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STUDY OF BIOLOGICAL EFFECTS OF DIFFERENT SOLUTIONS USED AS PRESERVATIVES IN DENTAL AVULSION: AN ANALYSIS *IN VITRO*

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ABSTRACT: *In case of dental avulsion the recommended treatment avulsion is an immediate replantation. Yet when this is not possible, the tooth must be placed in a storage media capable of maintaining the viability of the cells of the periodontal ligament and restores their physiology, what is extremely important for a favorable prognosis. The ideal storage media should be readily available at the time of the accident; it must have an adequate ph and osmolality and should still be able to maintain the vitality and the proper functioning of the cell with minimal toxicity and be able to avoid the presence and proliferation of microorganisms. Hank's balanced salt solution (HBSS) and milk have gained wide acceptance as storage media for avulsed tooth. However, the effect of the media and storage time on the periodontal ligament (PDL) cells involvement in the development of root resorption is still unclear. Among the various medias that have been successfully used, we may find HBSS, milk and propolis. This study aims to assess quantitatively and qualitatively the cytotoxic effects of storage medias, including propolis, coconut water, coconut milk, HBSS, saline with antibiotics and milk, used in cases of tooth avulsion through the microscopic observation of the cellular changes occurred in macrophages. In this study, the exposure of the macrophages to the storage medias used in dental avulsion, allowed the evaluation, through optical microscope, of the cytotoxicity based on the apoptotic behavior of the cells, that were subjected to these medias. Based on the analyses of the results, it may me suggested that the coconut milk was considered the best storage media in case of dental avulsion.*

KEYWORDS: coconut milk, macrophages, dental avulsion, cytotoxicity, apoptosis.

INTRODUCTION

Avulsion of a tooth caused by trauma, and its successful treatment is a challenging situation that a dentist encounters in clinical practice. There is a definitive treatment for the avulsed

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tooth, which depends on many factors. The immediate replacement and replantation of the avulsed tooth is necessary, but it cannot always be accomplished for a number of reasons. Thus, the tooth should be transported in a suitable medium to maintain the cell viability. The storage medium is one of the important factors that will help the dentist in rendering successful treatment for the avulsed tooth. There is a continuing search for an ideal storage medium. With a relatively small frequency among traumatic injuries, ranging from 1% to 16% tooth avulsion is defined as the complete tooth shift its alveolus and can be considered the worst of these traumatic injuries can lead to tooth loss. Falls, collisions, car accidents, sports and fights are just some examples of etiologic factors common to this type of traumatic injury (ANDREASEN & ANDREASEN, 2001).

The recommended treatment in this case is the immediate dental replantation and when this is not possible, you should put it in a conservative solution capable of maintaining the viability of the periodontal ligament cells and restore the physiology of it that is of fundamental importance for a favorable prognosis (LUSTOSA-PEREIRA *et al*., 2006).

Both the tooth of time out of the socket as the type of used storage medium may affect the long-term prognosis of re implanted tooth. The longer the time in which the avulsed teeth remain in a dry environment, the worse the prognosis. For this reason, many more storage media has been studied (ANDREASEN, 1993; CHAMORRO *et al*., 2008).

The ideal storage medium should be easily available at the time of accident must have appropriate pH and osmolality and must still be able to maintain the vitality and proper cell function with minimal toxicity as well as being able to avoid the presence and proliferation of microorganisms. Among various means which have been used successfully found the Hank's balanced salt solution, milk and propolis (TROPE, 2002; MARTIN & PILEGGI, 2004; LUSTOSA-PEREIRA *et al*., 2006).

Bai et al., 2015, reported that the extra-alveolar period significantly affects the prognosis of pulp in immature permanent teeth after replantation. When the extra-alveolar period is more than 30 minutes, the chance of pulp revascularization after replantation is very low, therefore pulp extirpation should be performed. The maintenance of cell morphology after exposure to preservatives means is another item that should be checked carefully. Solutions that have a high level of toxicity can damage the correct regulation and cell function that will directly affect the regenerative capacity of injured site after accident (CHAMORRO *et al.*, 2008).

Dental avulsion is the most severe type of traumatic tooth injuries because it causes damage to several structures and results in the complete displacement of the tooth from its socket in the alveolar bone. The ideal situation is to replant an exarticulated tooth immediately after avulsionbecause the extraoral time is a determinant factor for treatment success and for a good prognosis. However, it is not always possible. The success of replantation depends on a number of factors that may contribute to accelerate or minimize the occurrence of root resorption or ankylosis, among which is the type and characteristics of the medium used for temporary storage during the time elapsed between avulsion and replantation. Maintaining the tooth in an adequate wet medium that can preserve, as longer as possible, the vitality of the periodontal ligament cells that remain on root surface is the key to success of replantation. (Poi, *et al*., 2013).

Given these observations, it is believed to be appropriate to the *in vitro* study of some commonly used preservatives solutions and other recently used and tested, and a mixture of

those who were successful in previous studies to be able to observe the level of toxicity of these substances and their interference or not in the correct cell function which can even lead to cell death.

LITERATURE REVIEW

Healing following avulsion and replantation is dependent on the extent of pulpal and periodontal ligament (PDL) tissue damage. Therefore, immediate replantation is the recommended treatment of choice for an avulsed permanent tooth. To achieve a more favorable prognosis following tooth replantation, use of an appropriate interim transport medium is usually advocated. Numerous studies have researched and advocated the use of media like saliva, milk, Hank's Balanced Salt Solution (HBSS) and ViaSpan. However, current research has indicated the use of few newer media as promising interim transport media for an avulsed tooth.

1) Traumatology, avulsion and resorption

Traumatic lesions in front teeth are frequent accidents affecting many children at school age, 7-14 years old, with intense physical activity and no sense of danger. Most trauma, whether in adults or children, are often caused by falls and collisions or car accidents, sports and fights and 13% of subjects examined at the age of 18 years have been exposed to dental trauma during adolescence. Dental fractures seem more common in the permanent dentition as dislocations and especially intrusions predominate in the primary dentition (ANDREASEN, 1970; ANDREASEN, 1985; ANDREASEN & ANDREASEN, 2001; LUSTOSAPEREIRA *et al.*, 2006). These injuries can affect small parts of the crown or even cause the complete tooth displacement of its socket, featuring one tooth avulsion, which damages both the supporting tissues as the dental pulp and occurs most commonly between 7 and 9 years old due to the greater elasticity of the alveolar bone which generates minimal resistance to the extrusive forces. The role of the avulsed tooth repair after replantation will depend on the potential repair of each cellular component of the tissues involved, the procedure made the replantation and specific individual factors. The incidence of avulsion ranges from 1% to 16% of all traumatic injuries to the permanent dentition and is considered the worst of the dental-alveolar injury. The most frequently avulsed tooth is the maxillary central incisor. The recommended treatment is the dental immediate replantation is the act of replacing the avulsed tooth in its socket; and when this is not possible, you should put it in a conservative solution capable of maintaining the viability of the periodontal ligament cells and restore the physiology of the same. It is considered as an immediate replantation when the stay outside its tooth socket is up to 30 minutes. Thus, the late replantation has become a clinical reality for dentists considering the time the patient arrives at professional for care. When the avulsed tooth is replanted immediately or placed in a solution capable of preserving periodontal fibers attached to the root, the chances of successful replantation increase considerably. Intact restores esthetics and occlusal function soon after the injury and the replantation tooth can keep its function for a few years (CHO & CHENG, 2002; MARTIN & PILEGGI, 2004; LUSTOSA -PEREIRA *et al.*, 2006; ÖZAN *et al.*, 2007; POI *et al.*, 2007; CHAMORRO *et al.*, 2008; COSTA *et al.*, 2014). Avulsion, thus resulting in complete displacement of the tooth socket. In addition, the neurovascular supply is severely compromised, which usually results in loss of vascularization of the pulp. The pulp nutrition is achieved through an arteriole 50 mm in diameter which penetrates through the apical foramen. This arteriole is surrounded by several layers of muscle cells, which protect from breakage. However, any major tooth movement than twice the vessel diameter, can cause its rupture. This could result in pulp necrosis in open and closed summits. After avulsion, the

after replantation success is dependent on the immediately following treatment to injury. The pulp revascularization is started after four days and is usually complete after four to five weeks replanted in immature teeth. Revascularization is rare in mature teeth that have a narrow apical foramen (ANDREASEN, 1993; MARTIN & PILEGGI, 2004; LIN *et al.*, 2007). Resorptions are traditionally classified as inflammatory and replacement, that occurs according to the mechanism for maintenance and evolution. Root resorption is grouped into four main groups that have their own subdivisions (CONSOLARO, 2013).

One of the consequences of tooth replantation and subsequent avulsion is the root resorption and occurrence depends on factors such as extra-alveolar period, conservative solution, microbial contamination and stage of root formation. In 1925, the American Dental Association resorption defined as the loss of products or the bodies own tissues. The root resorption initiated by a mineral or exposed surface area can be extended by mechanical irritation of the tissue of the dentin and root canal infection and certain systemic diseases. Along with additional stimulus resorption cells, it becomes progressive and can lead to the destruction of the root. The sequelae that lead to failure after replantation are inflammatory root resorption, Ankylosis and replacement resorption. The presence of an intact and viable periodontal ligament root surface is the most important factor to ensure the healing of the periodontal ligament without root resorption. The root resorption are the main limiting factor involved in determining the prognosis of transplants and dental replantations (TRONSTAD, 1988; ANDREASEN, 1993; KEUM *et al.*, 2003; MARTIN & PILLEGI, 2004; CONSOLARO, 2005; LUSTOSA PEREIRA *et al*., 2006; MANFRIN *et al.*, 2007; ÖZAN, 2007).

The mineralized tissues of permanent teeth are not normally reabsorbed. They are protected in the root canal by pre-dentin and odontoblasts and the root surface by the pre-cementum and cementoblasts. The pre-dentin, the pre-cementum and the osteoid tissue help protect the mineralized tissues of resorptive action. In traumas occur focal displacements pre-dentin and odontoblast layer. If the pre-dentine or cementum become pre-mineralized or pre-cement is mechanically damaged or lost, multinucleated cells will colonize the surface and unprotected or mineralized bone resorption will occur. Such elimination may be referred to as inflammatory root resorption. This can occur in the wall of the root canal (internal resorption) and the root surface (external resorption) and can be transitory or progressive. Thus, in human's permanent teeth no normal root resorption, always being pathological (TRONSTAD, 1988; CONSOLARO, 2005; CONSOLARO, 2013).

In teeth which have undergone displacement, external root resorption may become extensive. Tooth intrusion or extrusion and subsequent replacement procedures inevitably cause root damage resulting in areas of denuded root surface to be chemotactic for cells in hard tissue resorption. The root resorption will follow. In addition, the tooth displacement leads to the rupture of blood vessels in the apical foramen and ischemic necrotic pulp. Microorganisms will then fill the root canal through the dentin-enamel junction and exposed dentinal tubules and establish an infection in a period of 2-3 weeks. At this time, the root resorption induced denuded areas of the root surface may have exposed tubular root dentin. Bacterial products from infected root canals will occupy the resorption lacunae on the root surface through the dentinal tubules and sustain root resorption (TRONSTAD, 1988; ANDREASEN, 1993; TROPE, 2002). In addition, dislocated teeth, root resorption is initiated by mechanical trauma resulting in removal cementoblasts, pre-cement and cement sometimes in areas of the root surface. With the loss of cementum, the inflammatory response results in root and bone resorption. The process of resorption is then maintained by microbial stimulus from the

infected root canal which provides the required continuous stimulation of cell elimination. After a few weeks the condition can be recognized as radiographically periradicular radiolucent areas, usually involving the root and areas adjacent alveolar bone. If this progress, the resorption process can destroy the tooth completely in a few months. In cases of avulsion the initial damage to the cement layer after trauma is limited. However, if the remaining periodontal ligament cells remain dry at the root, this will cause an inflammatory response throughout the root surface which results in damage to the protective coating of cement. Inflammatory resorption may develop a week after replantation and follows a progressive path, unless the endodontic treatment is done. This type of damage is related to the absorption associated with the inner layers of the periodontal ligament in the root surface and the presence of necrotic pulp tissue infected (ANDREASEN, 1993; TROPE, 2002).

Dental alveolar ankylosis occurs after extensive necrosis of the periodontal ligament to bone formation in the area of bare root surface. Clinically, this condition is seen more often as a complication to injury with dislocation, especially in avulsed teeth were out of the mouth for a long enough period to the cells of the root surface to dry and die. If less than 20% of the root surface is involved, the reversal of ankylosis may occur. If not, the ankylosed tooth is embedded in the alveolar bone and becomes part of the normal process of bone remodeling. Consequently, it will be gradually resorptions and replaced by bone, hence the term replacement resorption (TRONSTAD, 1988). The resorption of cells in replacement resorption are osteoclasts normally involved in bone remodeling. However, despite the replacement resorption lead to complete destruction of the tooth, must not be understood as a disease process. This occurs as an "error" by the cells involved in bone remodeling are not able to distinguish between cementum, dentin and bone. The osteoclasts will resorb the tooth tissue in the same way as resorb bone, and are not able to form dentin or cementum, osteoblasts replace the reabsorb areas of bone root. If cementoblasts continue to cover the damaged root surface, the healing process will occur and the result will be favorable. On the other hand, if osteoblasts cover the root surface, the conditions for healing will occur and unfavorable ankylosis. Ankylosis can be demonstrated histologically two weeks after replantation (TRONSTAD, 1988; TROPE, 2002).

During avulsion and subsequent replantation is the injury of the periodontal ligament with cell necrosis resulting in injury repair processes where the necrotic periodontal ligament is removed by macrophages or cementum by osteoclastic activity. The latter will lead to the elimination of surface or inflammatory depending on the state of the pulp, the patient's age and stage of root development. Inflammatory resorption can be demonstrated histologically one week after replantation and the root is more common in immature teeth and replanted teeth mature young mature than in older teeth. When large areas are traumatized periodontal ligament, competitive wound healing begins between cells derived from bone marrow bone and intended to form programmed cell derived from periodontal ligament to form the fibers of the ligament and cementum. The result of this competition can be a ankylosis transitional or permanent. The absence of ERM in the periodontal ligament resection for extended extra-alveolar periods can lead to the occurrence of ankylosis because he is responsible for maintaining the periodontal space (ANDREASEN, 1993; LUSTOSA-PEREIRA *et al*., 2006).

