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Evaluation of coconut water neutralized by different agents on the viability of human fibroblasts: an in vitro study

Avaliação da água de coco neutralizada por diferentes agentes na viabilidade de fibroblastos humanos: estudo in vitro

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Resumo

Objetivo: Avaliar a eficácia de quatro tipos de substâncias usadas para ajuste do pH da água de coco (AC) sobre a viabilidade de fibroblastos humanos (HFF). **Material e método:** O pH da AC natural e industrializada foi ajustado para pH 7,0 utilizando: (1) Hidróxido de Sódio (NaOH), (2) bicarbonato de sódio (NaHCO3), (3) Trietanolamina (C6H15NO3), (4) 2-Amino-2- Methil-1-propanol (C4H11NO). Células HFF foram plaqueadas em 2×10^4 células/poço em placas de 96 poços e mantidas nas diferentes soluções de AC acima durante 2 h e 4 h. Controle positivo foi representado por HFF mantidas em DMEM e o controle negativo por água da torneira. A viabilidade celular foi avaliada pelo método de MTT Formazan. Os dados foram analisados por 3-way ANOVA seguido pelo teste de Tukey e Dunnett. **Resultado:** A viabilidade celular não é influenciada pelo período de avaliação, e as interações entre AC e período de avaliação, AC e método de ajuste de pH, método de ajuste de pH e período de avaliação ($p>0,05$). **Conclusão:** O produto utilizado para ajuste do pH não interfere na viabilidade de FH, embora, haja uma tendência de melhor desempenho em AC natural.

Descritores: Avulsão dentária; água de coco; viabilidade celular.

Abstract

Objective: This study evaluated four types of pH adjustment of the coconut water (CW) on viability of human fibroblasts (HFF). **Material and method**: Natural and industrialized CW were adjusted to pH 7.0 using: (1) Sodium Hidroxide (NaOH), (2) Sodium bicarbonate ($NaHCO_3$), (3) Triethanolamine ($C_6H_{15}NO_3$), (4) 2-Amino-2-Methil-1-Propanol ($C_4H_{11}NO$). Fibroblasts were plated at 2×10^4/ well in 96 well plates and maintained in the CW solutions for 2 h and 4 h. Positive control was represented by HFF maintained in DMEM and the negative control by tap water. Cell viability was analyzed by MTT formazan method. Data were analyzed by 3-way ANOVA followed by Tukey's and Dunnet's test. **Result:** There are no significant effect on the cell viability regarding type of CW, period of evaluation, and the interactions between CW and period of evaluation, CW and pH adjustment method, pH adjustment method and period of evaluation ($p>0.05$). **Conclusion:** The product used for CW pH adjustment did not influenced HFF viability, thought there are a tendency of better performance in natural CW.

Descriptors: Tooth avulsion; coconut water; cell viability.

INTRODUCTION

Tooth avulsion is an injury characterized by the complete displacement of a tooth from its alveolar socket, which often results in damage to the periodontal ligament (PDL) cells and pulp necrosis[1]. The ideal treatment is immediate replantation[2,3], though it is not always possible[3-5]. The most critical factors affecting the avulsed tooth are the extra-alveolar period and the dry conditions to which the tooth is subject until treatment is rendered[3-5]. Such conditions affect the survival of PDL cells resulting in ankylosis and subsequent replacement resorption[6].

Hence, the choice of a storage medium, for maximum PDL cell survival until replantation, is crucial for a good prognosis[7]. The ideal storage media should present biocompatibility, sterility, pH 6.0-7.0, physiologic osmolarity, and nutrients[5] which maintain cell viability. Great effort has been made by the scientific community to find an optimum storage medium for avulsed teeth. In this sense, various storage media have been investigated for their ability to maintain the viability of PDL cells for as long as possible. These storage media include milk, culture media, Viaspan, Hank's Balanced Salt

Solution (HBSS), Gatorade®, propolis[8,9], soy milk[10,11], and coconut water (CW)[11-15].

