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ROLE OF CELL FREE DNA AND HMGB-1 IN POSTMORTEM INTERVAL DETERMINATION

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Abstract: Postmortem interval (PMI) determination is essential for criminal and civil cases concerning forensic death. Today, many methods that are in practice are subjective, without a conclusive result. With the increasing use of molecular studies in forensic sciences, key studies have been conducted especially in the field of Forensic Genetics. This study aimed to determine PMI by utilizing early postmortem changes in cell free DNA and serum HMGB-1 protein levels that particularly increase in serum with cell necrosis.

The study was carried out on 96 Wistar rats, weighing 230–260 g. The rats were kept at two different temperatures, i.e. +4°C and +24°C, after anesthesia and cervical dislocation. Blood samples were collected during autopsy at hours 0, 3, 6, 9, 12, 24, 48 and 72. The blood was centrifuged for five minutes at 5000 rpm to separate the serum. HMGB-1 concentration measurement in serum samples using HMGB-1 ELISA kit and SYBR Gold Nucleic Acid Gel Stain nucleic acid staining protocol was followed by optical density measurement with a luminometer for cell free DNA.

The results obtained in the study were converted into concentration values by equations obtained from standard samples. It was found that there was an increase in cell free DNA and HMGB-1 level in serum in parallel to the increasing length postmortem interval at +4°C ([$r = 0.751$ $p < 0.001$] and [$r = 0.698$ $p < 0.001$], respectively). At +24°C, postmortem interval was found to have a negative correlation with the amount of cell free DNA in serum ($r = -0.213$ $p = 0.15$) and a weak positive correlation with serum HMGB-1 concentration ($r = 0.313$ $p = 0.030$).

In conclusion, our study was the first experimental study to investigate cell free DNA and serum HMGB-1 levels together in postmortem interval determination. Our findings suggest that serum cell free DNA and serum HMGB-1 levels at +4°C, where decay is particularly less effective, can be used in the determination of PMI.

Keywords: Postmortem interval, HMGB-1, Cell Free DNA, Time of death, ELISA.

INTRODUCTION

One of the common requests faced by forensic experts during the investigation of forensic deaths is to determine the exact and accurate time of death [1]. Unless there is an eyewitness to death, the time from the actual time of death to the time when the body was found cannot be determined precisely. However, with the investigation of postmortem changes, a time interval can be estimated, which includes the actual time of death [2]. After death, there are many physical, metabolic, autolytic and biochemical processes occurring in the body. The changes that are observed as a result of these processes continue regularly until body integrity is disrupted and are of great importance in determining the postmortem interval [3]. Various methods such as algor mortis, i.e. cooling of the body after death, livor mortis, i.e. discoloration of the skin after death, rigor mortis, stiffening of the muscles after death, ophthalmological changes, changes in gastric contents, plasma and blood cells, biochemical changes in body fluids such as the vitreous fluid, pericardial fluid, synovial fluid and cerebrospinal fluid, bone marrow changes and entomological findings have been used in Postmortem Interval (PMI) prediction. These methods are influenced by many unforeseen factors and based on subjective findings, which cause challenges in determining the exact time of death by taking advantage of these findings. Therefore, in recent years, various studies have been carried out in the molecular field in

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order to make a more objective determination [4-6].

Nucleic acids that freely circulate in peripheral blood due to cell death caused by apoptosis and necrosis have been used as a potential marker of tissue damage [7]. Under normal circumstances, healthy individuals have a small amount of cell free DNA in their blood. However, high values have been reported in patients with various clinical conditions such as infection, inflammation, cancer, connective tissue diseases, ischemic stroke, myocardial infarction, pregnancy-related disorders and hemodialysis [8, 9]. In the postmortem process, DNA protected by the nucleus and cell membranes is expected to be released as a result of cell necrosis. Particularly within the first 72 hours of postmortem interval, the time-dependent increase in cell necrosis can cause possible changes in the amount of cell free DNA and the amount of cell free DNA can be an important marker in postmortem interval determination.

