ORIGINAL ARTICLE

Estimation of time since death based on light microscopic, electron microscopic, and electrolyte analysis in the gingival tissue

Sivagami Muthukrishnan, Malathi Narasimhan, Sampath Kumar Paranthaman[1], Thamizhchelvan Hari[2], Pushpa Viswanathan[3], Sharada T. Rajan[2]

Department of Oral Pathology and Microbiology, Faculty of Dental Sciences, Sri Ramachandra University, Departments of [1]Forensic Medicine and Toxicology and [2]Oral Pathology and Microbiology, Sri Ramachandra Medical College, Sri Ramachandra University, [3]Department of Electron Microscopy, Cancer Institute, Chennai, Tamil Nadu, India

Address for correspondence:
Dr. Sivagami Muthukrishnan, Department of Oral Pathology and Microbiology, Faculty of Dental Sciences, Sri Ramachandra University, Porur, Chennai - 600 116, Tamil Nadu, India.
E-mail: sivagamibds@yahoo.co.in

Abstract

Background: Estimation of time since death is an important parameter in forensic science. Although there are various methods available, precise estimation is still to be established. **Aim:** The present study aimed to evaluate the histological and ultrastructural changes in the gingival tissue along with the changes in electrolyte levels (sodium, potassium, calcium, and magnesium) among the three groups which included normal, 2, and 4 h since death. **Materials and Methods:** For light microscopic examination and electrolyte analysis, five normal gingival tissue samples were collected from patient following impaction procedure and five gingival tissue samples were obtained from postmortem specimen at 2 and 4 h since death. Each sample was divided into two parts. The first part was fixed in 10% formalin solution for the light microscopic analysis, and microscopic changes were observed between the groups. The second part was snap frozen at −80°C, until measurement of electrolyte using inductively coupled plasma-optical emission spectrometer, and the values were compared among the groups using Kruskal–Wallis test. For electron microscopic examination 2 and 4 h postmortem, gingival tissue samples were collected from the same individual and immediately fixed in 2.5% buffered glutaraldehyde, and the ultrastructural changes were compared with the normal gingival tissue. **Results:** The light microscopic changes were observed as early as 2 h since death, but there was no significant difference observed between 2 and 4 h postmortem samples whereas ultrastructurally significant difference in morphology was observed between 2 and 4 h postmortem gingival tissue. Our results can confirm histomorphological changes within 2 and 4 h since death.

Key words: Electrolyte, gingival tissue, histological, postmortem changes, ultrastructural

Introduction

Postmortem interval (PMI) is the time between death and the attempt to determine time since death.[1] Determination of time since death is not only important in criminal cases but also in civil cases where they have legal

Access this article online	
Website: www.jfds.org	Quick Response Code
DOI: 10.4103/jfo.jfds_36_17	

How to cite this article: Muthukrishnan S, Narasimhan M, Paranthaman SK, Hari T, Viswanathan P, Rajan ST. Estimation of time since death based on light microscopic, electron microscopic, and electrolyte analysis in the gingival tissue. J Forensic Dent Sci 2018;10:34-9.

implications in issue of insurance and inheritance.[2,3] The main objectives for estimating time since death is to give a primary idea of time of assault to the police and to check whether it is reliable with the alibi of the suspect.[4]

The estimate of the time since death by scientific methods after the first 24–48 h is determined by routinely applying conventional methods of corpse examination and detecting the postmortem changes.[5] Despite its great importance, precise estimation of time of death is a persistent problem in forensic science.[6] This is because the postmortem changes are inevitable and vary depending on a number of intrinsic and extrinsic factors. A very precise but unreliable result might mislead and alienate the police investigations. A variable number of changes based on duration and circumstance of demise occur in the body after death.[7] Cells die by the process of enzymatic digestion. Dissolution of cells and tissues becomes apparent microscopically.[8]

The present study was conducted to assess the cellular changes in gingival tissue at specific time intervals since death. However, there are studies that correlate the degree of histological changes with PMI in oral mucosal tissue, but perhaps, there are no ultrastructural studies done so far. The present study was perhaps first to assess the ultrastructural changes and electrolyte variation in the gingival tissue in order to determine the time since death.