Andreasen (1970) studied the etiology and pathogenesis of traumatic dental injuries by collecting 1298 patients of a hospital data (908 men and 390 women). A total of 3026 traumatized teeth were treated, including 787 primary teeth and permanent teeth in 2239. Repeated dental injuries were found in 24% of cases. All injuries were classified according to the type of injury affects lips, oral mucosa, dental support structures and hard dental tissue.

The type of trauma appears to be related to the teeth with dental trauma involving structures support predominantly in the primary dentition. The source of the trauma was distributed into 9 groups, according to the impact energy and resistance. Statistical analysis revealed significant differences in the causes of injuries among different groups of trauma. The relationship between injuries on the lips and injuries to the teeth or supporting structures were analyzed separately. It seems through this analysis that the lip can act absorbing the impact, reducing the chance of coronary fracture and increasing the risk of dislocation and fracture of the alveolar process.

Andreasen & Ravn (1972) studied the epidemiology of traumatic injuries in the primary and permanent dentition in a sample of the Danish population consisted of 487 children, with 251 boys and 236 girls, aged 9 and 17 years. 30% of children have experienced trauma in the primary dentition, while 22% suffered trauma in the permanent dentition. The boys have experienced trauma more often in the permanent dentition compared to girls, while in the primary dentition was found very little difference. Individuals showing trauma in the primary dentition did not show a significantly higher frequency of injuries in the permanent dentition when compared to those without a history of trauma in the primary dentition. The annual incidence of injury was determined for the examined population. Among boys, the highest incidence occurred in the following age groups: 2-4 years and 9-10 years. In girls only seen a high incidence in the age group of 2-3 years.

Anderson *et al.* (2006) studied the degree of knowledge of students from Kuwait, the emergency measures to be taken in the event of dental avulsion. Altogether 221 students were interviewed. We evaluated the knowledge of principles of treatment in trauma, avulsion and tooth replantation, permanent tooth avulsion and deciduous avulsed tooth cleaning before replantation, extra-alveolar period and preservative. A child older than 10 years had a greater knowledge of the management of trauma, but regardless of age, on the avulsion, replantation, extra-alveolar period and preservative was low. Costa *et al.* (2014) conducted a study with 47 educators from public daycare centers in order to evaluate the knowledge towards the dental trauma in childhood. In both cases it can be concluded that knowledge generally is low which could affect the prognosis of patients with trauma.

Lustosa-Pereira *et al*. (2006) assessed the efficacy of alendronate sodium to avoid the occurrence of resorption of avulsed teeth. Fifty-four maxillary central incisors of rats rights were divided into three groups with extra-alveolar periods of 15, 30 and 60 minutes. Alendronate sodium has been used as a topical substance in the treatment of the root surface. The results indicated that the sodium alendronate was effective in reducing the incidence of root resorption, but not the tooth ankylosis. There was no significant difference between the extra-alveolar periods.

Poi *et al.* (2007) assessed the changes in the periodontal ligament healing replanted teeth after the use of Emdogain®. The central incisor 24 Wistar rats were extracted and left to dry for 6 hours. After that, the dental papilla and each tooth enamel organ was sectioned for pulp removal retrograde and the canal was irrigated with sodium hypochlorite 1%. The teeth were divided into two groups: in group I, the root surface was treated with 1% sodium hypochlorite for 10 minutes (by changing the solution after 5 minutes), rinsed with saline solution and immersed for 10 minutes in sodium phosphate fluoride acidulated 2% for 10 minutes; in group II, the root surfaces were treated in the same manner as described except by applying Emdogain® instead of sodium fluoride. The teeth were filled with calcium hydroxide and replanted. All animals received antibiotic therapy. The rats were killed by anesthetic overdose

10 and 60 days after replantation. The pieces containing the replanted teeth were removed, fixed, decalcified and embedded in paraffin. Serial sections of 6μ were obtained and stained with hematoxylin and eosin for histological and histometric analysis. It was observed that the use of acidic sodium phosphate fluoride generated more areas of resorption and substitution by the use of more areas. Emdogain® resulted in ankylosis and was not able to prevent dental alveolar ankylosis. It can be concluded that neither the acidulated phosphate fluoride nor sodium Emdogain® neither were able to prevent root resorption in later replanted teeth of rats.

Santos (2010) had the aim to prevent and / or delay the installation of root resorption and preserve the integrity of the cement layer is proposed to study the repair process in teeth of rats delayed replantation after using the papain solution and sodium fluoride treatment root surface. Rights upper incisors 40 rats were divided into 4 groups, in Group I the teeth were immediately replanted in their respective sockets. In group II, III, IV, extracted teeth were kept dry for 1 hour period. In group II, the teeth were immersed in a solution of papain at 50% for 20 minutes, rubbed with gauze and immersed for 1 minute in a sodium fluoride 2% acidulated phosphate, pH 5 at 20 minute period. In group III, the teeth were stored in saline solution for 20 minutes, root surfaces were rubbed for 1 minute, immersed in sodium fluoride 2% acidulated phosphate, pH 5, for a period of 20 minutes. In group IV, the teeth were replanted in their respective sockets, without any treatment the root surface. After replantation, the animals received systemic antibiotic therapy and 60 days later were euthanized. The results showed a higher incidence of ankylosis in group II compared to group I. The replacement resorption committed more group IV teeth when compared to groups I and II. When the sum of resorbed areas was seen, it was observed that the teeth of group IV were absorbed more than the teeth in group I and group II. The results show that the root surface treatment with papain solution and sodium fluoride may be an option which will be replanted into the teeth.

Buck *et al.* (2013) examined clinically and radiographically using the combination of calcium hydroxide, 2% *Chlorhexidine* gel and zinc oxide as the obturator folder without periodic replacement in replanted teeth. Single-rooted teeth of 18 patients were examined, of both genders, aged 7-25 years. The average length of stay of obturator folder was 2 years and was no clinical and radiographic signs before and after insertion into the root canals. Were studied: presence of spontaneous pain, pain on vertical and horizontal percussion, pain on palpation, infraposition, presence of the hard layer, inflammatory root resorption and replacement, areas of periapical radiolucency and the insolubility of the obturator folder after a period of up to 4 years without periodic exchanges. It was found that there was a statistically significant difference from the spontaneous pain, percussion and palpation results also showed a significant reduction in peri root radiolucency and inflammatory resorption. It follows that the obturator binder can be an alternative for the treatment of teeth were replanted.

Costa *et al.* (2014) analyzed the knowledge of the public day care educators about what behaviors would take against the dental trauma in childhood. A questionnaire to 23 educators was applied to evaluate the approach in the emergency situations of dental trauma in school, Data analysis, was performed using the statistical program SPSS version 18.0. It was observed that most educators (91.3%) was not qualified and not being able to make the first call in case of dental trauma. Pertaining to the dental avulsion 60.9% of Educators do not know what to do in case of avulsion of the permanent tooth and 40.3%, of deciduous. Before the avulsed tooth, 26.1% would wash the tooth briefly with tap water, would store in a

napkin, handkerchief or cotton (21.7%) and would take the child to the Dental Surgeon. Front tooth crown fracture, 34.8% would keep the fractured piece, while 39.1% would not know how to proceed. Most educators are not prepared to deal with dental trauma in childhood, with little or no knowledge about.

2) Conservative solutions after avulsion

In most clinical cases prior to replantation of the avulsed teeth have been stored in the oral cavity or other means, such as saline or tap water. Since these storage media differ considerably, especially in relation to the concentration of electrolyte, it can be assumed that the choice of storage medium can influence the pulp wound healing and the development of different types of root resorption (ANDREASEN, 1993). The means of physiological storage is one of the key factors for the successful treatment of avulsed teeth. An ideal medium should be moist, contain glucose, calcium and magnesium ions, pH and osmolarity have compatible. Able to preserve viability and mutagenicity of damaged periodontal ligament in order to facilitate placement of the new denuded root surface preventing root resorption (ANDREASEN, 1993; PEIXOTO *et al.*, 2013). In 1994, the American Association of Endodontists recommended Hank's balanced salt solution as the medium of choice for avulsed teeth. Found a strong relationship between the dry storage and storage in non-physiological means such as tap water and periodontal healing and root resorption (ANDREASEN, 1993).

Poi *et al.*, (2013) reported that there is no single product or solution able to preserve the vitality of the cells and cellulose, pH and osmolarity, clonogenic capacity, antioxidant properties, or no minimum microbial contamination, high availability, accessibility ready in accident sites, homes, schools, and hospitals dental offices, and low cost and that, so far, as well as solutions specifically designed for storage and culture, regular pasteurized milk is the most suitable and the best prognosis among other solutions that may be available on the site of an accident, such as water, saline or saliva.

It seems that the pulp revascularization depends on the storage conditions. If the avulsed teeth are stored damp, revascularization occurs in approximately one third of cases stored up to three hours. Grafting occurs after dry storage, in general, in about half the cases, when the storage period is less than five minutes. Then the revascularization rate drops to about one-third, from six to twenty minutes and then continues to decrease with increasing periods in which the tooth is dry. Moreover, it has been noticed that revascularization occurs only in the teeth with an apical foramen diameter exceeding one millimeter (ANDREASEN, 1993; CHAMORRO *et al.*, 2008).

Both the tooth of time out of the socket as the type of used storage medium may affect the long-term prognosis of replanted tooth. The goal of treatment of the patient who suffered tooth avulsion is the immediate replantation, but as this is not always possible, put the tooth in solutions that preserve the vitality of the periodontal ligament cells helps prevent inflammatory root resorption and replacement resorption. The remaining periodontal ligament in root after injury, is dependent on a supply of vital metabolites, otherwise the cellular destruction begins. To preserve the optimal cellular metabolism, the supply must be renewed after 60 minutes from the time of injury. If the cells survive, they will promote the reproduction of new cells which can differentiate and recover the supporting tissues (TROPE, 2002; MARTIN & PILEGGI, 2004; LIN *et al.*, 2007).

Conservative solutions such as milk, for example, favor the successful replantation procedure, while leaving the tooth in an echo prediction significantly compromising the environment. Replanted teeth 30 minutes show a success rate better than those with a longer period extra-oral two hours and the dry cell is not found in the periodontal ligament life. Many methods have been suggested to preserve the vitality of the cells of the periodontal ligament to the tooth vary from place under the patient's tongue by means such as milk, saline solution or even more suitable Hank's balanced salt solution. The saliva showed worse than milk because of its low osmolarity, high risk of bacterial contamination, while preserving the vitality of periodontal ligament cells for up to 2 hours. Tap water, saliva and saline are all ineffective in maintaining the viability of the cells of the periodontal ligament. These means are not recommended by their hypotonic properties (tap water and saliva) and high incidence of bacterial contamination leading to rapid cell death of the periodontal ligament. Some studies have begun to suggest the use of propolis as a conservative means of the avulsed tooth (TROPE, 2002; MARTIN & PILEGGI, 2004; ANDERSON *et al.*, 2006; POI *et al.*, 2013). Propolis is a resinous product bee hive with antibacterial and anti-inflammatory, antioxidant, anti-fungal, anti-viral and repairing tissues, among others. Oxygen radicals and oxygen tension modulate activity of osteoblasts and osteoclasts. Oxidative damage can promote resorption of the root surface by toxic effects on the cells of the periodontal ligament or cementum damaged mechanically or by increasing the resorption activity of clastic cells. It has been suggested that placing the avulsed teeth in a medium containing one or more antioxidants may increase the success of replantation. Propolis has a great antioxidant capacity. The most important components of propolis are flavonoids that are powerful antioxidants. Antibiotics show favorable effects both topically and systemically to prevent the resorption after replantation. Penicillin reduces the occurrence of tetracycline resorption and has properties that act against resorption in addition to its antimicrobial effects. The root surface treatment with stannous fluoride solution at 1% and also reduces reabsorption tetracycline. As propolis has antimicrobial activity, this makes it a favorable preservation medium. The milk has been studied extensively and has gained acceptance as a means capable of maintaining the viability of the cells of the periodontal ligament probably due to physiological osmolarity which is not overly harmful to these cells, the presence of neutral pH and nutrients. Its fat content affects this viability, and milk with low fat content more appropriate. It is a practical way and found near most of the sites of accidents (TROPE, 2002; ANDERSON *et al.*, 2006). American Association of Endodontists (1995) accept milk as conservative solution for avulsed tooth transport. The Hank's balanced salt solution (HBSS) normal saline is widely used in biomedical research to support the growth of various cell types. It has no toxicity, shows ideal osmolality, pH is unbalanced and contains many essential nutrients, preserving the vitality of the fibroblasts by 72 hours. A conservative solution for dental transport using HBSS was developed and marketed under the name Save-A-Tooth (Save-A-Tooth Inc., Pottstown, PA, USA), but is not yet available in pharmacies or drugstores in Brazil. ViaSpan is a conservative medium used in the transportation of organs, but has a shelf life of only a few months along with their high cost makes its rare use. A container called Dentosafe® was introduced and distributed in Germany and Austria schools. (TROPE, 2002; POHL *et al.*, 2005; MARTIN & PILEGGI, 2004; LUSTOSA-PEREIRA *et al.*, 2006; ÖZAN *et al.*, 2007; LIN *et al.*, 2007).

Blomlof (1981) preservatives studied different ways in the case of tooth avulsion. The periodontal ligament cells survived well in milk, 50% of vital cells showed after 12 hours of exposure, while no viable cells were found after 3 hours storage saliva. Brief storage in saliva followed by storage in milk was better than the store only in saliva.