Another molecular marker studied in the determination of PMI is the High Mobility Group Box -1 (HMGB-1) protein [10]. High mobility group (HMG) B proteins, which are intranuclear and cell regulatory proteins, regulate numerous activities such as transcription, replication and repair [11]. HMGB-1 protein is actively released from immune cells such as macrophages, monocytes, NK cells, dendritic cells, endothelial cells and platelets, in addition to their passive release from necrotic or damaged cells and apoptotic cells [12]. Similar to the amount of cell free DNA, this suggests that HMGB-1 protein can be used as a marker in PMI determination.

As a result of the literature review, it was found that there were a few molecular studies about the determination of PMI. In this study, it was aimed to investigate the amount of cell free DNA and HMGB-1 protein, the time-dependent change thereof and the correlation of cell free DNA and HMGB-1 protein levels for PMI determination.

MATERIAL AND METHOD

This study was an experimental study and it was approved by Ankara University Animal Experiments Local Ethics Committee (number 2014-6-35). This study was also supported by Ankara University Scientific Research Projects Coordination Department with Project Number 14L0230005.

Sampling Method

In the study, adult Wistar rats weighing 230-260 g were used as a model system. The rats were reproduced in the Experimental Animals and Research Laboratory of Ankara University School of Medicine and grown under 12 hours of artificial day/night cycle in the presence of sufficient water and feed. 120 mg/kg ketamine+10 mg/kg xylazine was administered intraperitoneally for anesthesia. The rats were sacrificed by cervical dislocation after providing a sufficient level of anesthesia. Cervical dislocation was preferred to prevent affecting the cell free DNA and HMGB1 protein levels in serum, which constitute the main objective of our research.

Sample size

It was estimated that the standard deviation in the calculation of the sample size would be around ± 0.05. The sample size was calculated by taking into account that the significance level of the difference between calculated mean values would be 0.03 and below. Power = 0.80, alpha = 0.05 and p (1-Beta) = 0.20 values were used with the formula $n > (2X (z_1+z_2)^2 \sigma^2)/d^2$ = to calculate $2x7.8x (0.05)^2 /(0.03)^2 = 44$. Considering a 1% experimental animal attrition rate, 48 experimental animals were included for each temperature value, i.e., a total of 96 experimental animals.

Collection of Serum Samples

For the determination of PMI, the rats were sacrificed by cervical dislocation after providing a sufficient level of anesthesia. To investigate whether temperature had an impact on the degradation of cell free DNA and HMGB-1 protein and whether there would be any difference in terms of PMI determination at different temperatures, the sacrificed experimental animals were kept at two different ambient temperatures, i.e. 4˚C and 24˚C.

At 4˚C and 24˚C, the sacrificed rats were divided into 8 separate groups and blood was collected from intracardiac and/or large veins during autopsy at hours 0, 3, 6, 9, 12, 24, 48 and 72. The collected blood was centrifuged for 5 minutes at 5000 rpm without waiting for more than 15 minutes and the serum was separated. The obtained serum samples were kept at -80 C until analyzed. The grouping of experimental animals according to ambient temperature and postmortem interval is provided in Table 1.

Four serum samples were excluded from the study in the calculation of cell free DNA levels, because a sufficient amount of blood could not be collected from one rat at +4 °C at hour 9, from another rat at hour 12, from another rat at hour 24 and from one rat at 24 °C at hour 72.

