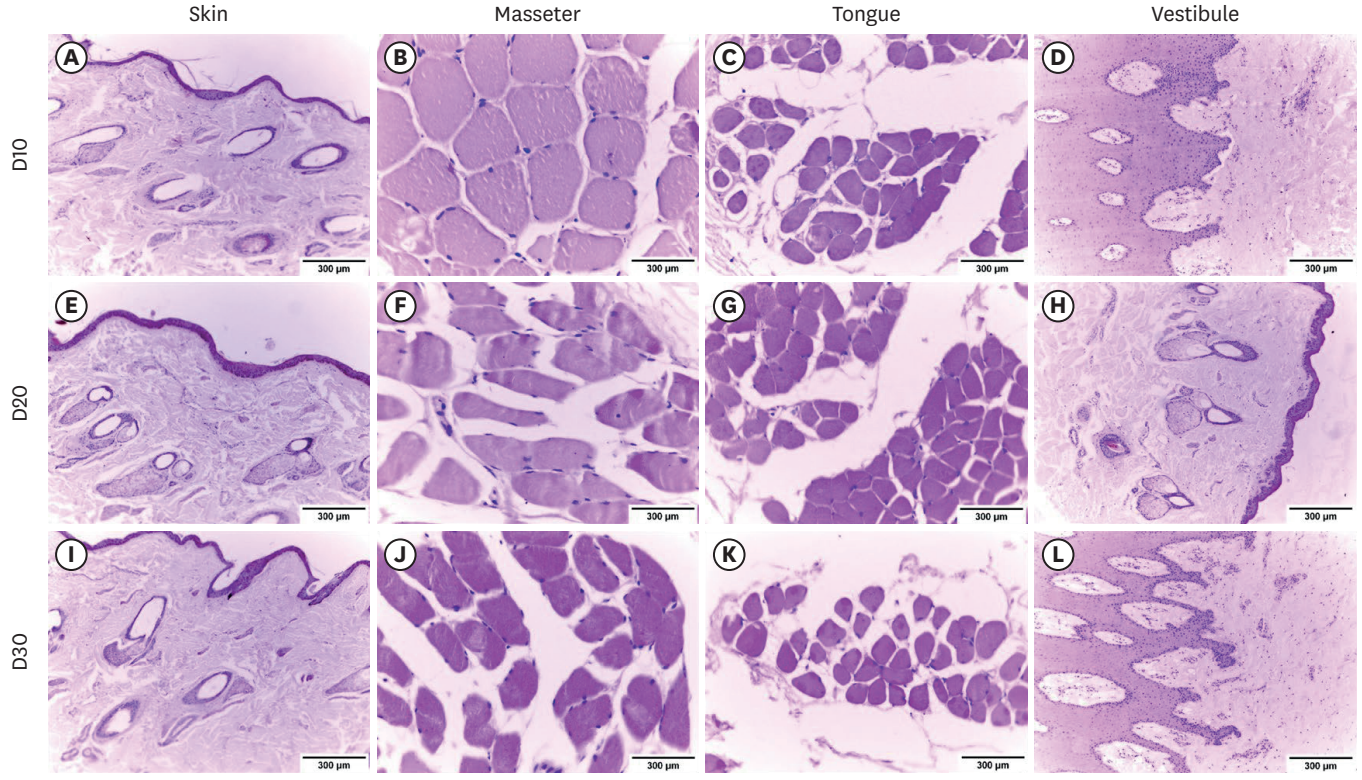

Fig. 4. Photomicrograph of histologic sections of skin, masseter muscle, tongue and oral vestibule tissues preserved in curing salt (hypertonic solution of sodium chloride, sodium nitrite, sodium nitrate and 99.8% alcohol in 98% glycerin). Preserved organ morphology (hematoxylin and eosin stain; 10× magnification; scale bar 300 μm). Skin preserved for 10 (A), 20 (E) and 30 (I) days; masseter muscle preserved for 10 (B), 20 (F) and 30 (J) days; tongue preserved for 10 (C), 20 (G) and 30 (K) days; oral vestibule preserved for 10 (D), 20 (H) and 30 (L) days. Scale bars: 300 μm.

The oral vestibule had a similar microscopic appearance to the skin and masseter muscle. Epithelial lining detachment was observed in four out of six samples (specimens No. 2, 3, 4, and 5) with loss of superficial cells at all time points (**Fig. 5**).

In two samples (specimens No. 2 and 4; **Fig. 5**), the sweat glands underwent moderate autolysis over time (particularly on D30). Cellular enucleation and other severe cellular changes were also detected in these samples. In the remaining samples, tissue structure was sufficiently preserved.