Recently, some studies[11-15] have proposed CW as a promising storage medium for avulsed teeth. The natural, fresh and tender CW is a sterile solution, with physiological osmolarity used in the past as a blood plasma substitute[16]. It can replace fluids, amino acids (lysine, cystine, phenylalanine, histidine, and tryptophan), sugars (fructose and glucose), and electrolytes (potassium, calcium, and magnesium)[12]. All these properties may explain its ability to preserve cell viability[12,13,17]. Despite the favorable characteristics of CW, studies involving this solution as a storage medium have shown contradictory results. Some studies report that CW showed better performance than other storage media such as HBSS or milk in terms of maintaining PDL cell viability after avulsion[11-13,15,18]; another study addressed low capability to preserve cells[14]. The pH adjustment of this solution seems to be an indispensable stage for its production at an industrial scale[9]. The agents used to neutralize the pH of coconut water must be stable over time and have no negative influence on the osmolarity. Another important factor is the possible difference regarding properties of natural versus industrialized coconut water, which may influence the final results[19].

Therefore, the purpose of this in vitro study was to evaluate the influence of different coconut water pH control agents on the viability of human fibroblasts (HFF), and their long-term stability. Two hypotheses were tested: 1) natural coconut water will provide better results because it does not have the preservatives used in the process of industrialized coconut water; 2) triethanolamine and amino methyl propanol provide more promising results than sodium hydroxide and sodium bicarbonate because they are weak alkaline compounds and, consequently, may not have a significant effect on the osmolarity, resulting in less pH variation over time.

MATERIAL AND METHOD

Natural (NCW) and industrialized coconut water (ICW) (Ducoco®, Linhares, ES, Brazil) were adjusted to pH 7.0, measured with a digital pH meter (mPA-210; MS TECNOPON, Piracicaba, SP, Brasil), at room temperature using: (1) Sodium Hydroxide (NaOH) 1M (Fmaia, Zilquimica, Ribeirão Preto, SP, Brazil), (2) Sodium bicarbonate ($NaHCO_3$) 1M (Fmaia), (3) Triethanolamine (TEA) ($C_6H_{15}NO_3$) 1M (Bothanica, Uberlândia, MG, Brazil), and (4) Amino methyl propanol (AMP) ($C_4H_{11}NO$) 1M (Farmácia de Manipulação Oriente, Uberaba, MG, Brazil).

Immortalized human skin fibroblasts (HFF) (Cell Bank of Rio de Janeiro, Rio de Janeiro, RJ, Brazil) were cultured in T-25 cell culture flasks containing Dulbecco's Modified Eagle Medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Branchburg, NJ, USA), 100 units mL^{-1} of penicillin/streptomycin (Sigma) in a humidifier incubator with 5% CO_2, and 95% air at 37 °C. Growth was permitted until the cells achieved confluence. These cells were detached, counted using a hemocytometer, and plated in 96-well plates (Coastar Corp., Cambridge, MA, USA) at an initial density of 1×10^4 cells/well in 100 µL of culture medium. The plates were returned to the incubator for 24 h. Subsequently, the culture medium was drained from each well and the cells were exposed for 2 hours and 6 hours to 100 µL of CW, DMEM or tap water, and divided into the following experimental groups: NCW; ICW; NCW neutralized with NaOH (NCW+NaOH); ICW neutralized with NaOH (ICW+NaOH); NCW neutralized with $NaHCO_3$ (NCW+$NaHCO_3$); ICW neutralized with $NaHCO_3$ (ICW+$NaHCO_3$); NCW neutralized with Triethanolamine (NCW+TEA); ICW neutralized with triethanolamine (ICW+TEA); NCW neutralized with amino methyl propanol (NCW+AMP); and ICW neutralized adjusted with amino methyl propanol (ICW+AMP). The positive control corresponded to cells maintained in DMEM 10% of FBS, and the negative control group corresponded to cells maintained in tap water.