Table 1. Grouping of experimental animals according to ambient temperature and postmortem interval

Group No:	4˚C postmortem time	24˚C postmortem time	Number of animals per group
1	4˚C-0	24˚C-0	6
2	4˚C-Hour 3	24˚C-Hour 3	6
3	4˚C-Hour 6	24˚C-Hour 6	6
4	4˚C-Hour 9	24˚C-Hour 9	6
5	4˚C-Hour 12	24˚C-Hour 12	6
6	4˚C-Hour 24	24˚C-Hour 24	6
7	4˚C-Hour 48	24˚C-Hour 48	6
8	4˚C-Hour 72	24˚C-Hour 72	6

Determination of HMGB-1 Protein Levels in Serum

HMGB-1 ELISA (Enzyme-Linked Immunosorbent Assay) (Code No: ST51011, IBL International GmbH, Hamburg, Germany) kit was used to measure HMGB-1 protein levels. Standard solutions and the contents of the kit that were in lyophilized form prior to the test were prepared in accordance with the procedure. All steps were carried out in accordance with the protocol. 10 microliters of the positive control, standards and each serum sample were added to the respective wells. Optical density was measured with the Eliza reader using a 450 nanometer wavelength and a reference wavelength of 600-650 nanometers.

Determination of the Amount of Cell free DNA from Serum

In this study, SYBR Gold Nucleic Acid Gel Stain (Catalog no: S11494, Life Technologies, CANADA) nucleic acid staining method, which is commonly employed in molecular biology and genetics, was used for the determination of cell free DNA level in serum. In the preparation of DNA standards, 0.02 grams of Salmon Sperm (Product code: D1626-250MG, Sigma-Aldrich Co. LLC, 3050 Spruce Street, St. Louis, MO USA) was mixed with 10 mL of PBS. 16 salmon sperm standard DNA solutions were prepared by diluting the resulting solution in certain proportions. DNA isolation was performed after collecting 1 tube of blood sample for human DNA sample. DNA levels were detected by a NanoDrop spectrophotometer. 16 human DNA standard solutions were prepared by diluting the resulting solution. SYBR Gold was diluted with Dimethyl sulfoxide (DMSO) at a ratio of 1:1000. The resulting solution was diluted with phosphate buffered saline (PBS) at a ratio of 1:8. 10 microliters of serum samples and standards were placed in the corresponding wells of a 96-well plate. Optical density measurement was performed at an emission wavelength of 535 nm and excitation wavelength of 485 nm using a 96-well luminometer.

A prediction equation Level = a (Optical Density) + b was obtained by applying a linear regression analysis to the optical densities and standards with known quantities using Microsoft Office 2016, in order to convert the optical densities that were read for HMGB-1 and cell free DNA levels into nanograms. The predictive power of the prediction equation (R^2) was found to be 99.81% in HMGB-1 standards and 96.36% in cell free DNA standards. Prediction equations were applied to the optical density values of each serum sample and HMGB-1 protein and cell free DNA levels were calculated in ng/mL.

Statistical Analysis

The data obtained in the study was evaluated using IBM SPSS 20 (Statistical Package for the Social Sciences) software package.

Mann Whitney U and Kruskal Wallis tests were used in the analysis of the data. The time-dependent change in HMGB-1 protein and cell free DNA levels was analyzed with the Spearman correlation test. $P < 0.05$ was considered statistically significant.

RESULTS

In the study, serum HMGB-1 protein levels exhibited an increase in parallel to the postmortem time at +4 and +24°C and there was a weaker correlation at +24°C ($r=0.698$ $p<0.001$; $r=0.313$ $p=0.030$, respectively). The relationship between serum HMGB-1 protein levels at +4°C and +24°C with postmortem time was presented in Figure 1 and Figure 2, respectively.

In the postmortem period, serum HMGB-1 protein levels in blood samples taken at the same time after incubation at two different temperatures did not exhibit a significant difference at hour 3, 6, 9 and 24 ($p=0.485$, $p=0.394$, $p=0.937$ and $p=0.394$, respectively). Serum HMGB-1 protein levels at hour 12 were higher at 24°C ($p=0.004$) and higher at 4°C at hours 48 and 72 ($p=0.026$ and $p=0.041$ respectively). Comparison of the changes in serum HMGB-1 protein levels at 4°C and