Blomlof *et al.* (1983) evaluated the periodontal conditions after 8 weeks, having been exposed teeth of monkeys endodontically treated milk and saliva for 2 to 6 hours. The teeth stored in milk by 2:06 hours and teeth stored in saliva for 2 hours showed periodontal repair nearly as good as the teeth replanted immediately. The teeth stored in saliva for 6 hours or left to dry showed extensive replacement resorption. The milk can therefore be recommended as a preservative for avulsed teeth.

Marino *et al.* (2000) assessed the ability of long-life milk to serve as a preservative solution for avulsed teeth cell viability by maintaining the periodontal ligament exposed to this medium. Cells were plated in culture medium for 24 hours, then medium was replaced with pasteurized milk (cooled), long-life milk or Save-A-Tooth disease. The plates were incubated at 37°C for 1, 2, 4 or 8 hours. After eight hours the viability of the periodontal ligament cells in pasteurized milk and UHT milk was significantly higher than in the Save-A-Tooth. There was no statistically significant difference between milk. These results suggest that the long-life milk, which has the advantage of not requiring refrigeration, is as effective as pasteurized milk and more effective than the Save-A-Tooth.

Schwartz *et al*. (2002) examined the effects of temperature retention some teeth means during periods of time of packaging variables on the pulp and periodontal ligament repair after tooth replantation in monkeys. Lower incisors side with complete root formation were extracted and left to dry at 22, 4 and -18ºC; in saline to 37, 22, 4, and -18ºC; or saliva at 37 for 60 or 120 minutes before replantation. Animals were sacrificed 8 days after replantation and 125 teeth were replanted histometrically examined. The histological parameters were: normal periodontal ligament, surface resorption, inflammatory resorption, replacement resorption, periodontal pocket depth, periapical inflammatory changes and extension of pulp vitality. Packaging saliva at 37 °C showed similar amount of periodontal ligament compared to the normal saline at 60 and 120 minutes. The saline solution for 60 or 120 minutes showed no difference in the extent of the periodontal ligament when the medium was compared to 37, 22 and 4°C. However, the medium at -18°C showed significant less loss of periodontal ligament that means in other temperatures. The dry medium for 60 minutes showed significant lower resorption at 4°C compared to 22°C. The dry medium at -18°C showed significantly lower loss of periodontal ligament that the medium at 4°C. Weather in dry medium 120 minutes, did not differ between 22.4 and 18°C. It is concluded that the high temperature (above 0°C) of the method of preservation is important only for the dry medium and in situations where extra-cellular short periods of time up to 60 minutes and 120 minutes; when extensive destruction of the periodontal ligament has occurred. It is suggested that 4°C may result in less evaporation of the periodontal ligament and less damage their cells. The pulp wound healing in all cases was restricted to the channel input and no pattern was found between the means of storage, time, and temperature.

Martin & Pileggi (2004) an assay performed with collagenase dispase to investigate the potential of a new dental storage means, propolis, maintaining the viability of cells in the periodontal ligament avulsed teeth. Seventy freshly extracted teeth were divided into five groups and two control groups. This study compared two different concentrations of propolis (50% and 100%) to HBSS, saline and milk. The number of viable cells was counted with a hemocytometer and analyzed. Statistical analysis showed that the propolis groups remained significantly more viable periodontal ligament cells when compared to milk, saline or HBSS proving to be a better alternative. The Propolis 100% was not significantly different from Propolis 50%, indicating that the concentration showed no difference in toxicity to the cells

of periodontal ligament. HBSS, physiological serum and milk were not significantly different.

Propolis is a bee-metabolized resinous substance (bee glue) from plant sap and gums. It has been in usage as a healing agent since antiquity, yet has not garnered global popularity as a health promoter. Its biological effects, which range from antimicrobial, antioxidant, anti-inflammatory, antidiabetic, dermatoprotective, anti-allergic, laxative and immunomodulatory to anticancer, have been validated. Propolis has shown efficacy against brain, head and neck, skin, breast, liver, pancreas, kidney, bladder, prostate, colon and blood cancers. The inhibition of matrix metalloproteinases, anti-angiogenesis, prevention of metastasis, cell-cycle arrest, induction of apoptosis and moderation of the chemotherapy-induced deleterious side effects have been deduced as the key mechanisms of cancer manipulation. The components conferring antitumor potentials have been identified as caffeic acid phenethyl ester, chrysin, artepillin C, nemorosone, galangin, cardanol, etc. These compounds target various genetic and biochemical pathways of cancer progression. Depending on the botanical sources and the geographical origin, biological activities of propolis vary (Patel, 2015).

Pohl *et al.* (2005) studied healing after avulsion and replantation. We evaluated 28 permanent teeth in 24 patients. Immediately after avulsion, 6 teeth were placed in a container containing a culture medium and termed Dentosafe® for 1-53 hours and the periodontal ligament was not compromised teeth 16 teeth were left in non-physiological situation temporarily teeth 6 in non-physiological conditions for long periods. In 14 teeth, antiresorptive regenerative therapy (ART) with local application of glucocorticoids and derived from the enamel matrix and systemic administration of doxycycline was used. In all of the teeth was carried extra-oral endodontic treatment via retrograde pin insertion. The teeth were observed for about 31 months. It was observed that the predominant influence on the physiological healing was immediate packaging of the avulsed teeth. So for a good prognosis, the avulsed tooth should be put immediately in a cellular environment compatible. These means should be distributed in accident risk places and the TRA only has the potential to improve the prognosis when the periodontal ligament is not compromised.

Fagade (2005) a literature review with the objective to study the various means used in transplantation and replantation. A literature review with the objective to study the various means used in transplantation and replantation. The most favorable were HBSS, the patient's plasma Eagle culture medium, saline and pasteurized milk.

Özan *et al.* (2007) evaluating the effectiveness of propolis as a means for the temporary preservative maintaining the viability of cells in the periodontal ligament avulsed teeth. Periodontal ligament cells from human third molars were obtained and cultured in DMEM. The four groups were compared: propolis solution of 10% propolis solution of 20% long-life milk with a low fat content, Hank's balanced solution besides tap water as negative control, and DMEM as a positive control. The plates were incubated at 37 °C for 1, 3, 6, 12 and 24 hours. The results indicated that 10% propolis 3, 6, 12 and 24 hours was significantly better than HBSS and milk. In 1 hour there was no significant difference between them. In 1 hour propolis 20% HBSS was worse than the contrast of 3 to 6 hours. Propolis 20% was significantly better than HBSS and milk. At 12 and 24 hours, propolis was 20% better than HBSS only. When propolis solutions were compared, there was no significant difference in 1, 3 and 6 hours. At 12 and 24 hours to 10% propolis was significantly better than 20%. In general, propolis 10% was the most effective way and found that it can be recommended as a suitable means of transport of avulsed teeth.

Chamorro *et al.* (2008) in vitro investigated the degree of apoptosis periodontal ligament cells after different storage conditions. Human periodontal ligament cells were placed in culture media for 24 hours on plates. The cells were then exposed for one hour to milk, Hank's balanced salt solution (HBSS), Soft Wear solution for contact lenses and Gatorade® at room temperature or frozen. Culture medium was used as negative control. The degree of apoptosis was evaluated after 24, 48 and 72 hours of treatment by direct immune fluorescence. Total cell number and the total number of apoptotic cells were counted. The results indicated that at 24 and 72 hours, the treated periodontal ligament and Gatorade® solution for contact lenses showed the highest percentage of apoptotic cells compared to other groups at room temperature. Finally, frozen, treated cells showed significantly lower levels of apoptosis compared with treatment at room temperature. In conclusion, the results indicated that apoptosis plays a major role in cell death in cells treated with Gatorade® and solutions for contact lenses compared to other storage solutions and storage by freezing can inhibit programmed cell death.

Gopikrishna *et al.* (2008) investigated the potential of a new storage medium after tooth avulsion, the coconut water, as compared with propolis, Hank's balanced salt solution (HBSS) and milk in maintaining the viability of the cells of the periodontal ligament (PDL). Seventy freshly extracted human teeth were divided into 4 experimental groups and two control groups. Positive and negative controls were: 0 minutes and 8 hours of the time in a dry environment. The teeth of the experimental groups were stored dried for 30 minutes and then immersed in one of 4 ways (coconut water, propolis, HBSS and milk). The teeth were treated with dispase grade II and collagenase for 30 minutes. The number of viable cells in the periodontal ligament was counted with a hemocytometer and analyzed. Statistical analysis showed that coconut water showed higher significant amount of viable periodontal ligament cells compared with propolis, HBSS or milk. The coconut water can therefore be used as a transport medium for avulsed teeth preservative.

Souza *et al.* (2009) studied the feasibility of human periodontal ligament fibroblasts (FLPH) after 24 hours of contact with skim milk, whole milk, Hank's balanced salt solution (HBSS), Save-A-Tooth®, propolis, egg white and coconut water, 5 and 20°C, and verifying the cell proliferation ability after incubation in Minimum Essential Medium (MEM) at 37°C for 24, 48, 72, 96 and 120 h. Cells maintained in MEM and tap water served as positive and negative control, respectively. The evaluation of the viability and proliferative capacity was performed by means of statistical analysis as the Kruskal-Wallis test and showed that both 5 and 20°C, the skimmed milk and full maintained higher percentage of viable cells and when kept at 5°C gave higher proliferative capacity. When kept at 20°C, cells exposed to HBSS, skim milk and whole milk have shown, for periods of 24 and 48 hours, similar proliferation and higher than that of other media tested. From 72 hours the proliferation of cells maintained in HBSS was maintained higher than that of skim and whole milk. It was concluded that, at both temperatures, the most effective means to maintain cell viability (0h) were skimmed milk and whole milk. When the media were kept at 5˚C, skimmed milk and whole milk showed higher proliferative capacity to FLPH. When kept at 20C in HBSS showed the best results.

Martins (2013) examined the effectiveness of Dragon's Blood sap as a means of storage for avulsed teeth through the measurement of functional cell viability and metabolic. A 10% dilution of Dragon's Blood sap was tested as a control we used the phosphate buffer saline (PBS) ultrapasteurized full milk was used as a comparative medium, positive control used was DMEM and the negative control distilled water. The assessment of viability was made by the exclusion of trypan blue test and the *Tetrazolium*-based colorimetric (MTT) and

determined at times 1, 3, 6, 10 and 24h of incubation. The tests were repeated 4 times in triplicate for MTT. The Dragon's Blood sap showed good results. For the methodology with Trypan Blue, the Dragon's Blood sap was similar to milk, both with the best viability values, and the MTT showed superior results to all media, including milk, then the Dragon's Blood sap was as effective as milk, semi traditionally used for storage of avulsed teeth, showing a good performance while maintaining the integrity of the membrane of different cell types and functional viability of the cells of the periodontal ligament.

3) Cellular reactions before certain substances

The function of the normal cell requires a balance between physiological requirements and limitations of cellular structure and metabolic capacity; the result is a stable state or homeostasis. The cells can change their functional status in response to a moderate stress and maintain its stable state. More excessive or adverse physiological stresses pathological stimuli (lesion) adjustments result in reversible and irreversible damage injury or cell death. The adjustments take place when physiological or pathological stresses induce a new state altering the cell, but preserves viability in response to external stimuli, such as hyperplasia, hypertrophy, atrophy and intestinal metaplasia. The reversible cell injury denotes pathological cellular changes that can be restored to normal if the stimulus is removed or if the cause of the injury was not serious. The irreversible damage occurs when the stimulus exceeds the capacity of the cell to adapt and denotes permanent pathological alterations that cause cell death. There are two morphological patterns and mechanics of cell death: necrosis and apoptosis (HORTELANO *et al.*, 2001; AMARANTE-MENDES, 2003; TABAS, 2005; MITCHELL *et al.*, 2006). Necrosis is the most common type of cell death, involves large cellular edema, denaturation and coagulation proteins, degradation of cellular organelles and cell disruption, loss of integrity of the plasma membrane, cytoplasmic and nuclear disorganization dissolution. In general, a large number of cells in adjacent tissue is affected. Apoptosis occurs when the cell dies due to the activation of a "suicide" program-controlled internally, which involves an orchestrated disorder of cellular components; is a minimum disruption of the surrounding tissue. Morphologically, condensation occurs and the fragmentation of chromatin degradation of genomic DNA fragments in oligonucleosomal, volume loss and increased cellular granularity, maintaining the structure of the organelles, forming "folds" in the plasma membrane and subsequent cell fragmentation into apoptotic bodies. In different cell populations, there is a great similarity in phenotype of apoptosis, even in very different physiological situations. This phenotype is the result mainly from the cascading of members of a particular family of cysteine-aspartate proteases called *caspases*. These are divided into two groups, with the executing responsible for implementing the process itself. Through *caspases*, will be generated fragments oligonucleosomal characteristic of apoptosis. Cells can die by apoptosis mechanisms or non-apoptotic (HORTELANO *et al.*, 2001; AMARANTE-MENDES, 2003; TABAS, 2005; MITCHELL *et al.*, 2006). If the stress signal is very violent, which usually occurs in pathological situations, a given cell has no choice but to undergo necrotic death, impossible to control genetic or pharmacological, and quickly lose the integrity of its cell membrane, releasing their cytoplasmic contents and inducing an inflammatory response in the affected tissue. On the other hand, when the stress is warmer, the generated signal will be analyzed by the group of mitochondria, which act as a sensor and have the responsibility of deciding whether this cell will continue to live or be eliminated by apoptotic mechanisms. In this case, the uncoupling of mitochondria undergo a respiratory chain that will result in the release, in particular cytochrome c into the cytosol, which activates the *caspases* cascade that will execute the program packaging cell known as apoptosis. In certain situations where apoptotic cells were not eliminated by phagocytosis, cell necrosis may occur called post-secondary necrosis or apoptosis. In this case, the

membranes of apoptotic cells are ruptured to release intracellular components such as proteases which induce the inflammatory response and tissue damage. In macrophages, the apoptotic phenotype shows a sequence of changes in various parameters, such as cell adhesion and loss of asymmetry of the plasma membrane, cytoskeleton disruption and internucleosomal DNA fragmentation, *caspases* activation with. General physical and chemical properties of the cell are modified during apoptosis (HORTELANO *et al.*, 2001; AMARANTE-MENDES, 2003; TABAS, 2005; MITCHELL *et al.*, 2006).