In one sample (specimen No. 5), the epithelial lining of the tongue was missing (detached or lost during processing) on D10, D20, and D30. This may have reflected poor tissue fixation.

DISCUSSION

Studies investigating alternative methods of cadaver preparation for use in anatomical and surgical teaching are essential and have been carried out globally [9,13,19,20].

Anatomical specimens are traditionally preserved by freezing. However, in spite of the appropriate retention of anatomical characteristics, frozen specimens have several use limitations [12]. Freezing and thawing procedures (i.e., the time elapsed between death and specimen collection, thawing in a water bath or by exposure to room temperature, etc.) are

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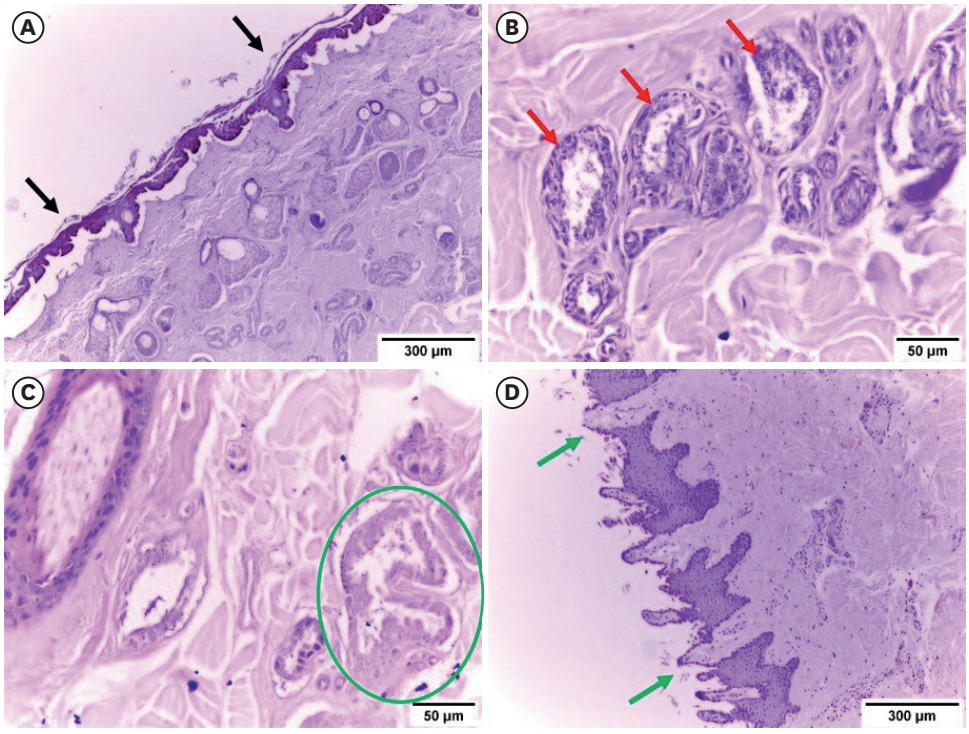

Fig. 5. Photomicrograph of histologic sections showing changes in skin and tongue tissues collected from equine head specimens and preserved in curing salt (hypertonic solution of sodium chloride, sodium nitrite, sodium nitrate and 99.8% alcohol in 98% glycerin; hematoxylin and eosin stain). (A) Skin collected from head specimen No. 2 at 30 days (D30). Note epidermal detachment (I, black arrows; 10× magnification; scale bar 300 μm). (B) Skin collected from head specimen No. 2 at 30 days (D30). Note sweat gland autolysis (red arrows; 40× magnification; scale bar 50 μm). (C) Skin collected from head specimen No. 4 at 20 days (D20). Note sweat gland autolysis (green circle; 40× magnification; scale bar 50 μm). (D) Tongue tissue collected from head specimen No. 5 at 30 days (D30). Note destruction of the superficial epithelial layer (green arrows; 10× magnification; scale bar 300 μm).

not standardized. As a result, physical and organoleptic characteristics may differ between specimens and change from one practical training session to the next. Secondly, and more importantly, specimens comprising digestive system structures, such as the oral cavity, undergo rapid microbial deterioration [20,21]. The standardization of freezing and thawing procedures proposed in this study allowed satisfactory specimen preservation.