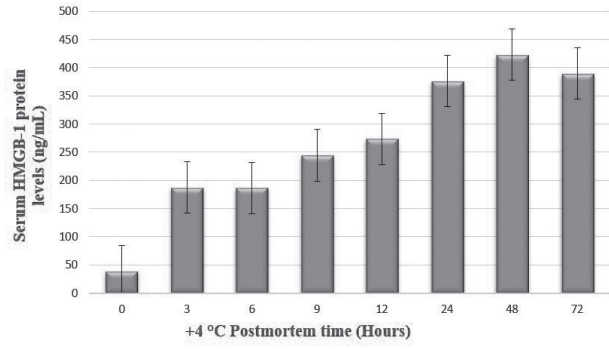

Figure 1. Relationship of serum HMGB-1 protein levels with postmortem time at +4°C.

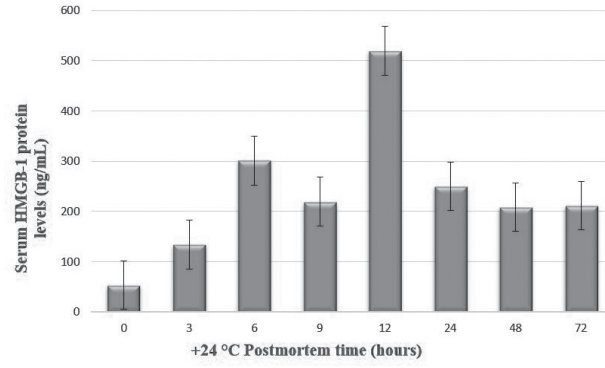

Figure 2. Relationship of serum HMGB-1 protein levels with postmortem time at +24°C.

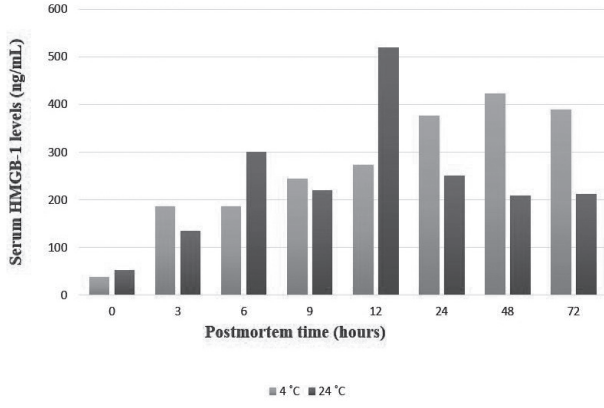

Figure 3. Comparison of serum HMGB-1 protein levels at +4 and +24 °C.

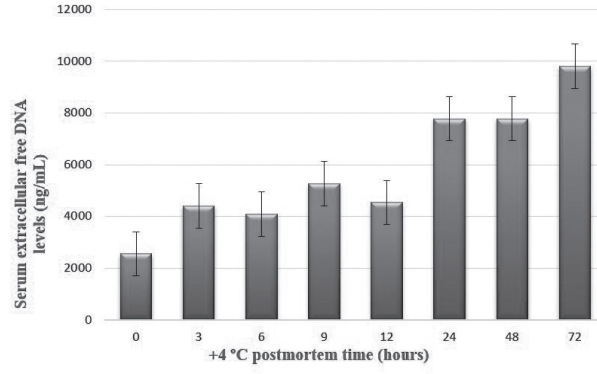

Figure 4. Relationship between cell free DNA levels and postmortem time at +4 °C.

24°C with postmortem time is provided in Figure 3.

In the study, cell free DNA levels at +4°C showed a strong positive correlation with time (r=0.751 p<0.001). The relationship between postmortem time and cell free DNA levels at +4°C is provided in Figure 4.

It was found that cell free DNA levels increased at +24 °C between postmortem hours 3-9, but a longer postmortem interval led to lower levels of cell free DNA (r = -0.213 p=0.15). The relationship between cell free DNA levels and postmortem time at +24 °C is provided in Figure 5.