PROPOSITION

This study aims to evaluate quantitatively and qualitatively the cytotoxic effects of preservative solutions, including propolis, coconut water, coconut milk, HBSS, saline with antibiotics and milk, used in cases of tooth avulsion through microscopic observation of cell disorders suffered in macrophages.

MATERIALS AND METHODS

1) Preparation of culture medium

This work was carried out in toxicology laboratory of the Federal Rural University of Rio de Janeiro. Macrophages were used obtained from Wistar rats (*Rattus norvegicus albinos*). The animals used in this study were obtained from the company Biocampo 2000 Produtos Biológicos Ltda (Bom Jardim, Rio de Janeiro). First we performed the preparation of Eagle's minimum essential medium (MEM) with a pH 7 was weighed 9.60 g of MEM (GIBCO 145000-083) and placed in a conical beaker. Demineralized water was placed to complete 900 ml. Dissolved with a bar and a magnetic stirrer. The pH was adjusted with sodium bicarbonate and the volume adjusted to 100 mL with distilled water. Positive filtration was carried out through the medium Eagle sterile membrane porosity 0.22 μm. Was weighed 100 mg of benzathine penicillin G and 100 mg streptomycin sulfate. At the end of Obtained 121 mg/L penicillin and 230 mg/l streptomycin (both gives trademark Sigma-Aldrich, St Louis, USA). Antibiotics were added to the culture medium as an additional safety measure, to avoid potential contamination. A concentration of 1 mL of the antibiotic per 50 mLl of medium (50 fold concentration) was utilized.

2) Obtaining macrophages

After obtaining the culture medium, the animals were sacrificed by inhalation of chloroform vapors. After sacrificing the animals, the application of the culture medium in the peritoneum of mice was performed. The swelling obtained by this maneuver attracts macrophages. Macrophages were aspirated with a sterile syringe and transferred to conical tubes with culture medium. These tubes were conditioned on ice. Aliquots of the contents were transferred to a plate for observation of cells. For microscopic analysis, we used the blade Neubauer. The counting was performed using a hemocytometer. At the end there was obtained 25 ml solution of macrophages. 0.8 x 10^5 cells/mL were observed. Were obtained from 2 x 10^7 cells of rats within 5 minutes. Fetal bovine serum 20%, containing adhesins that facilitate the adhesion of macrophages in the plate was added. Aliquots were placed in the culture medium containing the macrophages in culture wells of the plates. The plates were placed in a desiccators with a CO_2 environment (distilled water, sodium bicarbonate (Na_2HCO_3) and sulfuric acid) creating an anaerobic environment. The desiccant (Sciencor® Scientific, Sao Paulo, Brazil) was left in an oven at 37°C. Test 3 was used for cell culture microplate with 6 holes (Zellkultur Testplatte from Biochrom AG, Berlin, Germany).

3) Experimental procedure
In a second step, we left for the exposure of macrophages to preservatives channels as dental avulsion. Preservatives media used were: coconut water (1), milk (2), HBSS (3) coconut milk (4), propolis (5) saline containing antibiotics (6) positive control was used the culture medium with fetal bovine serum negative control we used a culture medium containing Antimycin A (lethal substance).

Coconut water was obtained from fresh coconut breaking it with a machete. The milk used in the experiment was of skimmed Elegê mark (Eleva Alimentos S/A, Rio Grande do Sul, Brazil). How preservative 3 was used as a preservative in Hank's balanced salt solution. The preservative 4 was the commercial coconut milk (Bom Coco®, Rio de Janeiro, Brazil). The preservative was 5 propolis sprays (Makrovit, Rio de Janeiro, Brazil). The preservative consisted of six 10 mL sterile PBS (phosphate buffered saline) with 0.2 mL of contend antibiotic means (100 mg benzathine penicillin G and 100 mg streptomycin sulfate, both the trademark Sigma-Aldrich, St. Louis, USA). For the negative control was used antimycin A (Sigma-Aldrich, St. Louis, USA) which inhibits the cellular oxidation, inhibits cytochrome (Complex I NADH dehydrogenase) and therefore no ATP production in mitochondrial respiration of macrophages causing cell death. The toxic dose of Antimycin A ($C_{20}H_{40}N_2O_4$) is 2 μg / mL and the lethal dose is 200DL_{50}.

The plates had their media collected and placed in test tubes and placed in numbered wells preservatives 1 to 6 in the plate respectively with its own pre-set numbers. These plates were numbered 1 to 3 and the top 4 to 6 at the bottom. One of these plates was placed for 30 minutes and another 60 minutes in the desiccator and left in an oven at 37°C. The third plate contained controls that were placed in each of 3 wells and left for 120 minutes. After the respective times of 30, 60 and 120 minutes, preservatives were collected and washed with PBS the plates quickly. The Blue Trypan (SP Labor, São Paulo, Brazil) 0.5%, was added to each well and left for 1 minute. The wells were washed with water and subsequently added methanol (Lab House, Belo Horizonte, Brazil) and Panotic (Lab House, Belo Horizonte, Brazil) to fix the dye. After stained and fixed, the plates were observed under a microscope. As evaluation was considered that little stained or lighter cells showed up alive and more stained bluish or well were killed.

RESULTS

In this study it was possible to obtain results that allowed a quantitative and qualitative analysis. Quantitatively obtained a certain percentage of live cells in each well of the plate to be subjected to each solution. These values were statistically nonparametric Kruskal-Wallis test. After this analysis a qualitative observation was made through the images obtained by optical microscopy that allowed the observation of cellular changes directly.

First were observed in 6 wells plate 30 minutes. It was observed that within 30 minutes, the well containing the coconut 17% live cells; The milk had a well containing 69% of living cells; The well containing Hank's solution (HBSS) showed 40% of living cells; the well containing coconut milk had 93% of living cells; The well containing propolis showed 71% of living cells; and the well saline containing antibiotics showed 83% live cells (Table 1).
In the first 30 minutes, it was observed that the coconut water showed unsatisfactory results with large numbers of dead cells showed features of apoptosis, DNA fragmentation by nuclear fragmentation that induces natural death (Figure 1). Milk preserved cellular

conformation of macrophages that had intact and clearly visible with a reasonable percentage of vital cells. The Hank's solution (HBSS) gave a median amount of vital cells. Coconut milk had a surprising result, with 93% of vital cells with adequate cellular conformation. Propolis the first 30 minutes showed considerable amount of vital cells and cellular architecture suitable normal (Figure 2). The salt solution containing antibiotics within 30 minutes showed a large amount of vital cells.

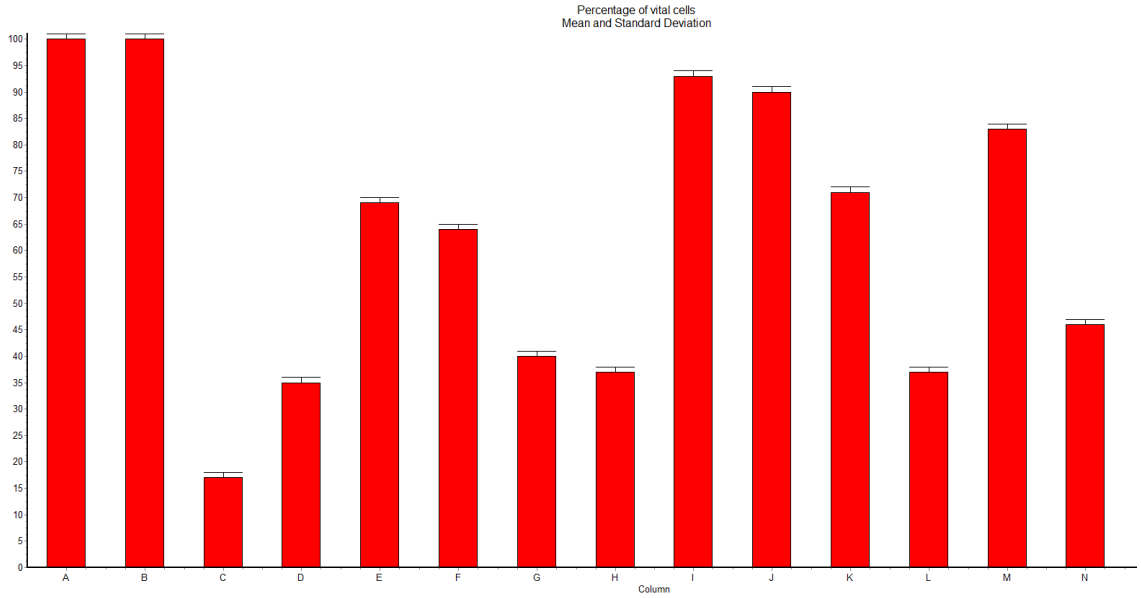
Thereupon, 6 wells were observed from 60 minutes plate. It was observed that in 60 minutes the well containing the coconut with 34% live cells (relative increase of the sample after 30 minutes probably obtained by differential display field). The well containing milk introduced at 64% of vital cells in the well HBSS containing 37% vital cells in the well containing coconut milk and 90% of vital cells, propolis 37% vital cells with saline solution and antibiotics at 46% of vital cells (Table 1).

After 60 minutes it was observed that the coconut water continued to present unsatisfactory result, with apoptosis, cell degeneration and DNA fragmentation (Figure 7). Milk produced a small decrease in the amount of cells (macrophages) alive, but there was no significant change, the cell pattern remained within normal limits. The HBSS also generated small decrease in the amount of living cells, but kept the same characteristics of 60 minutes. Coconut milk is still maintained with a high percentage of living cells (90%) and surprising maintaining cell characteristics (Figures 8 and 9). Propolis and saline with antibiotics showed a very large drop in the percentage of live cells which makes them of little use after 60 minutes of exposure (Figures 10, 11, 12 and 13).

Table 1 Effect of various preservatives into macrophages. Percentage of vital cells after each exposure.

Preservatives means	30 mim	60 mim
Positive control (fetal bovine serum)	100%	100%
Coconut Water	17%	34%
Milk	69%	64%
HBSS	40%	37%
Coconut Milk	93%	90%
Propolis	71%	37%
Physiological serum and antibiotics	83%	46%
Negative control (Antimycin A)	0%	0%

Statistical analysis showed a statistically significant difference ($p < 0.01$) among most groups, especially the comparison between the positive control and the coconut so after 30 minutes and after 60 minutes and between coconut water and coconut milk after 30 minutes.
Graphic 1- Statistical analysis on the percentage of vital cells after each exposure.



In the graphic 1 the letters listed in the abscissa axis represent:
A= Positive control (fetal bovine serum) 30min
B= Positive control (fetal bovine serum) 60min
C= Coconut Water 30min
D= Coconut Water 60min
E= Milk 30min
F= Milk 60min
G= Coconut Milk 30min
H= Coconut Milk 60min
I= Propolis 30min
J= Propolis 60min
K= Physiological serum and antibiotics 30min
L= Physiological serum and antibiotics 60min
M= Negative control (Antimycin A) 30min
N= Negative control (Antimycin A) 60min

One-way Analysis of Variance (ANOVA)

The P value is < 0.0001, considered extremely significant.
Variation among column means is significantly greater than expected by chance.

Bonferroni Multiple Comparisons Test

If the value of t is greater than 3.938 then the P value is less than 0.05.

Comparison	Mean Difference	t	P value
PC 30 vs PC 60	0.000	0.000	ns P>0.05
PC 30 vs CW 30	83.000	108.67	*** P<0.001
PC 30 vs CW 60	65.000	85.105	*** P<0.001
PC 30 vs M 30	31.000	40.589	*** P<0.001
PC 30 vs M 60	36.000	47.135	*** P<0.001
PC 30 vs HBSS 30	60.000	78.558	*** P<0.001
PC 30 vs HBSS 60	63.000	82.486	*** P<0.001
PC 30 vs CM 30	7.000	9.165	*** P<0.001
PC 30 vs CM 60	10.000	13.093	*** P<0.001
PC 30 vs P 30	29.000	37.970	*** P<0.001
PC 30 vs P 60	63.000	82.486	*** P<0.001
PC 30 vs PSA 30	17.000	22.258	*** P<0.001
PC 30 vs PSA 60	54.000	70.703	*** P<0.001
PC 30 vs NC 30	100.00	130.93	*** P<0.001
PC 30 vs NC 60	100.00	130.93	*** P<0.001
PC 60 vs CW 30	83.000	108.67	*** P<0.001
PC 60 vs CW 60	65.000	85.105	*** P<0.001
PC 60 vs M 30	31.000	40.589	*** P<0.001
PC 60 vs M 60	36.000	47.135	*** P<0.001
PC 60 vs HBSS 30	60.000	78.558	*** P<0.001
PC 60 vs HBSS 60	63.000	82.486	*** P<0.001
PC 60 vs CM 30	7.000	9.165	*** P<0.001
PC 60 vs CM 60	10.000	13.093	*** P<0.001
PC 60 vs P 30	29.000	37.970	*** P<0.001
PC 60 vs P 60	63.000	82.486	*** P<0.001
PC 60 vs PSA 30	17.000	22.258	*** P<0.001
PC 60 vs PSA 60	54.000	70.703	*** P<0.001
PC 60 vs NC 30	100.00	130.93	*** P<0.001
PC 60 vs NC 60	100.00	130.93	*** P<0.001
CW 30 vs CW 60	-18.000	23.568	*** P<0.001
CW 30 vs M 30	-52.000	68.084	*** P<0.001
CW 30 vs M 60	-47.000	61.537	*** P<0.001

