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Post-mortem Interval and Its Relation with the RNA Degradation in the Dental Pulp in Submerged Teeth

زمن الوفاة وعلاقته بتدهور الحمض النووي الريبي من اللب السني في الأسنان المغمورة

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Abstract

Post-mortem interval is the time between death and the discovery of the body or human remains. Teeth are the most resistant structures of the human body, able to withstand extreme conditions such as high temperature, humidity, and post-mortem degradation. The objective of this study was to evaluate the applicability of the method of quantifying degradation of RNA extracted from dental pulps to estimate the post-mortem interval, by simulating drowning conditions with teeth submerged in fresh water and exposed to different time intervals.

The sample consisted of 80 human teeth (third molars), divided into eight groups, and placed in the aquatic environment, for pre-established periods of three days, and 1, 2, 3, 4, 8, 12 and 16 weeks respectively. After the stipulated time, the teeth were removed and the RNA was extracted form the dental pulp. Finally, the RNA was electrophoresed and its Integrity Number (RIN) was calculated for each RNA pulp sample.

After the analysis, significant amount of dental pulp degra-

Keywords: Forensic Science, Forensic Dentistry, RNA Stability, Nucleic Acids, Molecular Biology.

المستخلص

زمن الوفاة هو الفترة الزمنية الممتدة ما بين وقوع الوفاة واكتشاف الجثة أو الرفات البشرية. إن بنية الأسنان هي أكثر بنية مقاومة في جسم الإنسان، فهي قادرة على تحمل العديد من الظروف مثل ارتفاع درجة الحرارة والرطوبة وتحلل الجسم بعد الوفاة.

تهدف هذه الدراسة إلى تقييم مدى قابلية تطبيق طريقة القياس الكمي لتدهور الحمض النووي الريبي المستخرج من اللب السني لتقدير زمن الوفاة، وذلك بمحاكاة ظروف الغرق حيث تكون الأسنان مغمورة في المياه العذبة على مدى فترات زمنية مختلفة.

تألفت عينة الدراسة من 80 سنًا بشريًا (الأضراس الثالثة)، وتم تقسيمها إلى ثماني مجموعات ووضعها في بيئة مائية لفترات محددة مسبقًا مدتها ثلاثة أيام و 1، 2، 3، 4، 8، 12 و 16 أسبوعًا. بعد انقضاء الفترات الزمنية المحددة تمت استعادة الأسنان وإزالة اللب السني واستخراج جزيء الحمض النووي الريبي وعمل تحليل لتدهور الجزيء.

الكلمات المفتاحية: علم الأدلة الجنائية، طب الأسنان الجنائي، استقرار الحمض النووي الريبي، الأحماض النووية، علم الأحياء الجزيئي.

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dation was observed showing a RNA RIN of 6.50. The 18S/28S ribosomal RNA ratio was null (with a value of zero), and only in sample, it was extremely low (0.8). The fact that the samples were submitt to the environment associated with that the low proportion the18S/28S ribosomal RNA found in the samples, may be essential factors to justify the results obtained.

RNA degradation quantification method was not applicable, since it was not possible to establish a relation between the degradation of the RNA molecule and the estimation of the post-mortem interval.

أظهرت نتائج التحليل أن أعلى قيمة لعدد سلامة الحمض النووي الريبي هي 6.50، كما أظهرت النتائج وجود جزيئات متدهورة، مما يشير إلى أن وضع العينات في البيئة المائية مع محاكاة الظروف اليومية الحقيقية قد يكون عاملًا رئيسيًا في تعليل النتائج التي خلصت إليها هذه الدراسة.

لم تكن طريقة القياس الكمي لتدهور الحمض النووي الريبي قابلة للتطبيق، حيث لم يكن من الممكن إنشاء علاقة بين تدهور جزيء الحمض النووي الريبي وتقدير زمن الوفاة.