The pH was measured with a pH meter (mPA-210; São Paulo/SP, Brazil) in the NCW and ICW and, immediately after, substances to adjust the pH were added, and then for all the analyzed periods (2 hours and 4 hours), to provide long-term pH variation. Cell viability was determined by the MTT Formazan assay (Sigma). At the conclusion of the experimental periods, the storage solutions were replaced with 100 µL of DMEM 10% and incubated with MTT solution (5 mg. mL^{-1}) for 4 h. The same protocol was used for the positive and negative control groups. After this period, the MTT solution was removed and 100 µL of dimethyl sulfoxide (Sigma) were added to each well. Cell viability was determined by measuring the optical density at 540 nm using a microplate reader (Asyz UVM 340; Biochrom, Cambridge, England). The absolute values of absorbance obtained from each well, for each group, at all experimental times, were subjected to analysis of normality. The goal of the initial analysis was to determine the influence of the 2 factors involved in cell viability: the pH adjustment method and the period of evaluation. Osmolarity was measured using a cooling digital osmometer (Peltier; Roebling, Germany) immediately after pH adjustment substances were added. Cell viability and pH variation over the analyzed periods were performed using two-way ANOVA and, for the data of osmolarity, using one-way ANOVA. Dunnett's test was used to compare the control with the experimental groups. The statistical analysis showed significant difference, for $\alpha=0.05$.

RESULT

Table 1 shows the means and standard deviations of the number of viable cells for the different experimental groups and the periods analyzed. Two-way ANOVA showed no significant effect on cell viability regarding the type of CW ($p=0.116$ $p=1.00$), period of evaluation ($p=0.616$), and the interactions between the type of CW and period of evaluation ($p=0.453$). The same test also showed no significant effect on type of CW and pH adjustment method ($p=0.231$), pH adjustment method and period of evaluation ($p=0.431$). Finally, the two-way ANOVA showed no significant effect on the interaction among 3 study factors: the type of coconut water, the pH adjustment method, and the period of evaluation ($p=0.881$). However, the two-way ANOVA did show significance for pH adjustment method ($p<0.01$). The unadjusted pH had significantly lower cell viability than all other groups, irrespective of the CW type and period of evaluation. Dunnett's test showed significant differences between the experimental and control groups for the periods analyzed (Tables 2 and 3, $p<0.05$). Dunnett's test

Table 1. Means and standard deviations for the number of viable cells and statistical category determined by Tukey's test (α=0.05)

Period	Type of coconut water	Unajusted pH	pH adjusted to 7.0 using			
			NaOH	$NaHCO_3$	TEA	AMP
2 hours	Natural	0.42 (0.04) ^{Ba}	2.06 (0.09) ^{Aa}	2.21 (0.17) ^{Aa}	1.90 (0.18) ^{Aa}	2.00(0.18) ^{Aa}
	Industrialized	0.45 (0.04) ^{Ba}	1.92 (0.12) ^{Aa}	1.72 (0.19) ^{Aa}	1.65 (0.15) ^{Aa}	1.58 (0.14) ^{Aa}
4 hours	Natural	0.35 (0.04) ^{Ba}	2.00 (0.16) ^{Aa}	1.90 (0.10) ^{Aa}	1.93 (0.13) ^{Aa}	1.69(0.12) ^{Aa}
	Industrialized	0.34 (0.05) ^{Ba}	1.56 (0.13) ^{Aa}	1.72 (0.19) ^{Aa}	1.37 (0.16) ^{Aa}	1.40 (0.19) ^{Aa}

Different letters indicate significant differences verified by ANOVA two-way and Tukey's test ($P<0.05$). Capital letters represent comparisons between the pH adjustment method (in columns rows), and lower case letters represent comparisons between the type of coconut water (in rows columns). No significant difference was found between period of evaluation.

Table 2. Means and standard deviations (SD) of the number of viable cells for the control and experimental groups for the period of 2 hours, with *P* values calculated by the Dunnett's test