Materials and Methods

The study was conducted on the gingival tissue obtained from deceased individuals. Death due to unnatural reasons such as poisoning and suicide except road traffic accidents were not included in the study. This study was reviewed and approved by the Institutional Ethical Committee, and the consent was obtained from the relatives.

The study material consists of three groups: (for light microscopic examination and electrolyte analysis).

- Group I: Five normal tissues (control tissue) from clinically healthy individual following impaction procedure
- Group II: Five gingival tissue samples obtained from unrefrigerated postmortem specimen at 2 h since death
- Group III: Five gingival tissue samples obtained from unrefrigerated postmortem specimen at 4 h since death.

Each sample was divided into two parts: The first part was fixed in 10% formalin solution for the light microscopic analysis. The second part was weighted and immediately frozen at −80°C, until measurement of potassium (K), sodium (Na), magnesium (Mg), and calcium (Ca).

For electron microscopic examination 2 and 4 h postmortem, gingival tissue samples were collected from the same individual. Ultrastructural changes were compared with the normal gingival tissue which was obtained from clinically healthy individual following impaction procedure.

Light microscopic analysis

Gingival tissue samples were fixed in 10% formalin solution and subjected to routine processing. The embedded tissue was then excised into 4-micron thickness which was fixed on slides and stained with hematoxylin and eosin. The autolytic changes were then observed under light microscope.

Electrolyte analysis

Wet weight of tissue was used for the determination of K, Na, Mg, and Ca contents in each gingival tissue sample. Samples were placed in a Teflon container and predigesting was done using concentrated nitric acid for 45 min followed by microwave digestion first set at a temperature of 20°C and slowly the temperature was raised to 190°C for 1 h, and then, the solution was transferred to a glass container and placed over the hot plate to boil until the sample quantity was reduced to half. The solution was transferred to 10 ml volumetric flask and diluted to the mark in the flask with deionized water. Standard solution for each electrolyte was prepared and measurement was done using inductively coupled plasma-optical emission spectrometer (ICP-OES EXPERT II).[9]

Electron microscopic examination

The sample was taken from a 34-year-old male who died from a road traffic accident. Gingival tissue sample was taken at a PMI of 2 h and 4 h and immediately fixed in 2.5% buffered glutaraldehyde overnight at 4°C followed by fixing with osmium tetroxide. Tissue bits were subjected to dehydration in ascending grades of acetone and then embedded using Epon 812 resin mixture. The tissues were sectioned using Leica Ultracut R Ultramicrotome with diamond knives. The ultra-thin sections were stained with uranyl acetate and lead acetate. These sections were screened in JEOL JEM 1400 Transmission Electron Microscope at 80 kV. The micrographs were taken using the Olympus Keen view CCD camera attached to the microscope.

Results

Light microscopic examination

On comparing control and postmortem samples at different time intervals (2 and 4 h), there were significant changes observed.

Group I showed the normal gingival tissue with keratinized epithelium having four distinct strata.

The following changes were observed in Group II and Group III samples [Figures 1 and 2].

- Homogenization and increased eosinophilia were predominant in the granular and spinous layer

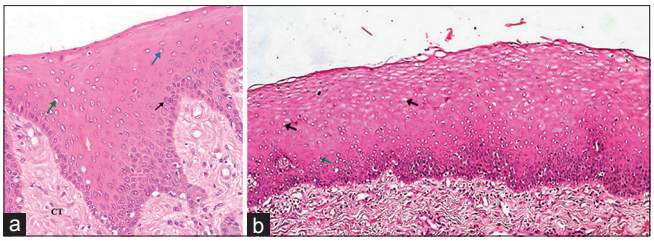

Figure 1: (a) Postmortem changes in gingival tissue at 2 h showing H and E, ×40: Homogenization and eosinophilia (yellow arrow) and nuclear change karyorrhexis (blue arrow) and chromatin clumping (black arrow). (b) Postmortem changes in gingival tissue at 2 h: H and E, ×10 showing cytoplasmic vacuolation throughout the epithelium (arrows)

- Cytoplasmic vacuolation was evident throughout the epithelium
- Chromatin clumping was seen predominantly in the spinous cell layer
- Pyknosis, characterized by nuclear shrinkage and increased basophilia, was predominantly observed in the spinous cell layer
- Karyorrhexis, fragmentation of the nucleus, was seen throughout the epithelium predominantly in the granular and spinous layer
- Few cells in the granular and spinous cell layer showed nuclear vacuolation
- Few samples showed disruption in the superficial layer and loss of desmosomes in the superficial layer and in focal areas in the spinous cell layer.