1. Introduction

Post-mortem interval (PMI) is the time between death and the discovery of the body or human remains. Studies in the field of Forensic Medicine seek to clarify information about the context of death [1], in order to assist in the reconstruction of information in criminal cases, as well as enabling exclusion and limiting the number of suspects [2].

Since the PMI estimation is subjected to change according to the environment where the body or its remnants are found [3,4,5], therefore, it is necessary to test new methods by simulating real conditions and their applicability in medicolegal death investigations.

Dental tissues are the most resistant structures in the human body which can withstand the most varied environmental hazards such as high temperature, humidity, charredness, and advanced putrifaction stage to post-mortem degradation. Under these circumstances when other sources of DNA are partially or completely compromised, dental pulp RNA may offer an alternate source for forensic human identification [6,7,8]. The dental pulp is rich in fibroblasts and odontoblasts, containing low levels of RNase activity [9]. The pulp chamber serves as protection against several environmental factors. Conde et al. (2012) showed in their study that dental pulp is a tissue that can be a potential source for RNA integrity analysis [10].

RNA is an unstable and complex molecule [1] and shows faster postmortem degradation due to RNases enzymes [11]. Its degradation can be influenced by external factors, and the influence of a prolonged post-mortem interval can vary according to the tissue used [12]. Recently, RNA has gained the potential to estimate the post-mortem interval, but its degradation in the post-mortem period is a complex process that requires further studies [13].

The aim of the present study was to evaluate the applicability of the method of quantifying the RNA molecule degradation from dental pulp to estimate the PMI in drowning conditions.

2. Materials and Methods

The research was approved by the Research Ethics Committee (Certificate Number of Presentation of Ethical Appreciation: 67257617.4.0000.5440). Participants (donors of teeth extracted for therapeutic indications) were informed about the research. Written informed consent was obtained from each donor prior to the surgical intervention (dental extraction).

The sample consisted of 80 human teeth, only third molars (upper and/or lower). The inclusion criteria were third molars extracted for therapeutic indication, intact and healthy, with no fragmentation during the surgical procedure. After extraction, the following exclusion criteria were applied: if the

Table 1- *Period in which each group of teeth was submitted to the aquatic environment.*

Group	Period
Group 0	3 days
Group 1	1 week
Group 2	2 weeks
Group 3	3 weeks
Group 4	4 weeks
Group 5	8 weeks
Group 6	12 weeks
Group 7	16 weeks

teeth had periapical pathologies, open apices, dental caries, fractures in crown and/or root regions, endodontic treatments, restorative treatments and/or prosthodontics. To obtain perfect samples, the researcher was present at the time of tooth extraction to apply the exclusion criteria correctly.

In order to standardize each group created, the sample was divided as follows: eight groups of ten teeth each, corresponding to the periods in the aquatic environment (Table-1). The teeth were stored in a -80°C freezer (Thermo Scientific Forma 8600 Series, Thermo Fisher ScientificTM, São Paulo, Brazil) to prevent the RNA degradation [1] until sufficient number of teeth was obtained to place it in its respective group, and subsequently placed in the aquatic environment. To simulate the conditions for drowning, the sample groups were submerged in a freshwater lake, and this condition was chosen because it was available for research at the time of the study in the university campus.

Polyester bags (16x13 cm) were made to store each group, in order to avoid material loss in the lake. The bags were identified by groups and periods and placed inside a birdcage tied by chains to the trees around the lake, and thrown into the lake.

After removal from the lake, the dental pulp was removed using dental carbide drills nº 701 and a high-speed motor to section the teeth. Then, a 2mm channel was made below the cementoenamel junction without completely separating the crown and the root, and the total separation was performed with the aid of surgical forceps nº 151, in order to expose the dental pulp. With endodontic files nº 10 and 15, and dentin digger nº 16, the coronal and root dental pulp of the teeth were removed and stored in individual microcentrifuge tubes containing 0.5 mL of RNAlater™ (Thermo Fisher Scientific™, São Paulo, Brazil). The microcentrifuge tubes containing the RNAlater solution and the respective dental pulp were stored at -20°C until the RNA extraction was performed.