Groups	Means (SD)	*P* value Positive Control	*P* Value Negative Control
Positive control group	2.76 (0.15)	-	P<0.01*
Negative control group	0.15 (0.00)	P<0.01*	-
Natural with unajusted pH	0.42 (0.04)	P<0.01*	P=0.41
Industrialized with unajusted pH	0.45 (0.04)	P<0.01*	P=0.40
Natural + pH adjusted with NaOH	2.06 (0.09)	P=0.45	P<0.01*
Industrialized + pH adjusted with NaOH	1.92 (0.20)	P=0.33	P<0.01*
Natural + pH adjusted with $NaHCO_3$	2.21 (0.27)	P=0.65	P<0.01*
Industrialized + pH adjusted with $NaHCO_3$	1.72 (0.19)	P=0.28	P<0.01*
Natural + pH adjusted with TEA	1.90 (0.28)	P=0.31	P<0.01*
Industrialized + pH adjusted with TEA	1.65 (0.15)	P=0.12	P<0.01*
Natural + pH adjusted with AMP	2.03 (0.18)	P=0.44	P<0.01*
Industrialized + pH adjusted with AMP	1.58 (0.14)	P=0.10	P<0.01*

* Indicates a significant difference between the experimental group and the control group.

Table 3. Means and standard deviations (SD) of the number of viable cells for the control and experimental groups for the period of 4 hours, with *P* values calculated by the Dennett's test

Groups	Means (SD)	*P* value Positive Control	*P* Value Negative Control
Positive control group	1.91 (1.14)	-	P<0.01*
Negative control group	0.15 (0.02)	P<0.01*	-
Natural with unajusted pH	0.35 (0.04)	P<0.01*	P=0.51
Industrialized with unajusted	0.34 (0.05)	P<0.01*	P=0.50
Natural + pH adjusted with NaOH	2.00 (0.36)	P=0.87	P<0.01*
Industrialized + pH adjusted with NaOH	1.56 (0.13)	P=0.65	P<0.01*
Natural + pH adjusted with $NaHCO_3$	1.80 (0.30)	P=0.74	P<0.01*
Industrialized + pH adjusted with $NaHCO_3$	1.72 (0.19)	P=0.69	P<0.01*
Natural + pH adjusted with TEA	1.93 (0.13)	P=0.97	P<0.01*
Industrialized + pH adjusted with TEA	1.37 (0.16)	P=0.23	P<0.01*
Natural + pH adjusted with AMP	1.65 (0.22)	P=0.71	P<0.01*
Industrialized + pH adjusted with AMP	1.43 (0.19)	P=0.34	P<0.01*

* Indicates a significant difference between the experimental group and the control group.

Table 4. Means and standard deviations for the pH variation over the analyzed periods of time and statistical category determined by Tukey's test (α=0.05)

Period	Type of coconut water	Unajusted pH	pH adjusted to 7.0 using			
			NaOH	$NaHCO_3$	TEA	AMP
0 hours	Natural	5.5 (0.5) ^{Aa}	7.0 (0.0) ^{B}	7.0 (0.0) ^{Bb}	7.0 (0.0) ^{Bb}	7.0 (0.0) ^{Bb}
	Industrialized	5.4 (0.4) ^{Aa}	7.0 (0.0) ^{B}	7.0 (0.0) ^{Bb}	7.0 (0.0) ^{Bb}	7.0 (0.0) ^{Bb}
2 hours	Natural	5.6 (0.4) ^{Ab}	7.1 (0.1) ^{B}	7.2 (0.2) ^{Bb}	7.1 (0.1) ^{Bb}	7.2 (0.1) ^{Bb}
	Industrialized	5.5 (0.4) ^{Ab}	7.1 (0.1) ^{B}	7.2 (0.2) ^{Bb}	7.2 (0.0) ^{Bb}	7.2 (0.0) ^{Bb}
4 hours	Natural	5.6 (0.4) ^{Ac}	7.3 (0.1) ^{B}	7.5 (0.2) ^{Bb}	7.3 (0.1) ^{Bb}	7.3 (0.1) ^{Bb}
	Industrialized	5.5 (0.3) ^{Ac}	7.3 (0.0) ^{B}	7.6 (0.1) ^{Bb}	7.4 (0.1) ^{Bb}	7.3 (0.1) ^{Bb}

Different letters indicate significant differences verified by ANOVA two-way and Tukey's test (P<0.05). Capital letters represent comparisons between the pH adjustment method (in columns rows), and lower case letters represent comparisons between the type of coconut water (in rows columns). No significant difference was found between period of evaluation.

also showed that NCW, ICW and ICW +TEA had significantly higher cell viability than the positive control group (Table 3, p<0.05). The negative control group showed the lowest level of cell viability among the experimental groups (Tables 2-4, p>0.05). The osmolarity values ranged from 442.3±21.4 for ICW+AMP to 496.7±17.0 for NCW+NaHCO3, with no significant differences among all tested groups.