CW 30 vs HBSS 30	-23.000	30.114	***	P<0.001
CW 30 vs HBSS 60	-20.000	26.186	***	P<0.001
CW 30 vs CM 30	-76.000	99.507	***	P<0.001
CW 30 vs CM 60	-73.000	95.579	***	P<0.001
CW 30 vs P 30	-54.000	70.703	***	P<0.001
CW 30 vs P 60	-20.000	26.186	***	P<0.001
CW 30 vs PSA 30	-66.000	86.414	***	P<0.001
CW 30 vs PSA 60	-29.000	37.970	***	P<0.001
CW 30 vs NC 30	17.000	22.258	***	P<0.001
CW 30 vs NC 60	17.000	22.258	***	P<0.001
CW 60 vs M 30	-34.000	44.516	***	P<0.001
CW 60 vs M 60	-29.000	37.970	***	P<0.001
CW 60 vs HBSS 30	-5.000	6.547	***	P<0.001
CW 60 vs HBSS 60	-2.000	2.619	ns	P>0.05
CW 60 vs CM 30	-58.000	75.940	***	P<0.001
CW 60 vs CM 60	-55.000	72.012	***	P<0.001
CW 60 vs P 30	-36.000	47.135	***	P<0.001
CW 60 vs P 60	-2.000	2.619	ns	P>0.05
CW 60 vs PSA 30	-48.000	62.847	***	P<0.001
CW 60 vs PSA 60	-11.000	14.402	***	P<0.001
CW 60 vs NC 30	35.000	45.826	***	P<0.001
CW 60 vs NC 60	35.000	45.826	***	P<0.001
M 30 vs M 60	5.000	6.547	***	P<0.001
M 30 vs HBSS 30	29.000	37.970	***	P<0.001
M 30 vs HBSS 60	32.000	41.898	***	P<0.001
M 30 vs CM 30	-24.000	31.423	***	P<0.001
M 30 vs CM 60	-21.000	27.495	***	P<0.001
M 30 vs P 30	-2.000	2.619	ns	P>0.05
M 30 vs P 60	32.000	41.898	***	P<0.001
M 30 vs PSA 30	-14.000	18.330	***	P<0.001
M 30 vs PSA 60	23.000	30.114	***	P<0.001
M 30 vs NC 30	69.000	90.342	***	P<0.001
M 30 vs NC 60	69.000	90.342	***	P<0.001
M 60 vs HBSS 30	24.000	31.423	***	P<0.001
M 60 vs HBSS 60	27.000	35.351	***	P<0.001
M 60 vs CM 30	-29.000	37.970	***	P<0.001
M 60 vs CM 60	-26.000	34.042	***	P<0.001
M 60 vs P 30	-7.000	9.165	***	P<0.001
M 60 vs P 60	27.000	35.351	***	P<0.001
M 60 vs PSA 30	-19.000	24.877	***	P<0.001
M 60 vs PSA 60	18.000	23.568	***	P<0.001
M 60 vs NC 30	64.000	83.796	***	P<0.001
M 60 vs NC 60	64.000	83.796	***	P<0.001
HBSS 30 vs HBSS 60	3.000	3.928	ns	P>0.05
HBSS 30 vs CM 30	-53.000	69.393	***	P<0.001
HBSS 30 vs CM 60	-50.000	65.465	***	P<0.001
HBSS 30 vs P 30	-31.000	40.589	***	P<0.001
HBSS 30 vs P 60	3.000	3.928	ns	P>0.05
HBSS 30 vs PSA 30	-43.000	56.300	***	P<0.001
HBSS 30 vs PSA 60	-6.000	7.856	***	P<0.001

HBSS 30 vs NC 30	40.000	52.372	***	P<0.001
HBSS 30 vs NC 60	40.000	52.372	***	P<0.001
HBSS 60 vs CM 30	-56.000	73.321	***	P<0.001
HBSS 60 vs CM 60	-53.000	69.393	***	P<0.001
HBSS 60 vs P 30	-34.000	44.516	***	P<0.001
HBSS 60 vs P 60	0.000	0.000	ns	P>0.05
HBSS 60 vs PSA 30	-46.000	60.228	***	P<0.001
HBSS 60 vs PSA 60	-9.000	11.784	***	P<0.001
HBSS 60 vs NC 30	37.000	48.444	***	P<0.001
HBSS 60 vs NC 60	37.000	48.444	***	P<0.001
CM 30 vs CM 60	3.000	3.928	ns	P>0.05
CM 30 vs P 30	22.000	28.805	***	P<0.001
CM 30 vs P 60	56.000	73.321	***	P<0.001
CM 30 vs PSA 30	10.000	13.093	***	P<0.001
CM 30 vs PSA 60	47.000	61.537	***	P<0.001
CM 30 vs NC 30	93.000	121.77	***	P<0.001
CM 30 vs NC 60	93.000	121.77	***	P<0.001
CM 60 vs P 30	19.000	24.877	***	P<0.001
CM 60 vs P 60	53.000	69.393	***	P<0.001
CM 60 vs PSA 30	7.000	9.165	***	P<0.001
CM 60 vs PSA 60	44.000	57.610	***	P<0.001
CM 60 vs NC 30	90.000	117.84	***	P<0.001
CM 60 vs NC 60	90.000	117.84	***	P<0.001
P 30 vs P 60	34.000	44.516	***	P<0.001
P 30 vs PSA 30	-12.000	15.712	***	P<0.001
P 30 vs PSA 60	25.000	32.733	***	P<0.001
P 30 vs NC 30	71.000	92.961	***	P<0.001
P 30 vs NC 60	71.000	92.961	***	P<0.001
P 60 vs PSA 30	-46.000	60.228	***	P<0.001
P 60 vs PSA 60	-9.000	11.784	***	P<0.001
P 60 vs NC 30	37.000	48.444	***	P<0.001
P 60 vs NC 60	37.000	48.444	***	P<0.001
PSA 30 vs PSA 60	37.000	48.444	***	P<0.001
PSA 30 vs NC 30	83.000	108.67	***	P<0.001
PSA 30 vs NC 60	83.000	108.67	***	P<0.001
PSA 60 vs NC 30	46.000	60.228	***	P<0.001
PSA 60 vs NC 60	46.000	60.228	***	P<0.001
NC 30 vs NC 60	0.000	0.000	ns	P>0.05

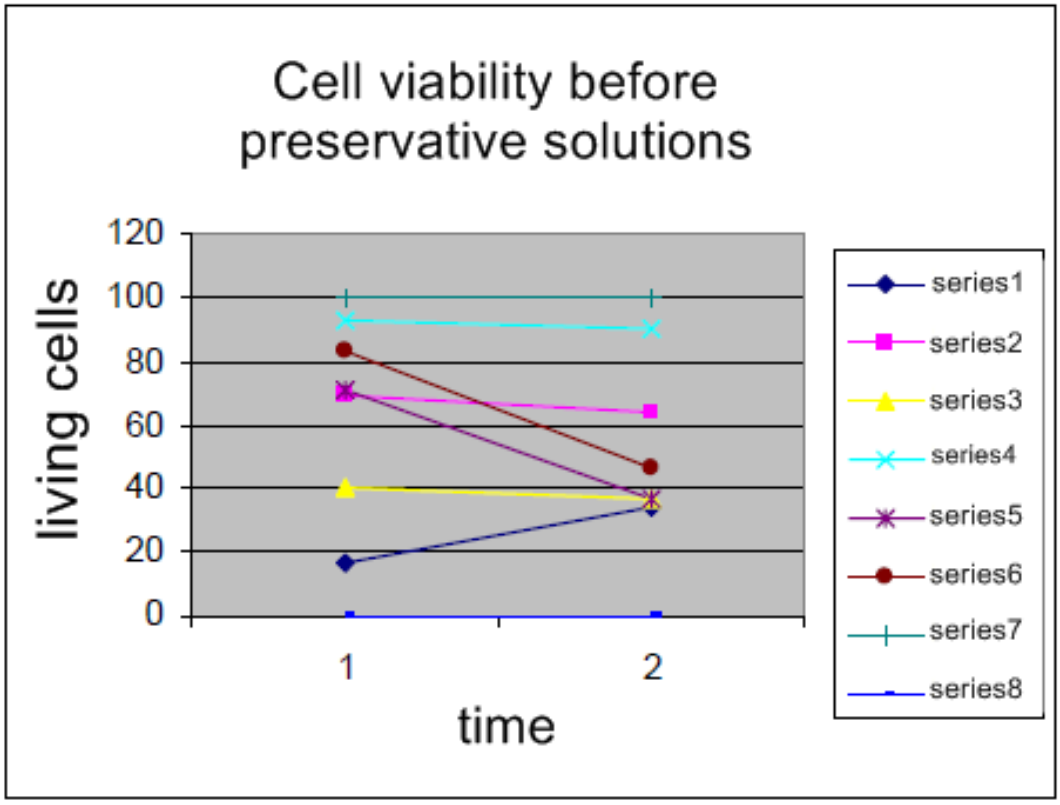


Figure 1: Graphical analysis of cell viability in the face of preservative solutions. Series: 1- Water coconut; 2- Milk; 3- HBSS; 4 Coconut milk; 5-Propolis; 6- saline with antibiotics; 7- positive control; 8 negative control. Time interval 30 minutes and 1- 2- 60 minutes.

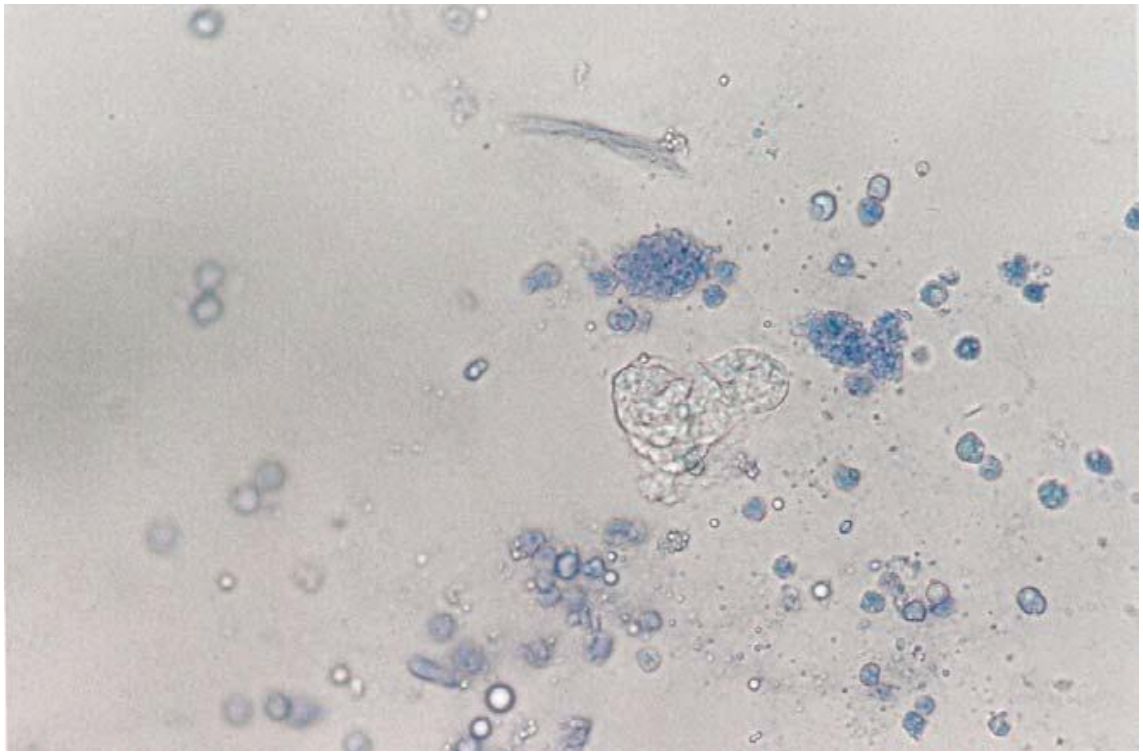


Figure 2: Photomicrograph of *Wistar* rats treated macrophages coconut water for 30 minutes and subjected to the survival test against Trypan Blue. It is observed dead cells degenerate, the presence of bodies' apoptotic. The blue color indicates cell death. Nikon, an increase of 400 x.

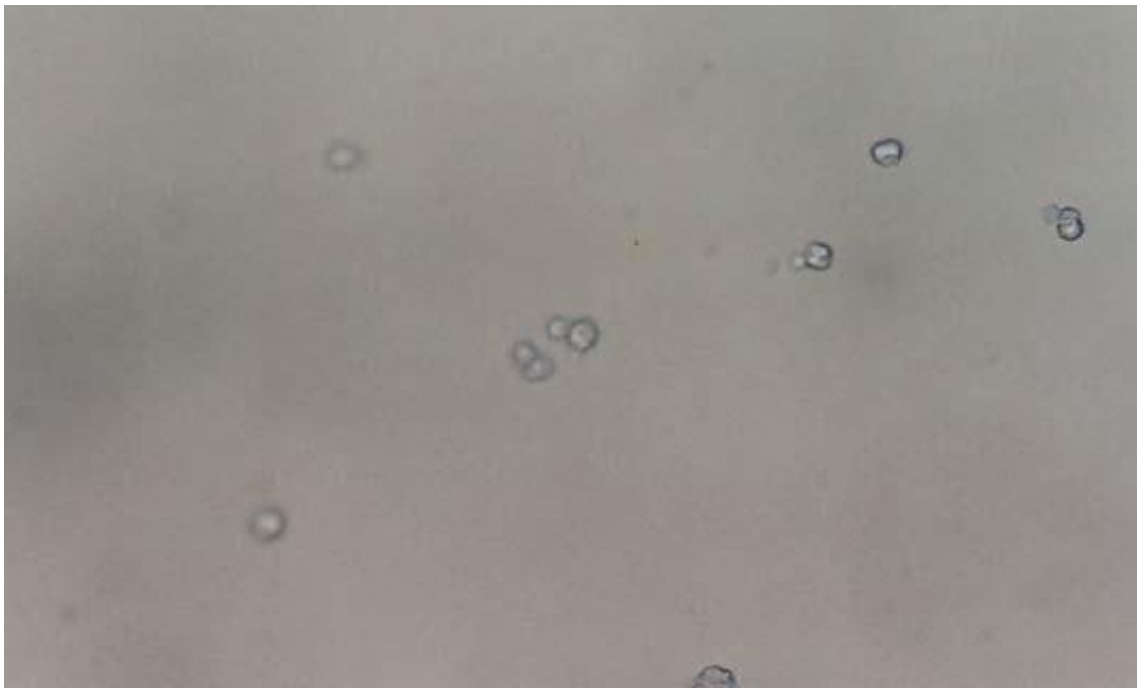


Figure 3: Photomicrograph of *Wistar* rats treated macrophages Propolis for 30 minutes and subjected to the survival test against Trypan Blue. Living cells are observed. Nikon, an increase of 400x.