The process of isolating the RNA molecule started with treating all instruments, materials and workbench treated with RNase Zap (Thermo Fisher Scientific™, São Paulo, Brazil) to prevent molecule degradation.

To enable the use of any piece of dental pulp that was present in the microcentrifuge tubes, the tubes were centrifuged at 4ºC for 5 minutes at 10.000g (gravitational force). The RNA molecule was extracted and isolated using the MagMAX™ mirVana™ Total RNA Isolation Kit (Thermo Fisher ScientificTM, São Paulo, Brazil) on the automated liquid handling platform Microlab NIMBUS (Hamilton CompanyR, Reno, United States of America).

The evaluation of the RNA integrity was done using RNA 6000 Nano kits (AgilentTM, California, United States of America) that uses electrophoresis

Table 2- *Samples that contained pulp visible to the naked eye in each group.*

Groups	Number of dental elements with pulp visible to the naked eye
0	10
1	7
2	3
3	5
4	0
5	0
6	2
7	5

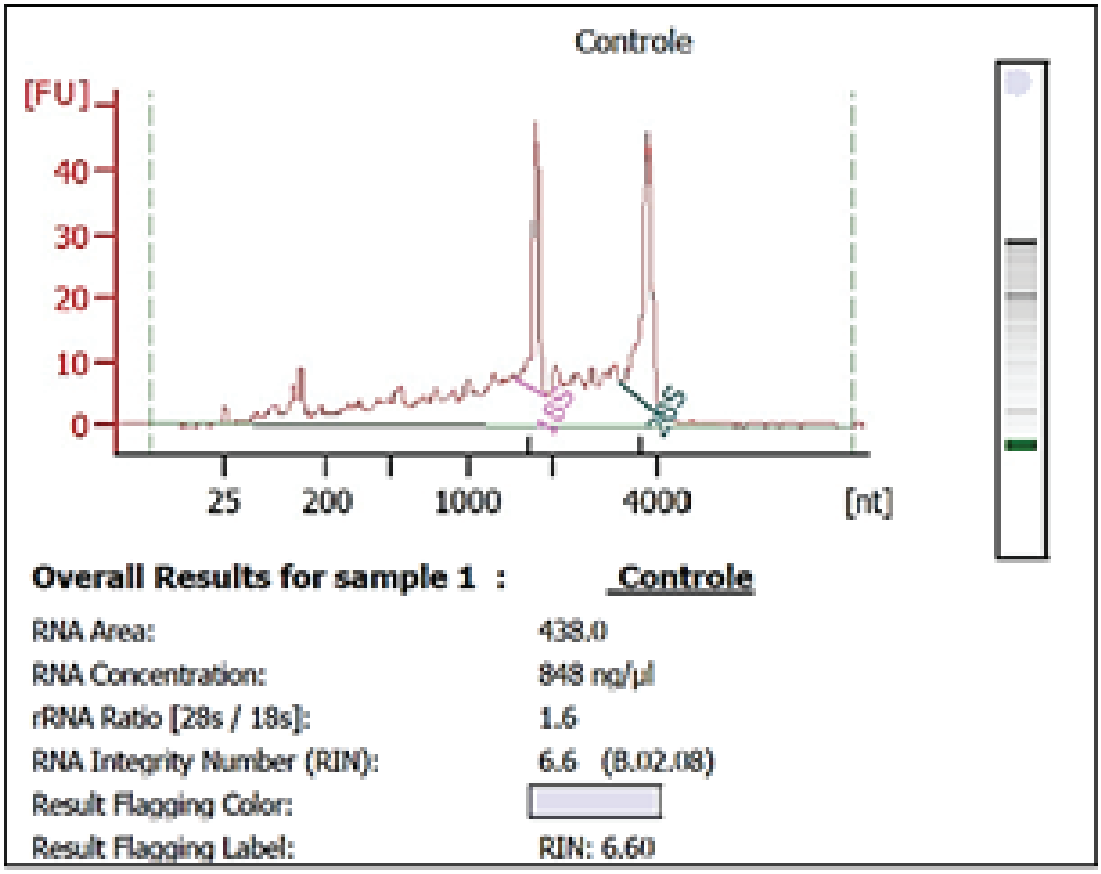

Figure 1- Result of the standard sample after analysis by the Agilent 2100 Bioanalyzer software (Agilent, California, United States).