In the study, considering serum cell free DNA levels assayed in blood samples incubated at different temperatures (+4°C and +24°C) at the same time during the postmortem period; while the said levels did not exhibit a statistically significant difference at hours 3, 12 and 24, cell free DNA levels were higher at 24°C at hours 6 and 9 (p=0.02; p=0.04, respectively). Moreover, cell free DNA levels at 4°C were found to be higher at hours 48 and 72 (p=0.026; p=0.04, respectively). Comparison of the change in cell free DNA levels in serum at +4°C and +24°C according to postmortem time is provided in Figure 6.

It was found that serum HMGB-1 protein and serum cell free DNA levels at showed a positive correlation +4°C (r=0.587 p<0.001), whereas this correlation was not observed at +24°C (r=0.093 p=0.533). Correlations of serum HMGB-1 protein and serum cell free DNA levels at +4°C and +24°C are provided in Figure 7 and Figure 8.

DISCUSSION

One of the important areas of forensic medicine is the determination of PMI. Today, traditional methods are more frequently used to determine PMI, and the fact that these methods can be influenced by many factors can lead to different results. This makes it clear that new methods are necessary for the determination of PMI. Therefore, this study aimed to investigate the amount of cell free DNA and HMGB-1 protein levels, the time-dependent change in these levels as well as the correlation of cell free DNA and HMGB-1 protein levels with time with the purpose of PMI determination.

It was suggested that the HMGB-1 protein played a key role in the coordination of cell damage

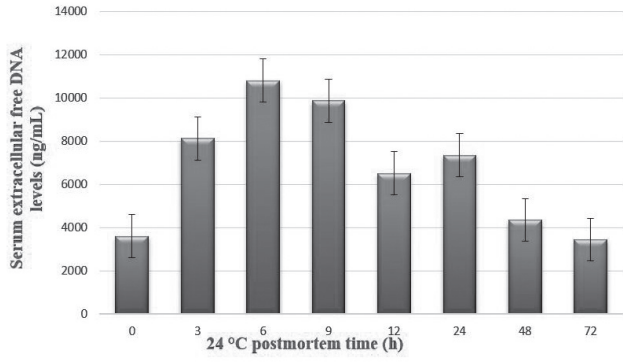

Figure 5. Relationship between cell free DNA level at +24 °C with postmortem time.

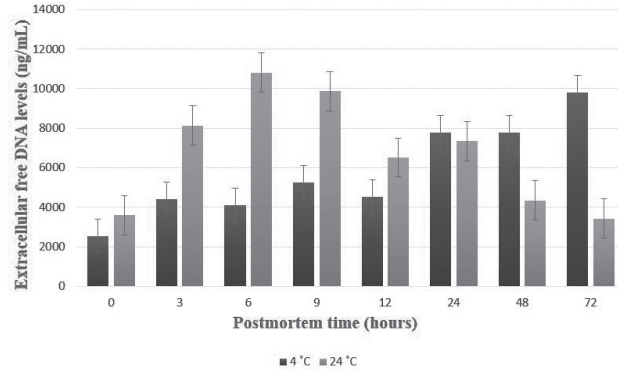

Figure 6. Comparison of serum cell free DNA levels at +4 and +24 °C.

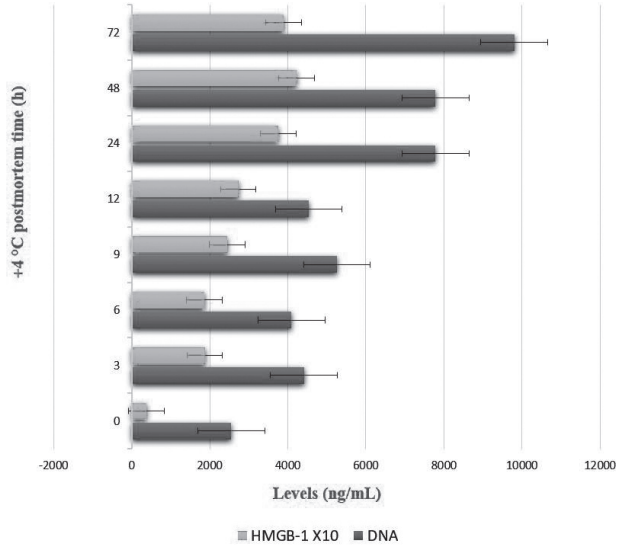

Figure 7. Correlation of cell free DNA and HMGB-1 protein levels with time at +4 °C.