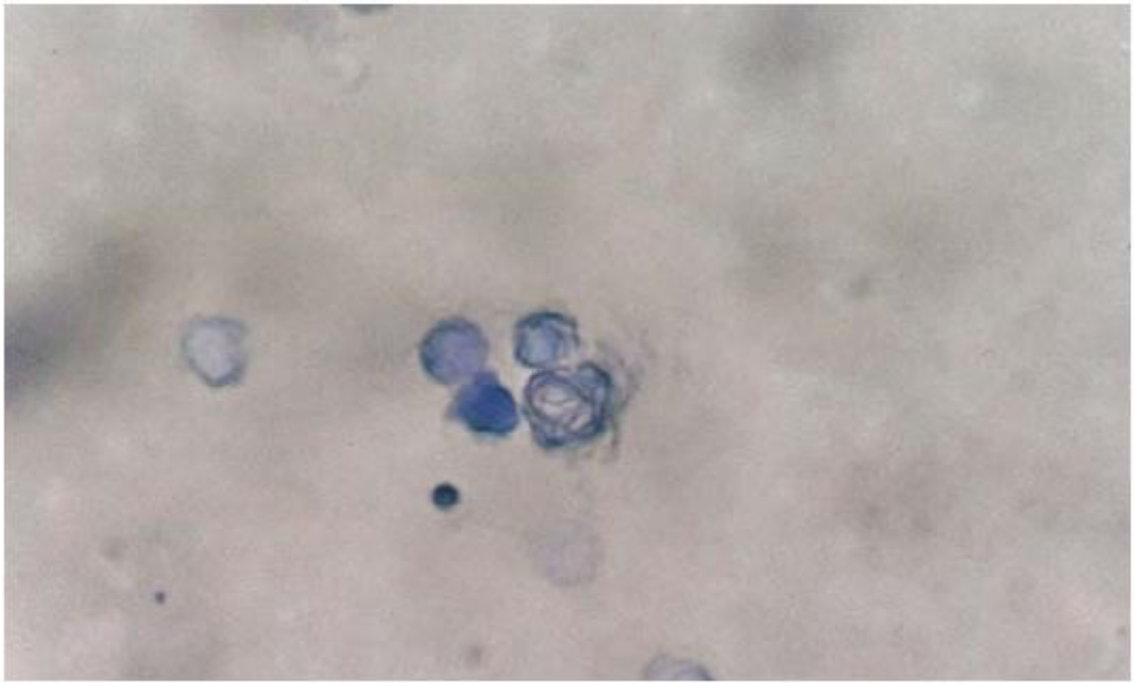


Figure 4: Photomicrograph of *Wistar* rats treated macrophages Propolis for 30 minutes and subjected to the survival test against Trypan Blue. We observe living cells with degenerated cells. The propolis caused by increased cell likely increase the permeability of the cell membrane. Nikon, 400x magnification.

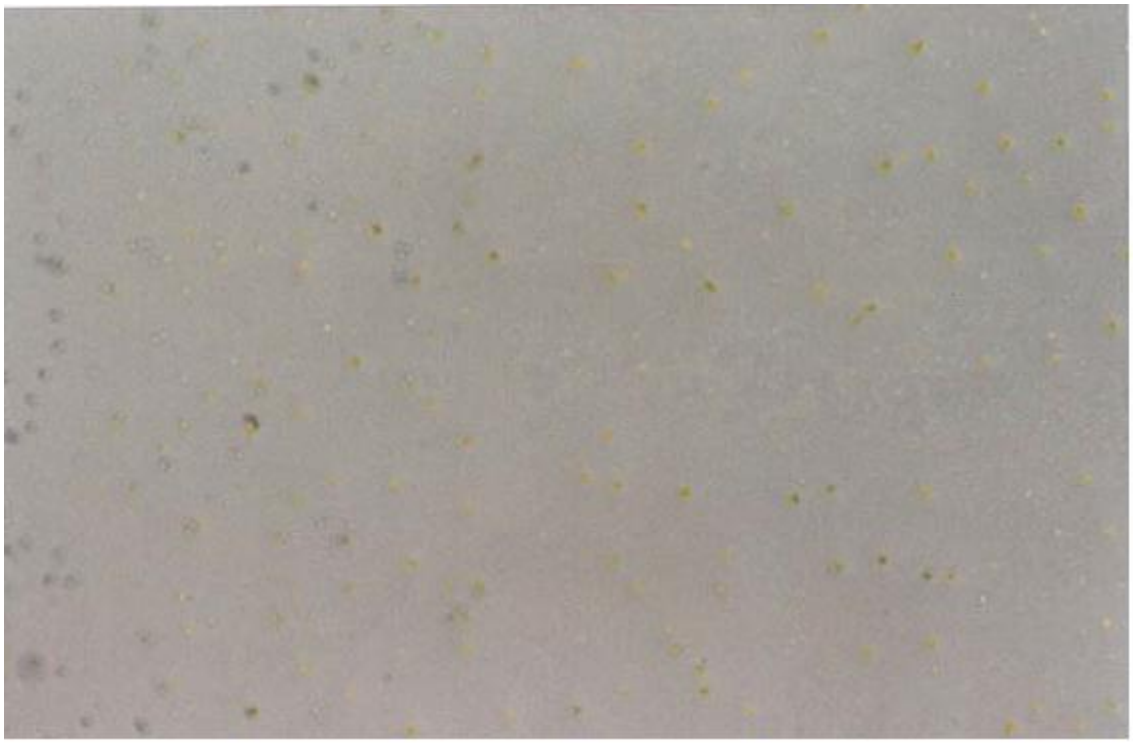


Figure 5: Negative control. It is observed that all cells are shown killed. Nikon, 100x magnification.

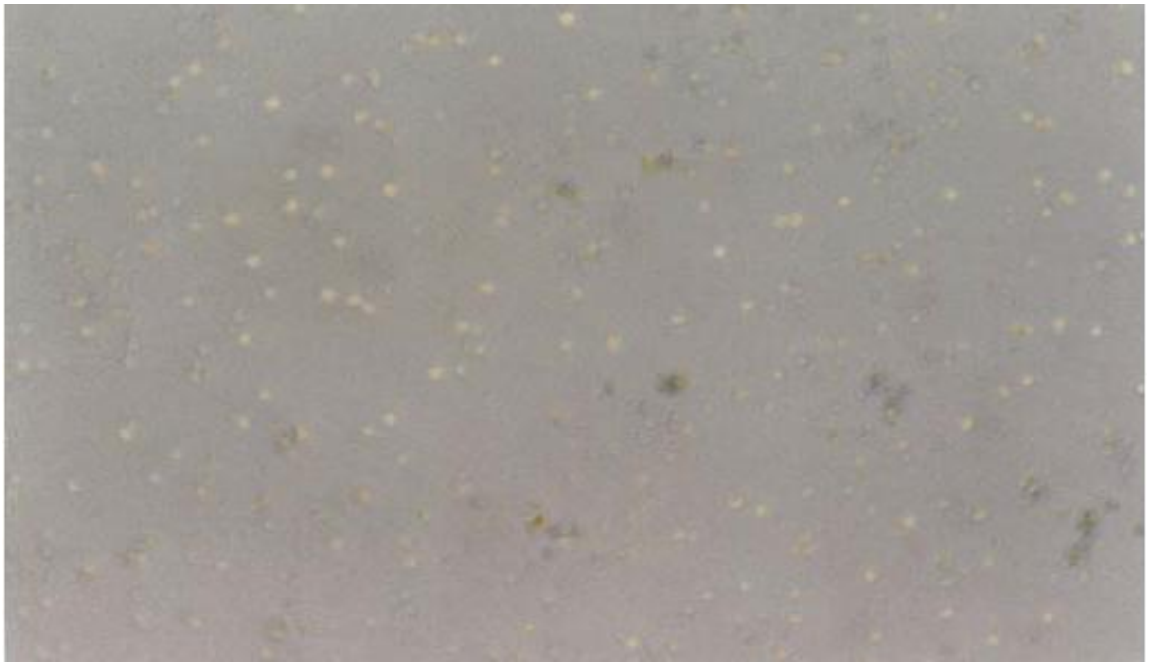


Figure 6: Positive Control. It is observed that all cells are shown live. Nikon, 100x magnification.

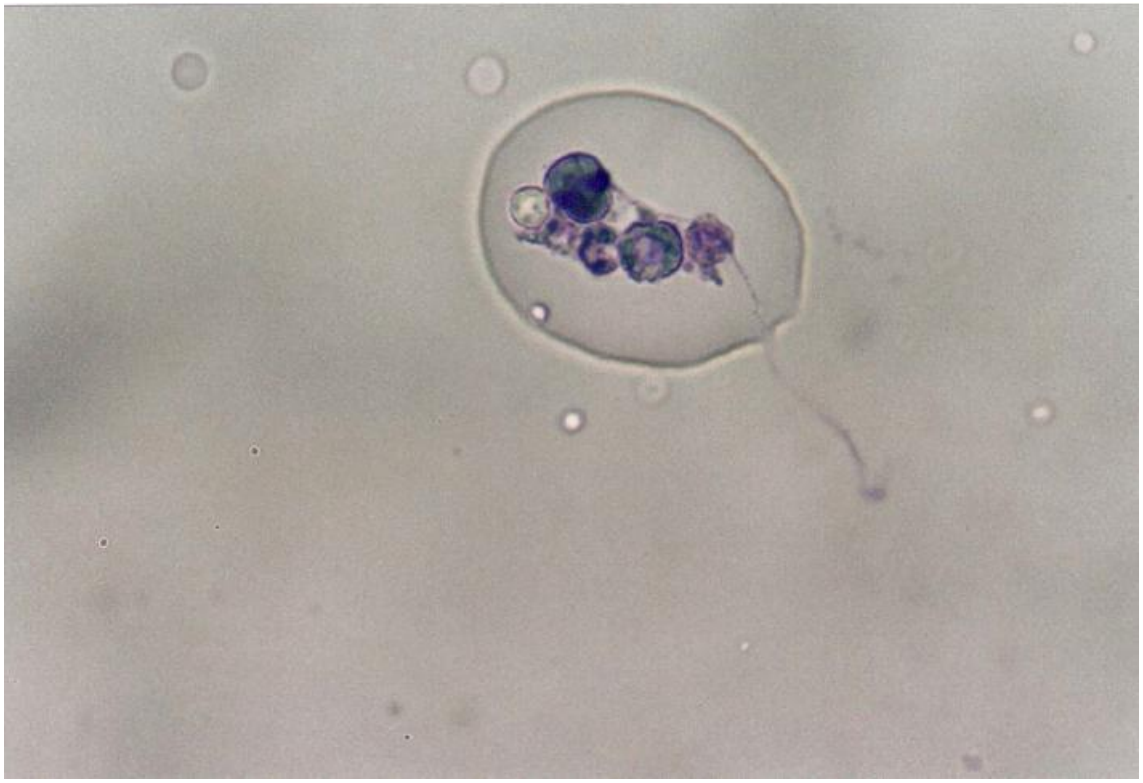


Figure 7: Photomicrograph of *Wistar* rats treated macrophages coconut water for 60 minutes and subjected to the survival test against Trypan Blue. It is observed apoptosis. Nikon, 400x magnification.

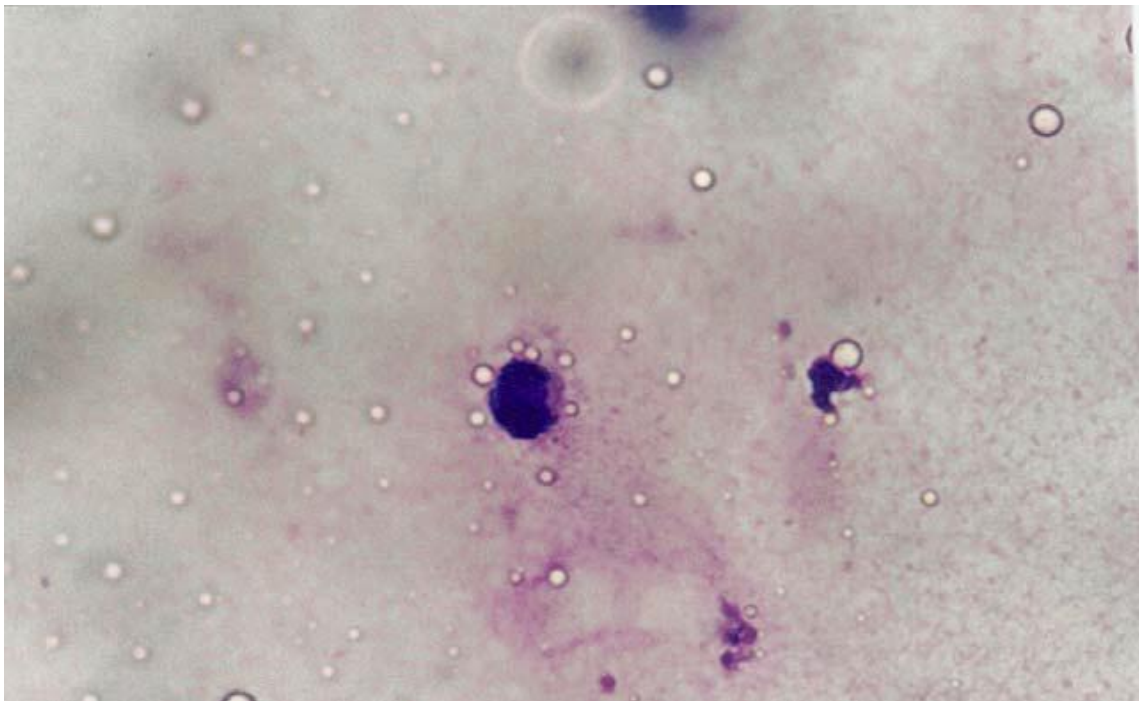


Figure 8: Photomicrograph of *Wistar* rats treated macrophages coconut milk for 60 minutes and subjected to the survival test against Trypan Blue. Note the presence of monocytes. Nikon, 400x magnification.