In light microscopy, there were not much significant changes seen between Group II and Group III samples. Hence, ultrastructural studies were carried out to find any significant changes exist between 2 and 4 h since death.

Electrolyte analysis

The mean and the median values were calculated and compared among the groups. Sodium and calcium levels were decreased in Group II and Group III samples when compared to Group I sample. Potassium and magnesium levels were increased in Group II and Group III samples when compared to Group I sample. The comparison of the median value in tissue electrolyte value for sodium, potassium, calcium, and magnesium among the various groups was analyzed by Kruskal–Wallis test (Hintze J (2006), NCSS, PASS, and GESS, Utah) which was statistically significance ($P < 0.05$) for sodium and potassium. Hence, intergroup comparison was carried out using Kruskal–Wallis multiple comparison Z value test for sodium and potassium. $Z > 1.9600$ was considered significant. Statistical significance was observed between Group I and Group III for sodium and between Group I and Group II for potassium [Table 1].

Transmission electron microscope analysis

Ultrastructure of the normal gingival epithelium showed compact arrangement of cells with intact desmosomes.

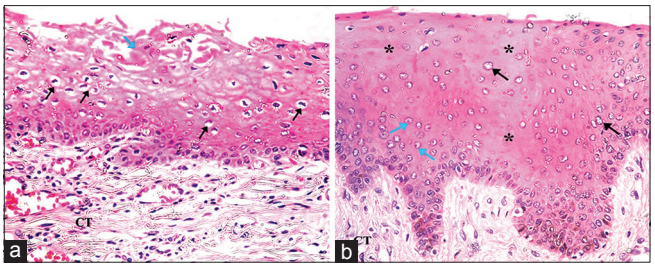

Figure 2: (a) Postmortem changes in gingival tissue at 4 h shows H and E, ×20 View: pyknotic nucleus (black arrow), cytoplasmic vacuolation, disruption in the superficial layer (blue arrow). (b) Postmortem changes in gingival tissue at 4 h. H and E, ×20 View: showing loss of desmosomes. Nuclear changes such as karyorrhexis (blue arrow) and karyolysis (black arrow) with chromatin condensation. Homogenization increased in the epithelium (*)

Table 1: Electrolyte analysis

Electrolyte	Groups	Mean (SD) (mg/kg)	Median (mg/kg)	*P*
Sodium	Group I	1831.6 (238.6)	1780	0.02
	Group II	1685 (474.1)	1484	
	Group III	1327.2 (154.1)	1301	
Potassium	Group I	712 (375)	584	0.02
	Group II	1530.8 (274)	1479	
	Group III	1315 (480)	1131	
Calcium	Group I	2170.4 (1394.6)	1932	0.07
	Group II	747 (578.7)	601	
	Group III	1041 (866.4)	825	
Magnesium	Group I	116.3 (40.4)	128	0.09
	Group II	216.6 (119.5)	178	
	Group III	135.5 (27.6)	137	

SD: Standard deviation

The cytoplasm of the basal and spinous cells contained cytoplasmic granules interspersed between loosely woven bundles of tonofilaments. Tonofibrils were relatively more in the spinous layer when compared to the basal layer. Mitochondria were seen around the nuclei in the basal and spinous layer and very few in the granular layers. Nucleus is rounded with nucleolus placed in center with dispersed chromatin. Rough endoplasmic reticulum was intact with ribosomes [Figure 3].

The following ultrastructural alterations were observed in the gingival tissue 2 h and 4 h since death. In 2 h tissue, lysis of desmosome was observed in the granular layer and prickle cell layer was intact. Nucleus with nucleolus was observed, and in few cells, nucleolus was absent. In few nucleuses, there was lysis of the inner nuclear membrane with peripheral condensation of chromatin. Numerous mitochondria with intact outer membrane and lysis of cristae were observed. Endoplasmic reticulum was lysed and slightly swollen, and thus, intracellular free ribosomes were increased. Numerous lysosomes were observed in intercellular areas and they are highly dense [Figure 4].