based microfluidic chip for their analysis. The samples were analyzed with the Agilent 2100 Bioanalyzer system (Agilent™, California, United States of America), and the RNA integrity number (RIN) was calculated using the Agilent 2100 Expert software (Agilent™, California, United States of America). The RIN values range from 10 (intact) to 1 (totally degraded) [14].

2.1 Statistical Analysis

A descriptive analysis was performed, showing the RIN values obtained through graphs and tables.

The relationship between the variables was demonstrated by the linear graph scatterplot.

3. Results

After removal of the teeth from the simulated drowning conditions, the dental pulp could be seen in few samples, as shown in Table-2. In the samples that did not have apparent pulp tissue, scraping was performed on the internal walls, with dentin curette nº 16, to collect any remaining pulp.

Initially, a standard sample was prepared to be used as a control to ensure that all experimental steps were carried out efficiently. The control was prepared and analyzed in the same way, using the same material and equipment as used for samples. The control sample consisted of rat gums, which were not exposed to the environment, and were kept in the RNAlater solution. This sample showed a low 18S/28S ribosomal RNA ratio of 1.6, but had a RIN of 6.60, showing more than 60% integrity of the RNA molecule (Figure-1).

From the 80 samples, RIN results were obtained from only 30 samples, whereas in the rest of the samples the pulp was not enough to quantify RNA degradation, even though of these teeth had pulp visible to the naked eye. The values of degradation obtained in each group and their respective periods are described in Table-3.

In the samples that presented higher RIN values in each group, the 18S/28S ribosomal RNA ratio was null (with a value of zero), and except one where it was extremely low (0.8), demonstrating no relation between them. The results obtained in the present study demonstrated that it was not possible to establish a relation between the RIN and post-mortem interval (Figure-2).

4. Discussion

Existing methodologies for PMI estimation are often constrained in their implementation due to a degree of inaccuracy [9,15], and since there are several variables that can influence post-mortem changes, they only convey an approximation of PMI [15].

In this research, it was decided to use dental pulp, which according to Poór et al. (2016) is considered having low levels of RNase activity and well-protected from environmental factors by a structural framework (enamel, dentin and cement) [9].

The teetth samples in the present study were stored at -80°C before being placed in the aquatic environment to avoid RNA degradation. Conde et al., (2012) tested four tooth storage temperature-based approaches prior to pulp removal and RNA integrity analysis - liquid nitrogen, -80 °C, -20 °C and 4 ºC, and demonstrated a well-preserved RNA integrity in all storage conditions [10].

Costa (2018) carried out a pilot study to assess

Table 3- RNA integrity numbers (RIN), obtained for each group according to the period of immersionin the water.

Period	RNA integrity number (RIN)					
3 days	1,7	1,9	2,1	2,4	-	-
1 week	1,5	1,7	3,1	6,5	-	-
2 weeks	1,2	5,5	-	-	-	-
3 weeks	2,3	2,4	3	-	-	-
4 weeks	1,4	1,5	2,1	2,1	-	-
8 weeks	1	1,2	1,2	1,4	1,5	2,1
12 weeks	1,3	1,4	-	-	-	-
16 weeks	1,5	1,9	1,9	2	2,3	-