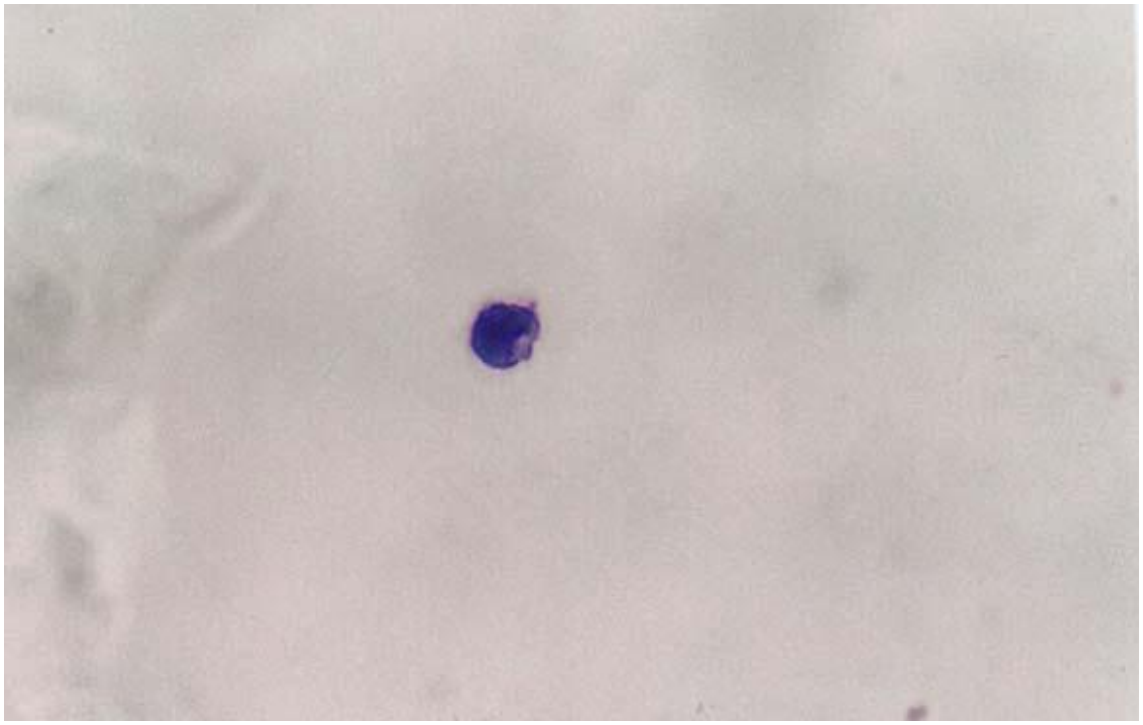


Figure 9: Photomicrograph of *Wistar* rats treated macrophages coconut milk for 60 minutes and subjected to the survival test against Trypan Blue. It is observed intact cell looking good, with adequate monocyte morphology, with kidney-shaped nucleus. Nikon, 400x magnification.

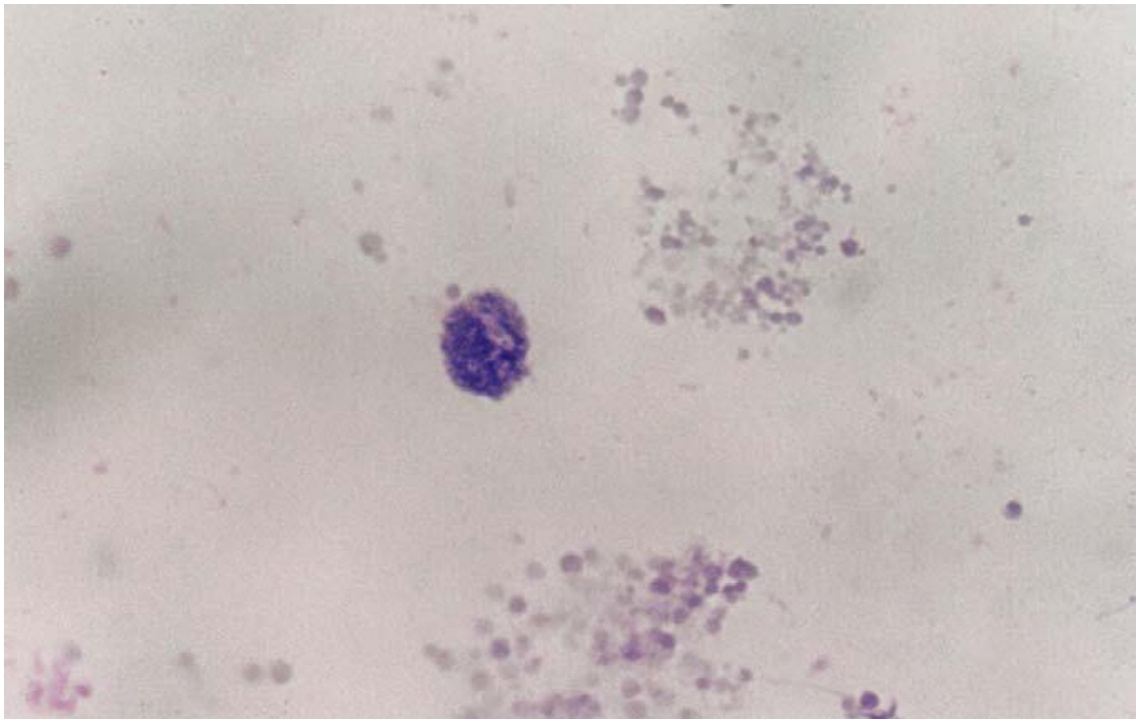


Figure 10: Photomicrograph of *Wistar* rats treated macrophages Propolis for 60 minutes and subjected to the survival test against Trypan Blue. Necrosis is observed, degenerated cells, apoptosis is observed with nuclear fragmentation. Nikon, 400x magnification.

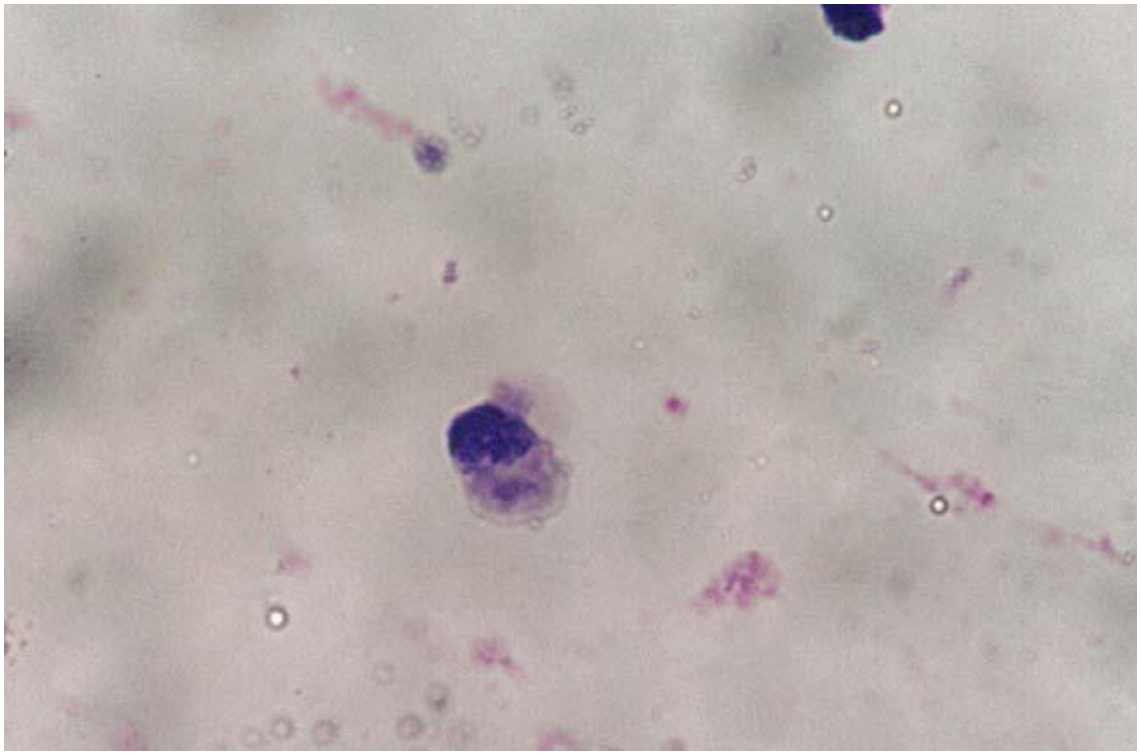


Figure 11: Photomicrograph of *Wistar* rats treated macrophages Propolis for 60 minutes and subjected to the survival test against Trypan Blue. It is observed cytoplasmic change; core starting to fragment; apoptosis. Nikon, 400x magnification.

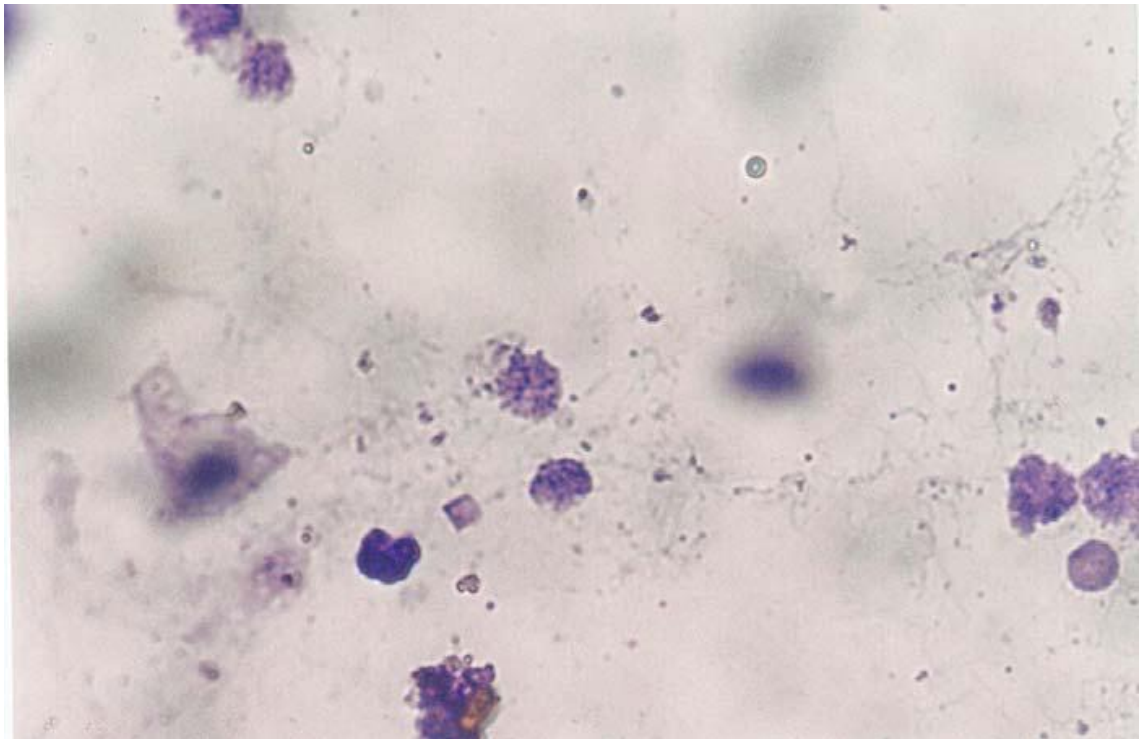


Figure 12: Photomicrograph of *Wistar* rats treated macrophages Propolis for 60 minutes and subjected to the survival test against Trypan Blue. It can be noticed cell necrosis. Scars occurring in the cell, causing necrosis. Necrosis stimulates the inflammatory process. Nikon, 400x magnification.

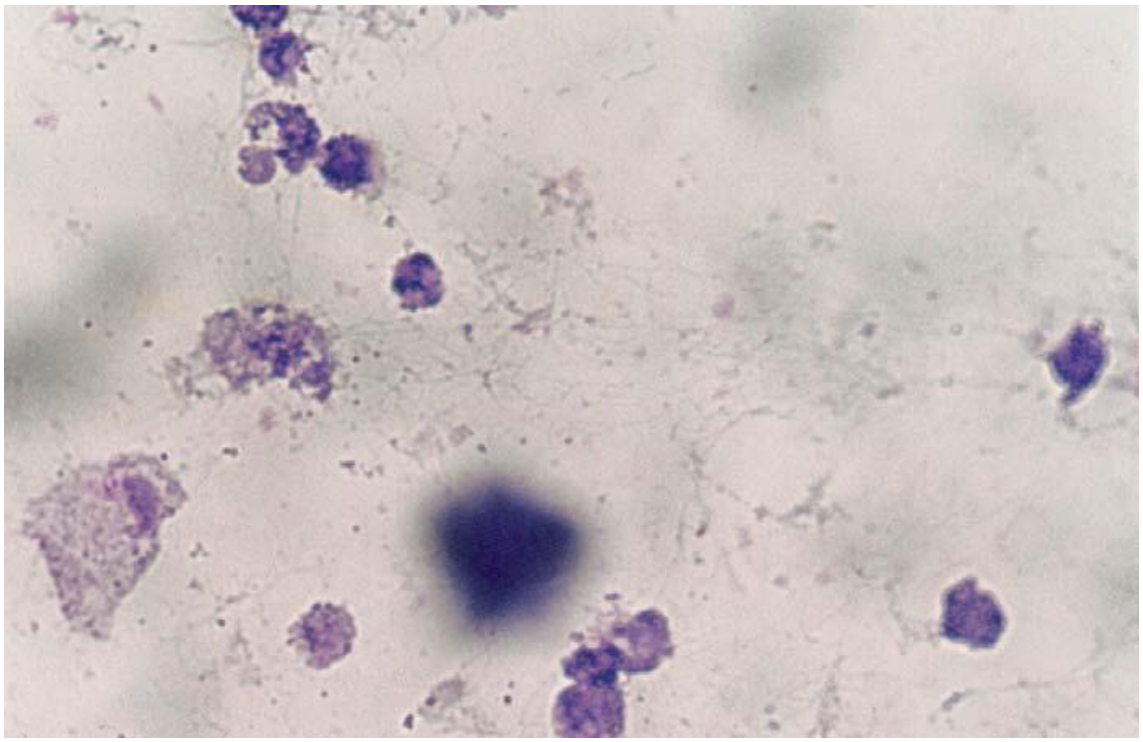


Figure 13: Photomicrograph of macrophages from *Wistar* rats treated with saline for 60 minutes and subjected to the survival test against Trypan Blue. It is observed cellular necrosis and apoptosis; Figures apoptosis (top left); various stages of apoptosis; advanced stage (lower left). Nikon, 400x magnification.