In 4 h tissue, lysis of desmosome was observed throughout the epithelium along with basement membrane disruption. Nucleus with nucleolus was observed, and in few cells, nucleolus was absent. In few nucleuses, there were crenations in the nuclear membrane and also disruptions were observed. Peripheral condensation of chromatin was present. The number of mitochondria was reduced along with lysis of the outer membrane. Endoplasmic reticulum was lysed and observed in small bits, and thus, intracellular free ribosome level was increased. Autophagic vacuole with dissolution of mitochondria and cytoplasmic organelles was evident [Figure 5].

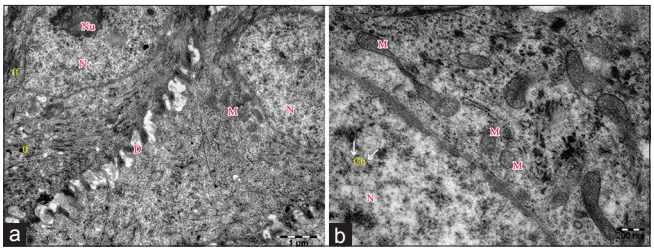

Figure 3: (a) Transmission electron micrograph of normal gingival epithelium showing intact desmosomes (D) with intact nucleus (N) and nucleolus placed in center (Nu) and mitochondria (M) present adjacent to nucleus (×700). (b) Transmission electron micrograph of normal gingival epithelium showing intact nucleus with dispersed chromatin (Ch) and numerous mitochondria (M) (×80,000)

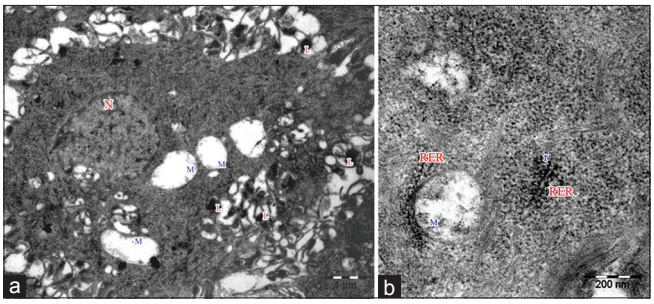

Figure 4: (a) Transmission electron micrograph of 2 h postmortem gingival tissue showing numerous swollen mitochondria with lysis of cristae (M) and numerous lysosome present at intercellular junction (L). Nucleolus absent within the nucleus (N) ×1000. (b) Transmission electron micrograph – Postmortem gingival tissue at 2 h shows rough endoplasmic reticulum lysed and slightly swollen and plenty of free ribosomes (r) ×3000

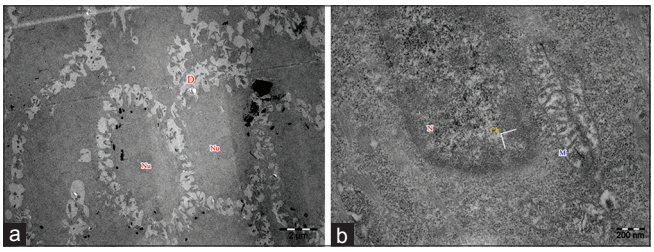

Figure 5: (a) Transmission electron micrograph – Postmortem gingival tissue at 4 h shows lysis in prickle cell layer and lysis of cytoplasmic organelles (×600). (b) Transmission electron micrograph of 4 h postmortem gingival tissue showing peripheral condensation of chromatin (Ch) and pleomorphic mitochondria (M) along with plenty of free ribosomes (r) (×3000)

Discussion

Estimation of time since death is an important part of medicolegal investigations. PMI is defined as "amount of time that has elapsed since the death of the decedent."[10]

Postmortem decomposition follows the arrest of biochemical process that develops, maintains, and preserves the integrity of cellular element. During decomposition, the tissue components leak and break up releasing hydrolytic enzyme. Decomposition may vary from individual to individual, from environment to environment, and even from one part of body to another part of same individual. Process of autolysis is temperature dependent and occurs earlier in some tissue which has high level of hydrolytic enzymes such as the pancreas whereas delayed in fibrous tissue such as uterus or skeletal muscles which have less amount of hydrolytic enzymes and few lysosomes.[11-13]

Like other tissue cells, oral mucosa also loses its normal morphology as a result of postmortem autolysis and putrefaction. The process from normal morphology to different degrees of cellular changes can be a useful criterion for estimating the postmortem interval.[14] However, there are studies that correlate the degree of histological changes in postmortem oral mucosal tissue, but there are no ultrastructural studies. The present study is a unique one done with a shorter PMI (2 and 4 h).