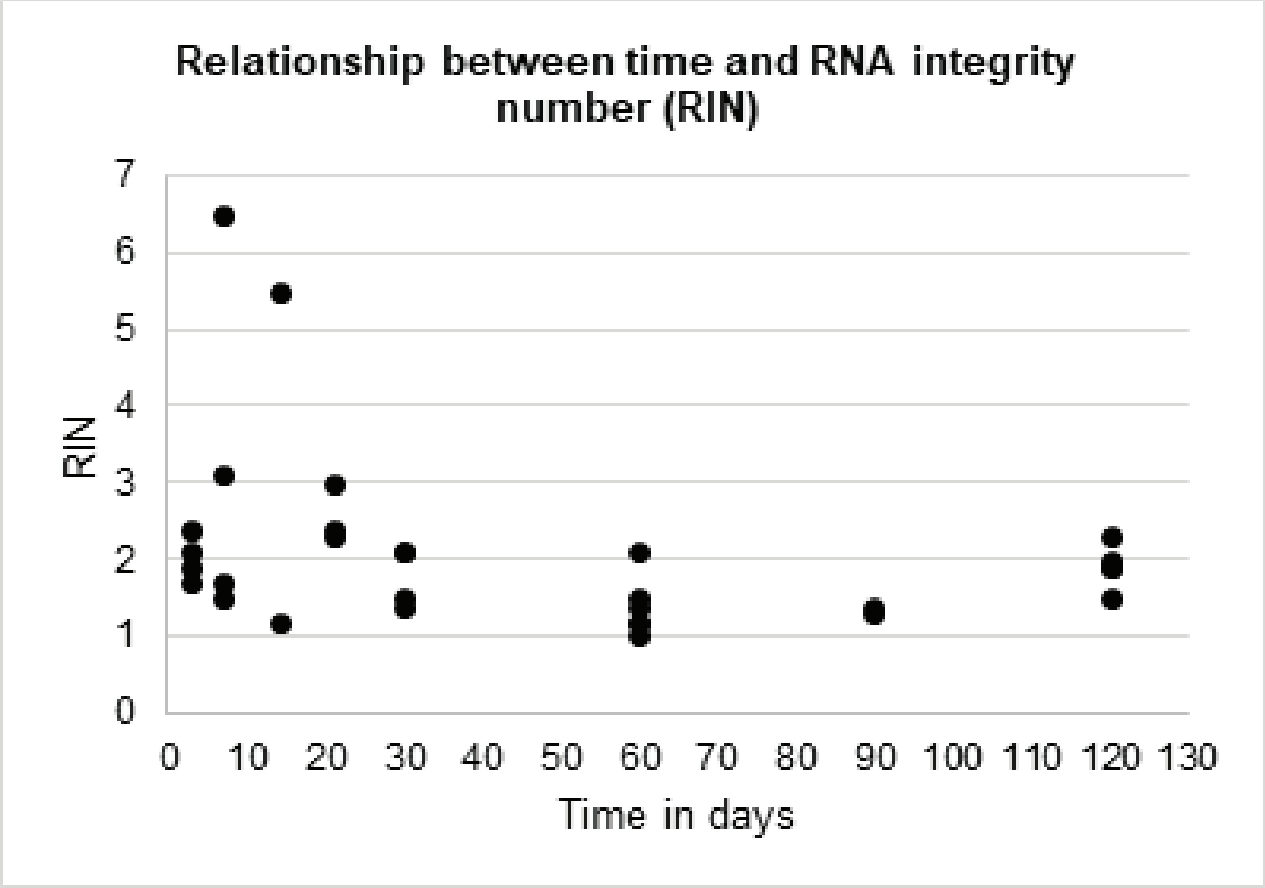

Figure 2- Graph of linear dispersion, showing the lack of linearity between the variables.

whether freezing teeth at -80°C would interfere with the behavior of the RNA molecule. The teeth were frozen for a period of three months before being compared to a series of teeth that were examined shortly following surgical extraction without being frozen. The findings were comparable between the two classes, with frozen samples reaching RIN=7.30 and the "fresh" sample RIN=8.0. As a result, it seems that freezing has no effect on the process [3].