DISCUSSION

In the present study was evaluated for the ability to maintain cell viability of macrophages six solutions (coconut water, milk, HBSS, coconut milk, propolis and saline solution containing antibiotics) used as preservatives after tooth avulsion. Sim *et al*. (2008) reported that the coconut water contains high levels of ribosídeos zeatínicos capable of expressing proteolytic activity possibly related to the sterility thereof. Gopikrishna *et al*. (2008) reported that coconut water is biologically pure and sterile, with rich presence of amino acids, proteins, vitamins and minerals and statistical analysis of the work showed that the coconut water was able to maintain significantly more viable periodontal ligament cells when compared with propolis, HBSS and milk and coconut water can be used as a suitable means of transportation for avulsed teeth. In this experimental study, however, found a small amount of viable cells when exposed to coconut water solution. In times of 30 and 60 minutes, the number of viable cells was found not greater than 34% of the samples from the wells. There was a high incidence of dead cells degenerate, and presence of apoptotic bodies indicating the induction of apoptosis in macrophages coconut water. This fact contraindicated the coconut water as a means of transport for avulsed teeth as described by Gopkrishna *et al.* (2008). As for whether the coconut water is biologically pure and sterile, this could be speculated. During handling, the coconut water may be easily contaminated and as described by Prabakaran *et al*. (2008) is a means of easy proliferation of *Bacillus thuringiensis var. israelensis* and shows excellent biological condition for the development of other types of contaminating microorganisms.
In another study, Abará *et al*. (2007) reported liver protective effect of coconut water showing that the ingestion of coconut water and coconut milk increased the values of the rates of protein and protein / RNA and decrease the activity of alanine and aspartate

aminotransferase (ALT and AST). These effects increase the induction of metabolic enzymes resulting in increased clearance and elimination of caffeine in the body by reducing the toxic effect on the liver. In addition, Ismail *et al*. (2007) showed that coconut water intake optimized better fluid and electrolyte balance in comparison to other commercial beverages. Possibly the debugger effect could be related to the stimulation of Kupffer cells which could express an increased production of nitric oxide and monocyte chemoattractant protein. According to the analysis of the results found in this study, the observed apoptosis levels appear to correlate with increased activity as purifying, Sandhya & Rajamahan (2006), described an increased conversion of cholesterol into bile acids rates and increased excretion of these neutral and acidic sterols observed in rats treated with the coconut water, from histopathological studies, which showed less accumulation of fat in the tissues evaluated. They found that treatment with coconut water resulted in increase in plasma L-arginine, the urinary levels of nitrite and activity of nitric-oxide synthase. These results indicate the beneficial effects of coconut water and analyzing the serum lipid parameters tissue. As suggested by Somers *et al*. (2008), calcineurin is associated with the production of nitric oxide-synthase, knowing that the coconut induces protein synthesis and increased rates of protein/RNA, this fact could be related to the increase in nitric oxide synthase activity, with the level of L-arginine, mechanisms that could potentially be deemed responsible for the apoptotic effect in isolated macrophages as observed in our in vitro study. Han *et al*. (2008) suggested that the increased nitric oxide-synthase synthesis is an inducer of apoptosis factor. According to Bhushan *et al*. (2007), *triterpenediol* (*TPD*), isolated Boswellia serrata induces an increase in reactive oxygen species and nitric oxide both the intrinsic and extrinsic signaling cascade. Nakamura *et al*. (2008) reported that lipopolysaccharide (LPS) isolated from roots of some plants studied, according to the dependent concentration expressed potent inhibitory effect on the expression of nitric oxide synthase. It could be suggested that the apoptotic effect associated with coconut water would be the tradeoff of TPD/LPS in relation to the expression of nitric oxide synthase-studied in macrophages.

Regarding propolis, Huang *et al*. (2007) described the isolation and characterization of G Propolina Taiwanese propolis first demonstrated that this component is a potent inducer of apoptosis in cancer cells in the brain and that the component and its extract showed a protective effect against oxidative stress in cortical neurons mouse. Complementing Inokushi *et al*. (2006) reported that the Brazilian propolis has a neuroprotective effect against damage to the retina in vitro and in vivo, and that inhibition of oxidative stress induced by propolis may be partially responsible for these neuroprotective effects.

Propolis is a resinous substance used by bees to repair and maintenance of their hives. It has over 180 components including flavonoids, phenolic acids and esters that have anti-inflammatory, antibacterial, antiviral, immunomodulatory, anti-proliferative and anti-oxidants. Propolis shown to inhibit cell division and protein synthesis, however, the exact mechanism behind the anti-tumor effect is not clearly described. According Gunduz *et al.* (2005), propolis apoptotic effect may be related to the inhibition of Telomerase expression. Hernandez *et al*. (2007) suggested that propolis samples showed a strong anti-proliferative activity in cancer cells. According to Chen *et al*. (2004a) propolis propolina is rich in A, B, C, D, E and F, which were able to induce apoptosis in human melanoma cell phenotype; this effect would be related to the high levels of propolina.

Chen *et al*. (2004b) showed that two *prenylflavonoids*, the Propolina A and B induced apoptosis in human cells melanoma and significant inhibition of xanthine oxidase activity. The isolation and characterization of Propolina C bee propolis enabled to evaluate this

compound is an excellent inducer of apoptosis in human cells melanoma. Oncag *et al*. (2006) showed that propolis has an excellent antibacterial activity, suggesting that it may be used as an alternative intra-channel medication.

Sabir *et al*. (2005) suggested that the direct pulp capping with flavonoids of propolis in mice can delay the pulp inflammation and stimulate the formation of reparative dentin.According Hayacibara *et al*. (2005) the cariostatic type 3 components of propolis and 12 are non-polar and H-FR is the fraction of choice to identify novel anticariogenic agents.Botushanov *et al*. (2001) have reported that Propolis is produced by bees with pronounced anti-inflammatory effect. It is an ingredient of many drugs and added to the toothpaste as a prophylactic component for periodontal diseases where this toothpaste showed excellent removal plate inhibitory effect and anti-inflammatory plate. Artepillin C has antibacterial activity and when applied to malignant tumor cells of humans and mice in vitro and in vivo, artepillin C exhibited a cytotoxic effect and the growth of tumor cells was clearly inhibited. The cytotoxic effects of artepillin C were documented in most carcinomas and melanomas. Apoptosis, necrosis, and paralysis of mitoses were identified by histological observation after intratumoral injection artepillin C. It was indicated by Kimoto *et al*. (1998) that the artepillin C activated immune system and has direct anti-tumor activity. She is an active ingredient of Brazilian propolis and when applied to human leukemic cells in vitro exhibited potent cytotoxic effect and induced marked apoptosis levels in all cells. The largest effects occurred in T cells. Cells were induced formation of apoptotic bodies and fragmentation of DNA. DNA synthesis in leukemic cells was clearly inhibited and cell disintegration was confirmed microscopically. The results suggest that artepillin C, an active ingredient of the Brazilian propolis has anti-leukemic effects.

Pinocembrin is the most abundant flavonoid in propolis and has been demonstrated to present anti-oxidant, anti-bacterial and anti-inflammatory. According to Gao *et al*. (2008), Pinocembrin protina could also regulate the expression of p53 and inhibit the release of cytochrome c from the mitochondria into the cytosol. Avci *et al*. (2007) found apoptotic and cytotoxic effects of CAPE (caffeic acid phenethyl ester). Chen *et al*. (2007) suggested that Propolina A and B may activate the mitochondria-mediated apoptosis, are potent antioxidants. Mishima *et al*. (2005) have documented that Brazilian propolis extract inhibited the growth of human leukemia cells, which was partly attributed to the induction of apoptosis associated with granulocytic differentiation.Gopikrishna *et al*. (2008) showed a large amount of viable periodontal ligament cells after exposure for 30 minutes propolis. In this work although quantitative analysis in the same time interval to indicate similar results, since the qualitative analysis indicates occurrence of apoptosis.Ozan *et al*. (2007) tested the ability of propolis in maintaining the vitality of the periodontal ligament cells in different time finding a high rate of viable cells within 3 hours of exposure which is not consistent with this work.
Martin & Pileggi (2004) demonstrated a significantly higher ability to maintain the viability of the periodontal ligament cells when exposed to propolis when compared with milk, saline or HBSS being the time of 45 minutes exposure.

In the present work, although initially (within 30 minutes), the quantitative analysis of apoptosis was not significant, qualitative morphological analysis indicates propolis ability induce apoptosis in macrophages. After 60 minutes the amount of dead cells as a consequence of apoptosis increased dramatically.

It is interesting to consider that Borges & Carvalho (2015) reported that Films containing the extract of propolis had good stability and effective antimicrobial properties against

Staphylococcus aureus, which shows that these films can potentially be used to release active compounds in the oral mucosa. We can suggest, based on this study, which possibly favor apoptotic action of propolis extract is stronger in prokaryotic cells.
As for milk, it is accepted by the American Association of Endodontists (1995) as a means of transport for avulsed teeth.

Ozan *et al*. (2007) agree with previous studies that indicate milk suitable as a means of maintaining the viability of the cells of the periodontal ligament is indicated milk low fat content.Martin & Pileggi (2004) obtained similar results to previous work with milk as a means of transport for avulsed teeth, showing no statistically significant difference in the HBSS.

Marino *et al*. (2000) showed that the long-life milk, which has the advantage of not requiring refrigeration, is as effective as most effective and pasteurized milk medium Save-A-Tooth avulsed teeth as a means of transportation in the maintenance of cell viability periodontal ligament. In a similar study, Pearson *et al*. (2003) compared several different commercial types and formulations of milk and the maintainability of the viability of the cells of the periodontal ligament, concluded that Enfamil, a formulation for babies rich in supplements, was more effective than the other formulations at least after 4 hours of exposure.

Blomlof *et al*. (1980) demonstrated that milk is capable of promoting periodontal ligament better conservation of the saliva or saline in monkeys. Again Blomlof (1981) showed that the periodontal ligament cells survive adequately in human milk, 50% of vital cells after 12 hours of storage and the combination of short storage saliva and subsequent storage of milk, was better than only in saliva. Blomlof *et al*. (1983) showed that after exposure of the periodontal ligament milk for 2 or 6 hours this had an adequate repair similar to those submitted to the immediate replantation. Ram & Cohenca (2003) look to the fact that milk is a recommended and easy way to be found anywhere.

In this study, the group treated with the milk had a significant amount of vital cells in 30 minutes and this result remained after 60 minutes indicating that the milk is in a stable means maintaining cell vitality with a variation of exposure time furthermore, the cell forming introduced generally suitable.

Regarding saline (or saline) & Cohenca Ram (2003) indicates that the third option would be after Hank's balanced salt solution (HBSS) and milk, but is not as easily found as milk. According Pileggi & Martin (2004) saline showed worse results than propolis.According Tanomaru *et al*. (2003) saline solution did not affect the inflammatory activity demonstrating that there was no induction of apoptosis, different from that observed in the present study.
Regarding Hank's balanced salt solution (HBSS), Khademi *et al*. (2008) demonstrated that both the egg and the HBSS showed best results in maintenance of the periodontal ligament cells milk and water, which differed from this work because the amount of vital macrophages was increased after exposure to the milk either HBSS after 30 and after 60 minutes.

Regarding coconut milk according Nneli & Woyike (2008), it was reported that coconut milk has played a major cytoprotective effect in comparison with the coconut water, at the level of the gastric mucosa of rats in this study, we observed that, in fact, the group of cells treated with the coconut milk extract showed higher viability over the different treatment times when compared with the group treated with the coconut.

According to Pryardarshani & Chandrika (2007), coconut milk has carotenoids, which exhibit anti-oxidant effect and can exert an anti-apoptotic activity.

CONCLUSIONS

Within the parameters of this study and according to the methodology used in this work, we suggest, based on the results, that with respect to the quantitative analysis presented significant difference between the positive control group and the group of cells exposed to both coconut water after 30 and after 60 minutes and between the group of cells exposed to the coconut water and coconut milk after 30 minutes. In the qualitative analysis, the group of cells exposed to the coconut apoptosis and loads of dead cells; the group of cells exposed to propolis and saline solution with antibiotics showed a large amount of cell vitality in the first 30 minutes but within 60 minutes showed a large decline in the amount of vital cells, which makes them of little utility in cases which require longer periods of storage; milk but kept smaller amount of vital cells the last two solutions in 60 minutes maintained the same pattern, showing more stable. The group exposed Hank's balanced salt solution showed low levels of vital cells in both ranges and coconut milk is the best solution for 30 and 60 minutes both in maintaining cell vitality and in the maintenance of cellular conformation.

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