In our study, the histopathological section using light microscope was viewed to analyze the cytoplasmic and nuclear changes in the gingival tissue.

Cytoplasmic changes were as follows:

- Homogenization and eosinophilia in the granular and spinous cell layer. These changes were observed in 2 and 4 h since death. These changes might be attributed due to increased binding of eosin to denatured cytoplasmic proteins and loss of glycogen particles
- Cytoplasmic vacuolation was observed due to enzymatic digestion of cytoplasmic organelles.

Nucleus also showed prominent changes such as:

- Karyolysis-fading of chromatin
- Pyknosis characterized by nuclear shrinkage and increased basophilia due to condensation of cleaved chromatin observed mainly in the spinous cell layers
- Karyorrhexis observed throughout the epithelium where the nucleus undergoes fragmentation.

With the above-mentioned findings, we could possibly conclude that the postmortem changes started as early as 2 h since death. These were observed in accordance with the result of other authors like Pradeep *et al*. (2009),[14] Yadav (2012),[15] and Gururaj and Sivapathasundaram.[16]

In light microscopy, there were not much significant changes seen between 2 and 4 h since death. Hence, ultrastructural studies were carried out to find any significant changes exist between 2 and 4 h since death.

Ultrastructure of the normal gingival epithelium in this study was observed in accordance with Listgarten[17] and Schroeder and Theiiade[18] studies where ultrastructure of the normal gingival epithelium was investigated.

In this ultrastructural study, there were significant morphological differences between 2 and 4 h postmortem gingival tissue.

The 2 h postmortem gingival tissue showed lysis of desmosomes in the granular layer with increased intercellular space and prickle cell layer was intact. The histological architecture was maintained, but in 4 h postmortem tissue sample, lysis of desmosomes was observed throughout the epithelium. Schaeffer[19] observed the similar change in the corneal epithelium after 3 days since death.

Numerous small spherical-shaped lysosomes were seen in intercellular spaces in 2 h postmortem sample, and they were highly dense which indicated it was in active stage wherein 4 h postmortem sample showed autophagic vacuole in which dissolution of cell organelles was evident. According to the review done by Turk and Turk,[20] there are three major morphologically distinct pathways of cell death and lysosomes have been found to be linked with all of them.

Nuclear membrane in 2 h postmortem tissue showed lysis of the inner membrane whereas 4 h postmortem tissue showed crenation and lysis of the outer membrane in some of the nucleus. Peripheral condensation and slight granularity of chromatin were observed in both the samples (2 and 4 h postmortem tissue). This massive crescent-shaped deposit of chromatin adjacent to the nuclear envelope was caused by minimal breaks of single strands of DNA by specific endonucleases without further proteolytic breakdown. This peripheral condensation of chromatin appeared to be a fairly early change that occurs in the nucleus after irreversible injury leading to cell death.[21] Nucleolus was dislocated in both the samples whereas dissolution of nucleolus was observed in 4 h postmortem gingival tissue. Karadzic *et al*.[22] observed similar changes in human hepatocytes at 6 h since death which was stored at 18°C.

Mitochondria which are considered as powerhouse of cell were found swollen in both 2 and 4 h of postmortem gingival tissue and were numerous in 2 h PMI tissue when compared to 4 h since death. Disruption of mitochondrial membrane and altered shape of mitochondria was evident in 4 h postmortem gingival tissue. Mitochondrial cristae disintegration is the hallmark of oxygen deficit and cell death. In accordance with the study done by Tomita *et al*.,[11] on different organs, shape deformation and progressive reduction of the number of mitochondria as well as vacuolization of sarcoplasm progressively grew with the increase of temperature and with the passage of postmortem interval.

Intracellular cations are important for various functions in epithelial cells. At a cellular level, maintenance of intracellular potassium and sodium ions is important to the physiologic functions of the cell membrane potential. When this system fails, sodium tends to accumulate inside the cells and potassium to diffuse out causing cellular swelling and dilation of the organelles.[23] Since Group II and Group III samples were collected from different individuals, no correlation could be established between these two groups. Hence, further research is required to refer this method.