The identification of the plexus arteriovenous is critical for injection of fixative solutions [1,20]. Injection under high pressure was required in order to eliminate blood clots and completely fill the vascular compartment. Infusion of other fluids, such as warm water, prior to fixative solution injection may help eliminate these clots [1,22]. However, despite the use of a different method, fixative solutions crossed the vascular barrier and impregnated tissues irrigated by the peripheral vascular system.

Different from this study, in which the CSS was infused 30 min before the alcoholic solution, in Fração et al. [20] and Queiroz et al. [19], alcoholic solution injection preceded CSS injection. Results were similar in dogs evaluated for up to 120 days [13] and cats evaluated for up to 90 days [20]. Hypertonic saline solution (20% sodium chloride or more) was used in all studies as recommended for effective cadaver preservation [23].

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Alcoholic fixation of cadaveric equine heads enabled appropriate tissue preservation and prevented degradation, as reported in dogs [13] and cats [20]. The findings of this study support the superiority of alcoholic fixation regarding tissue color and smell [21].

In this study, generalized head swelling was observed after fixative solution injection. Less dense tissues (subcutaneous compartment, tongue, lips, and eyelids) were particularly affected. The swelling decreased after a few days but was not completely resolved. Hence, a minimum interval of 10 days between specimen fixation and use is recommended.

Post-injection swelling has not been reported in prior studies [3,13,16,20,21]. In some of those studies, specimens became stiff following immersion in absolute alcohol, but stiffness subsided after completion of the fixation procedure [1]. In other studies [14], specimens were described as supple, but natural texture was lost. Results obtained with the use of a 95% alcoholic solution support the findings described by Guaraná et al. [4], namely effective preservation with no signs of deterioration.

In this study, the range of mouth opening was determined manually. Reduced mouth opening prevented appropriate manipulation of the oral cavity. However, this limitation was overcome using mouth gags such as the Gunther speculum. In canine, the cadavers preserved using Laskowski solution, the range of motion and flexibility remained unchanged and the mouth could be opened manually during practical training sessions [14].

Good to very good color and texture (mean scores, 4 to 5) confirmed appropriate tissue preservation. Prior to this study, the Larsen solution was thought to be the best alternative for natural color and texture retention [15]. Specimens prepared using the Laskowski solution have been described as dark and deep red [14,21]. Ideally, specimens should convey a similar sense of tissue resistance or fragility and reflect anatomical relationships and difficulties associated with different surgical approaches.

Peeling of the oral mucosa and tongue was detected on gross and histological examination. Similar changes have been described in the skin of dog cadavers preserved using the Laskowski solution [14]. As to the remaining physical and organoleptic characteristics, degenerative and sensory changes were not correlated with histological findings.

Skin, oral vestibule, and masseter muscle tissues were deemed well preserved at all time points. This is a positive factor in the training high-complexity procedures such as molar tooth extraction, as well as ancillary procedures such as tooth extraction via the minimally invasive transbuccal approach and interdental screw placement.

Epithelial lining detachment in oral vestibule samples is consistent with mucosal degradation, vacuolization, and detachment of large mucous membrane cells reported in porcine bladders submitted to alcoholic fixation [4].

Anatomical specimens were successfully preserved and retained appropriate gross and histologic characteristics for teaching activities. Physical characteristics (color, texture, odor, and flexibility) observed in this study support the prolonged use of preserved cadaveric head specimens in teaching. Consistency between gross characteristics and histologic findings indicates appropriate specimen preservation.

SUPPLEMENTARY MATERIALS

Supplementary Table 1
Composition of fixative solution

Supplementary Table 2
Specimen No. 4: mean scores assigned to physical and organoleptic characteristics at different time points

Supplementary Table 3
Specimen No. 5: mean scores assigned to physical and organoleptic characteristics at different time points

Supplementary Table 4
Specimen No. 6: mean scores assigned to physical and organoleptic characteristics at different time points

Supplementary Table 5
Specimen No. 1: mean scores assigned to physical and organoleptic characteristics at different time points

Supplementary Table 6
Specimen No. 2: mean scores assigned to physical and organoleptic characteristics at different time points

Supplementary Table 7
Specimen No. 3: mean scores assigned to physical and organoleptic characteristics at different time points

Supplementary Fig. 1
Specimen No. 3 right after fixative solution injection (D0). Eyelid and supraorbital swelling (A), submandibular swelling (B), large swelling of lips and labial commissures (C), swelling and enlargement of the tongue (D).

Supplementary Fig. 2
Macroscopic appearance of specimen No. 4 on D30. Note excellent tissue preservation at areas exposed during specimen collection (A), good overall specimen appearance (B) and appropriate structure and color of the masseter muscle at the time of sample collection (C).

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