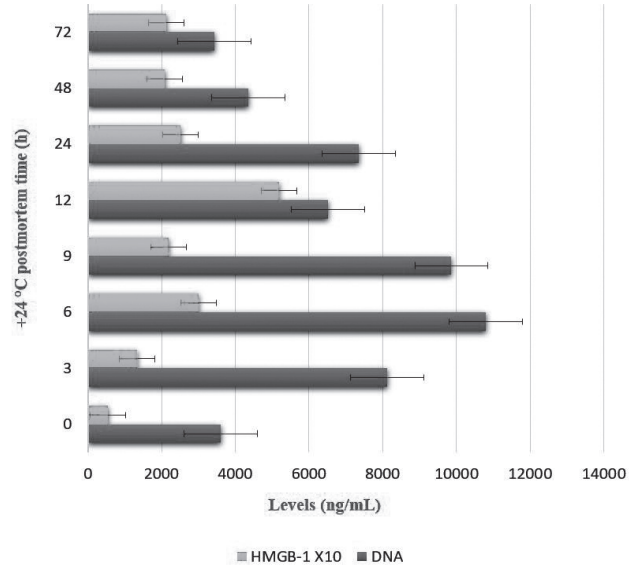

Figure 8. Correlation of cell free DNA and HMGB-1 protein levels with time at +24 °C.

signaling, inflammation and tissue repair. It is passively released from cells during cell damage and ischemia [13]. Taking into account this characteristic of the HMGB-1 protein; the level of serum HMGB-1 may also increase due to the increasing number of cells undergoing necrosis as a result of a longer postmortem interval, since tissues exhibit different sensitivity to oxygen during cell death.

In an experimental study where Chen *et al.* investigated the relationship of HMGB-1 release with time after hypoxic ischemia damage in newborn rats; it was reported that serum HMGB-1 levels were significantly elevated 3 hours after hypoxic ischemia, continued to increase up to 12 hours, began to decrease after 24 hours, and returned to normal levels between 24-48 hours [14]. In our study, it was found that serum HMGB-1 levels increased as the postmortem period was prolonged at +4°C. At +24°C, however, the said levels showed an increase until postmortem hour 12, then reached the peak value at 12 hours, after which they started to decline, wherein the rate of increase in HMGB-1 protein levels at both temperatures was the highest within the first three hours. Hypoxic ischemia can lead to cell death by various mechanisms, such as necrosis and apoptosis, just like cellular death that continues in the postmortem process. In a study by Chen *et al.*, serum HMGB-1 level was found to be around 25 ng/dL, while in our study the highest serum HMGB-1 level was 519.7 ng/dL at 24°C. This may stem from the fact that the amount of HMGB-1 released in cell necrosis is higher than that released in apoptosis, and the number of necrotic cells increases as cellular death continues in the postmortem process.

Similar to our study, in a study conducted in Japan by Kikuchi *et al.*, increased serum HMGB-1 levels were observed at +4°C in parallel to the postmortem

time, and it was reported that the said levels reached the peak value on day 2 at +24ºC, after which they started to decline until day 7 [10]. This suggests that the serum HMGB-1 level may be an important parameter for PMI determination. One of the most important external factors affecting decay is temperature [15]. Since the decay rate at 24ºC will be higher than the decay rate at +4ºC, the structure of the HMGB-1 protein may be disrupted, and its level may have declined after reaching its peak value within a shorter period of time.