By comparative analysis of the established changes between 2 and 4 h postmortem gingival sample, we can determine that the morphological changes are directly dependent on the length of postmortem interval.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

References

1. Dimaio V, Dimaio D. Forensic Pathology. 2nd ed. Boca Raton, Florida CRC Press; 2001.
2. Salam H, Shaat E, Aziz M, Moneim Sheta A, Hussein H. Estimation of postmortem interval using thanatochemistry and postmortem changes. Alex J Med 2012;48:335-44.
3. Sachdeva N, Rani Y, Singh R, Murari A. Estimation of postmortem interval from the changes in vitreous biochemistry. J Indian Acad Forensic Med 2011;33:171-4.
4. Madea B. Handbook of Forensic Medicine. 1st ed. Chichester: Wiley-Blackwell; 2014.
5. Poposka V, Gutevska A, Stankov A, Pavlovski G, Jakovski Z, Janeska B. Estimation of time since death by using algorithim in early postmortem period. Global J Med Res 2013;13:17-25.
6. Garg V, Oberoi SS, Gorea RK, Kaur K. Changes in the levels of vitreous potassium with increasing time since death. JIAFM 2004;26:136-9.
7. Krishan VI. Textbook of Forensic Medicine and Toxicology, Principles and Practice. 3rd ed. New Delhi Elsevier; 2005.
8. Dix J, Graham M. Time of Death, Decomposition and Identification. 1st ed. London: CRC Press; 2000.
9. Tavichakorntrakool R, Prasongwattana V, Sriboonlue P, Puapairoj A, Pongskul J, Khuntikeo N, *et al*. Serial analyses of postmortem changes in human skeletal muscle: A case study of alterations in proteome profile, histology, electrolyte contents, water composition, and enzyme activity. Proteomics Clin Appl 2008;2:1255-64.

10. Mathur A, Agrawal Y. An overview of methods used for estimation of time since death. Aust J Forensic Sci 2011;43:275-85.
11. Tomita Y, Nihira M, Ohno Y, Sato S. Ultrastructural changes during *in situ* early postmortem autolysis in kidney, pancreas, liver, heart and skeletal muscle of rats. Leg Med (Tokyo) 2004;6:25-31.
12. Sirbu V, Goldstein D. Cellular autolysis in intrapartum death. Some structural characteristic. Int J Crim Investig eISSN: 2247-0271;1:61-6.
13. Bardale R. Principles of Forensic Medicine & Toxicology. 1st ed. New Delhi: Jaypee Brother P Ltd.; 2011.
14. Pradeep G, Uma K, Sharada P, Prakash N. Histological assessment of cellular changes in gingival epithelium in ante-mortem and postmortem specimens. J Forensic Dent Sci 2009;1:61.
15. Yadav A, Angadi P, Hallikerimath S, Kale A, Shetty A. Applicability of histologic postmortem changes of labial mucosa in estimation of time of death – A preliminary study. Austral J Forensic Sci 2012;44:343-52.
16. Gururaj N, Sivapathasundharam B. Postmortem findings in the gingiva assessed by histology and exfoliative cytology. J Oral Maxillofac pathol 2004;8:18-21.
17. Listgarten M. The ultra structure of human gingival epithelium. Am J Anat 1964;114:49-69.
18. Schroeder H, Theiiade J. Electron microscopy of normal human gingival epithelium. J Periodont Res 1966;1:95-119.
19. Schaeffer EM. Ultrastructural changes in moist chamber corneas. Invest Ophthalmol 1963;2:272-82.
20. Turk B, Turk V. Lysosomes as "suicide bags" in cell death: Myth or reality? J Biol Chem 2009;284:21783-7.
21. Ghadially F. Ultrastructural Pathology of the Cell and Matrix: A Text and Atlas of Physiological and Pathological Alterations in the Fine Structure of Cellular and Extracellular Components. London: Butterworths; 1988.
22. Karadzic R, Ilic G, Antovic A, Kostic Banovic L. Autolytic ultrastructural changes in rat and human hepatocytes. Rom J Legal Med 2010;18:247-52.
23. Kumar V, Abbas A, Fausto N, Mitchell R. Robbins Basic Pathology. 7th ed. London: Elsevier Health Sciences; 2008.