Costa (2018) also demonstrated that samples which were not exposed to the enviroment presented higher RIN values as compared to the samples submitted to the simulated inhumation condition that produced further RNA degradation [3]. This fact corroborates the results of the present study, since the samples were placed in an aquatic environment, in order to simulate real conditions, and the results showed enhanced RNA degradation.

The RNA degradation occurs mainly by the enzymatic action of ribonucleases (RNases), both in cadavers and in different body parts, which are ubiquitous and have been considered responsible for the rapid degradation of RNA [16,17]. However, in humidity conditions, such as dehydrated tissues and dried bloodstains, it is possible that this activity could be altered, and the integrity of this RNA molecule prolong [3].

In the presence of cations such as Ca2+, transitional metals, and alkaline conditions, the RNA molecule is more vulnerable to hydrolysis. Several factors could limit the half-life of nucleic acids. Internal factors such as the nature of the bases, sugars, and phosphate, as well as external ones (pH, the

presence of oxygen and water), and these factors are distinct in each post-mortem situation [18].

Exposure of the samples to moisture and water can accelerate RNA degradation, which may be one of the reasons for the intense degradation observed. Other factors that contribute to RNA degeneration, such as pH and water salt content, should be studied.

Several research [3,9,16,17,19,20,21] used various methodologies and samples to explore changes in the RNA molecule and its use for PMI estimation. Unlike the current study, some of them reported positive results when they investigated the degradation of the RNA molecule [9,16,19,20]. However, we found studies that yielded unfavorable results [3,17,21]. The studies, however, used a variety of samples with varying storage, extraction, and analysis conditions.

The present study's findings revealed that there was no relation between the degradation of the RNA and PMI (Figure-2). Our results do not agree with Poór et al., (2016), who used premolar and third molar pulps to correlate RNA degradation with time, and reported that the method, despite its limitations, is promising with regard to PMI estimation [9]. This result may be associated with the fact that the samples in their study were kept at controlled environment and were not exposed to the natural environment.

Of all the values obtained, it was not possible to establish a relation with PMI, making it impossible to relate whether samples that remained for a shorter period in the established condition had a more intact RNA molecule, and those that were kept for a longer period contained more degraded molecules or vice versa. Poór and colleagues (2016) were able to correlate RIN values with PMI, but only with samples that were stored for periods of a maximum of 21 days [9].

The presence of visible pulp at the time of extraction did not affect the results. Some of the samples with visible pulp could not be read, and samples that did not contain visible material showed some RIN values.

Another important factor for determining good RNA quality is the 18S/28S ribosomal RNA ratio, which must be greater than or equal to 2.0 [22]. As a result, this characteristic also interfered with the research analysis, where we found a null relation (with value of 0.0) and only one extremely low relation of 0.8 in the samples that presented higher values of RIN in each group.

According to Poór et al. (2016), RINs ranging from 1.0 to 2.40 indicate that we are dealing with highly damaged molecules that can not be considered [9]. Thus, based on our findings, we could only rely on four feasible samples, and the overall time of research capability in the current study would be three weeks.

Great obstacles in the methodology used in this study are confirmed by the findings and in light of all the aspects examined. Among them are the use of third molars to extract dental pulp, the enzymatic activity of ribonucleases, and the fact that the samples were exposed to the environment, simulating real-world environments, as seen in other experiments [16,19,20]. This last factor, associated with the lack of an 18S/28S ribosomal RNA relationship (characterizing very poor-quality RNA), can be characterized as fundamental in justifying the findings of this study.

5. Conclusion

The RNA degradation quantification method was not applicable in estimating PMI, because it was not feasible to establish a relationship between the degradation of the RNA molecule extracted from third molar pulps placed in the aquatic enviroment for dif-

ferent periods and the PMI estimation.

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Conflits of interest

None

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