The level of HMGB-1 protein in blood was also shown to be elevated in clinical conditions such as sepsis, acute and chronic infections, hemorrhagic shock, trauma, pancreatitis, rheumatoid arthritis, mycobacterium tuberculosis infection, plasmodium falciparum malaria, cancer, myocardial and cerebral ischemia, autoimmune and chronic renal failure, in addition to the postmortem process [16]. In studies conducted on different groups of patients, patients with septic shock, and myocardial and cerebral ischemia were shown to have higher HMGB-1 protein levels in blood than healthy individuals and it was reported that the amount of passive HMGB-1 release in ischemic conditions was higher than that of active HMGB-1 release in inflammatory conditions [15,16]. This suggests that the level of HMGB-1 protein may be elevated due to prolonged PMI, as well as the underlying cause of death. In a study by Kikuchi *et al.*, the reported postmortem serum HMGB-1 levels were high, similar to the values found in this study, and it was stated that the cause of death did not affect serum HMGB-1 levels [13]. The reason for the different results reported in the literature may be the changes in employed temperatures, duration and environmental conditions, and the presence of different underlying unknown diseases. Therefore, more extensive studies are needed for the use of HMGB-1 protein in forensic practice.

Recently, it was discussed whether nucleic acids circulating freely in peripheral blood and resulting from cell death caused by necrosis and apoptosis could be used as a potential marker of tissue damage [7]. Under normal circumstances, there is a small amount of cell free DNA in the blood of healthy individuals, whereas the said levels can be elevated in clinical conditions such as infection, inflammation, cancer, connective tissue diseases, ischemic stroke, myocardial infarction, conditions associated with pregnancy and hemodialysis, which could be a negative prognostic marker for morbidity/mortality [8, 9]. In a study conducted by Gautschi *et al.* with subjects with and without lung cancer, it was reported that there was a positive correlation between increased plasma and serum DNA levels and the stage of the tumor [17]. The amount of cell free DNA has been reported to be used in the diagnosis of aneuploid conditions such as down syndrome and pregnancy complications such as preeclampsia, intrauterine growth restriction and preterm labor [18-21]. In a study conducted by Manokhina *et al.*, it was reported that the amount of cell free placental DNA in the mother's plasma was elevated in parallel to the increasing gestational age and rapidly dropped to zero after childbirth [22]. As the gestational age increases, the fetus will grow, the number of cells will increase, leading to a higher number of cells that pass through the placenta and a higher number of cells undergoing necrosis, hence the amount of cell free DNA is also expected to increase.

In a study, Vandewoestyne *et al*. investigated the amount of cell free DNA on various forensic biological samples, and reported that they could detect cell free DNA at a rate of 90.9% in blood samples, 50% on cigarette butts, and 100% on nail cleaners [23]. The literature review showed that our study is the first study regarding the utility of cell free DNA level in the determination of PMI.

In this study, it was found that serum cell free DNA levels exhibited a linear increase in parallel to the postmortem time from hour 0 to 72 in rats kept at +4ºC. On the other hand, serum cell free DNA levels reached the peak value at hour 6, after which they started to drop in rats kept at +24ºC. In both temperature conditions, cellular death reached the peak value in the first three hours of the postmortem period. At +24ºC, serum cell free DNA levels reached the peak value at hour 6, and then declined until hour 72, which was attributed to the rapid degradation of DNA at +24ºC and the detection of DNA in smaller quantities in analyzes conducted after 6 hours.

In conclusion, our study is a preliminary experimental study investigating the utility of HMGB-1 and cell free DNA levels in PMI determination. In particular, the increase in serum HMGB-1 protein and cell free DNA levels at +4 ºC in correlation with time in the postmortem process suggest that both materials can be used to determine PMI. At higher temperatures, further studies are needed in which environmental conditions are evaluated in more detail, as the effect of decay will be greater. Due to the importance of PMI determination in Forensic Medicine practices, the search for methods that provide more precise results is still ongoing.

Conflict of interest

The authors declare that they have no conflict of interest.

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