DISCUSSION

The first hypothesis was not supported because the NCW did not show better results than the ICW regardless of the neutralizing solution used. These findings indicate that the industrialization process does not have a negative impact on CW properties regarding long-term cell viability. The second hypothesis was not supported because the AMP and the TEA groups showed results of cell viability similar to the NaOH and $NaHCO_3$ groups.

Tender CW is sterile as long as it remains in the inner cavity of the nut. As soon as the nut is opened its biological composition and physical appearance begin to change[20]. That is why several methods have been used to preserve the CW, resulting in a viable solution for human consumption. In Brazil, the conservation methods of CW are applied in synergism (flash pasteurization + use of additives + cooling) to avoid deterioration and loss of quality during storage[21]. According to the manufacturer's specifications for the ICW used in the present study, fructose and sodium metabisulphite were added during the formulation process. Based on this information, it was hypothesized that the additives used in the manufacture of this CW could decrease its ability to maintain cell viability. However, the ability to maintain cell viability did not differ between groups using NCW and groups using ICW. This study postulated that the use of pH neutralizing agents may result in undesirable changes in osmolarity. This is a negative factor, since it is known that physiological osmolarity is one of the key parameters for the choice of a storage medium[22,23]. Osmolality values measured in this study were higher than in other studies[12,24], especially in the ICW groups. However, the osmolality values did not vary with the addition of neutralizing agents. Most studies evaluating CW as a storage medium for avulsed teeth did not investigate the pH neutralization[18,24]. To date, only three studies have been concerned with adjusting the pH to physiological conditions[11,14,15]. However, these studies have not compared the effects of other neutralizer agents, potentially applicable for this purpose, in order to better preserve the CW properties. In spite of the influence of pH and the industrialization process on CW characteristics, its ability to replace fluids and nutrients justifies the great interest in this solution in recent years[11,17,18,24].

The present study confirmed the need to neutralize the pH of CW so that it becomes an effective storage medium. In the present study, the substances used to neutralize pH are widely used in industrial and laboratorial scales. NaOH and $NaHCO_3$, in the presence of water, dissociate generating anions by an exothermic reaction. Thus, it was expected that the generation of anions may interfere negatively in the long-term stability of pH. This expectation was refuted in the present study because the solutions using NaOH or $NaHCO_3$ showed similar results to the AMP and TEA groups for both periods analyzed (2 and 4 hours). TEA and AMP are amines widely used as pH neutralizers in the cosmetics industry. Like other amines, they are weak bases that might promote low ionic dissociation which could influence pH stability. However, the results of the present study demonstrated that these amines show similar results to NaOH, $NaHCO_3$, and the control group. These results corroborate previous studies in which NCW+TEA showed viability values similar to the positive control group[1]. Regardless of the methodological limitations inherent in any *in vitro* study, these studies are useful in preliminary analyses, and for guiding further *in vivo* studies. The immortalized cell culture reduces the need for using large amounts of replicates for statistical validation of the results. Various *in vitro* studies evaluating interim storage media have used this experimental model, due to their ability to multiply rapidly and their unlimited lifespan[14,17,24].

Based on the results presented here, we can conclude that all the analyzed solutions can be used for the pH adjustment of CW since they present higher viability than the negative control group and similar results to the positive control group. As presented in the present study, the industrialization process did not influence

cell viability negatively. Thus, all the efforts should focus on the possibility of developing an industrial product made of CW with pH adjusted to 7.0 in order to facilitate access to this product by the population, which would not need to be concerned about the seasonal availability of the coconut or about the pH neutralization process.

CONCLUSION

The results of this study indicate that pH adjusted NCW tended to show better performance than the ICW regarding long-term cell viability, independently of the solution used for the pH adjustment.

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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