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Estimating the Postmortem Interval by the Difference Between Oxidant/Antioxidant Parameters in Liver Tissue

Określanie czasu zgonu za pomocą różnicy między wskaźnikami oksydacyjnymi i antyoksydacyjnymi w tkance wątroby

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. So far the authors could not find any published paper that shows the presence of a possibly significant relationship between oxidant/antioxidant parameters and time of death.
Objectives. This study sought to investigate whether the differences between the levels of endogenous parameters like superoxide dismutase (SOD), malondialdehyde (MDA), glutathione peroxidase (GPx), glutathione S-transferases (GST), total glutathione (GSH), catalase (CAT) and nitric oxide (NO) have a relationship with the early postmortem interval (EPI).
Material and Methods. A total of 60 Albino Wistar male rats weighing between 220–230 grams were obtained. The rats were divided into six subgroups (n = 10), fed at room temperature (22^{0}C) and then decapitated. The livers of the dead rats were extracted at intervals of one hour and biochemical examinations were performed and biochemical results obtained from the animal groups had been evaluated.
Results. The authors performed linear regression analysis in order to search for factors predicting the time of death. The time of death was taken as a dependent variable with SOD, MDA, GPx, GST, GSH, CAT, and NO as independent variables. This model resulted with a high predictive value (adjusted R square = 94.0%). SOD, GST and NO were found to be significant factors independently affecting the prediction of the death time.
Conclusions. In conclusion, the results obtained from the experiment showed that the oxidant and antioxidant parameters are important for estimating the EPI (**Adv Clin Exp Med 2012, 21, 6, 727–733**).

Key words: early postmortem interval, oxidants, antioxidants, liver, rats.

Streszczenie

Wprowadzenie. Dotychczas nie ukazała się publikacja, która opisywałaby potencjalnie znaczący związek między wskaźnikami oksydacyjnymi i antyoksydacyjnymi a czasem zgonu.
Cel pracy. Zbadanie, czy różnice między stężeniem wskaźników endogennych, takich jak dysmutaza ponadtlenkowa (SOD), aldehyd malonowy (MDA), peroksydaza glutationowa (GPx), glutation S-transferazy (GST), całkowity glutation (tGSH), katalaza (CAT) i tlenek azotu (NO) mają związek z wczesnym czasem zgonu (EPI).
Materiał i metody. Do badań włączono 60 szczurów rasy Albino szczepu Wistar płci męskiej o masie ciała 220–230 g. Szczury podzielono na 6 podgrup (n = 10), karmiono w temperaturze pokojowej (22°C) i uśmiercono za pomocą dekapitacji. Wątroby martwych szczurów ekstrahowano w odstępach godzinnych, przeprowadzono badanie biochemiczne i oceniono wyniki uzyskane w poszczególnych grupach zwierząt.
Wyniki. Przeprowadzono analizę regresji liniowej w celu znalezienia czynników, które pozwoliłyby przewidzieć czas zgonu. Czas zgonu przyjęto jako zmienną zależną, a SOD, MDA, GPx, GST, GSH, CAT i NO jako zmienne niezależne. Taki model miał dużą wartość prognostyczną (skorygowane R^2 = 94,0%). SOD, GST i NO były czynnikami niezależnymi pozwalającymi na przewidywanie czasu zgonu.
Wnioski. Wyniki uzyskane w doświadczeniu wykazały, że wskaźniki oksydacyjne i antyoksydacyjne są przydatne do oceny wczesnego czasu zgonu (**Adv Clin Exp Med 2012, 21, 6, 727–733**).

Słowa kluczowe: wczesny czas zgonu, utleniacze, przeciwutleniacze, wątroba, szczury.

The questions the authors are attempting to answer deal with identifying the corpse, cause of death and estimation of the time since death in forensic autopsy; however, reliably determining the time of death is the primary task for forensic medicine [1]. An estimation of the time since death is commonly inferred from the evaluation of physiological and physical postmortem changes, such as the distribution and amount of rigor mortis, death stains, changes in body temperature, changes in potassium concentration of the vitreous humor, the degree of decay in the body, proliferation of bugs on the corpse and their developmental stages [2].

In fact, there are several internal factors: age of the deceased, gender, physical and pathological state; and external factors: air temperature, humidity, and bug and animal activity affect the postmortem process, rendering the determination of time of death more complicated [3, 4]. Nonetheless, it was emphasized that the time of death is impossible to determine by investigating postmortem changes only, especially during periods of decaying process [5].

Indeed, several studies showed that there are many methods used to determine the time of death based on postmortem changes of biochemical markers over time, such as calcitonin, insulin in pancreatic beta cells; potassium and hypoxanthine in vitreous humor; and calmodulin-dependent kinase II and protein phosphatase 2A, myristoylated alanine-rich C-kinase substrate, calcineurin A, DNA and RNA degradation, DNA quantification in multiple tissues postmortem [4, 6–12]. However, all of these methods are still waiting for confirmation by other large studies, as no study has determined a significant relationship between the time of death and the oxidant/antioxidant parameters; therefore, the authors have attempted to investigate this relationship.

Under physiological conditions, the tradeoff between the oxidant and antioxidant balance is in favor of antioxidants [13]. However, the balance between oxidant and antioxidant after death (postmortem interval) is not clearly mentioned in literature. Furthermore, changes in oxidant and antioxidant parameters are evaluated as biochemical disturbances after death, as it cannot be said that the oxidant/antioxidant balance is sufficiently controlled by the body, even though cells show liability during the early hours of death. For this reason, the difference between oxidant and antioxidant parameter levels seems to be more significant than oxidant/antioxidant balance in determining the time since death.

The aim in this study is to investigate whether the differences between endogenous chemical parameters in rat livers known to be oxidant and antioxidant, such as superoxide dismutase (SOD), malondialdehyde (MDA), glutathione peroxidase (GPx), glutathione S-transferases (GST), total glutathione (GSH), catalase (CAT) and nitric oxide (NO) bear any relation in determining the early postmortem interval (EPI).

No study regarding whether a significant relationship exists between oxidant and antioxidant parameters and the early postmortem interval (EPI) has been found in a literature search.

Material and Methods

Animals

In this study, a total of 60 Albino Wistar male rats weighing between 220–230 grams were obtained from the medical experiment practice and research center in Ataturk University. Animal experiments were performed in accordance with national guidelines for the use and care of laboratory animals and approved by the local animal care committee (Ethic No: 53/ 05.08.2011) of Ataturk University.

Procedure of the Experiment

The rats were divided into six subgroups (n = 10), fed at room temperature (22^0C) and then decapitated. The livers of the dead animals were extracted at intervals of one hour (0–1, 1–2, 2–3, 3–4, 4–5, 5–6 hours intervals) and biochemical examinations were performed. Biochemical results collected from the rats groups were evaluated after being compared to each other.

Biochemical Analyses of Liver Tissue

Superoxide Dismutase Analysis

Superoxide dismutase (SOD) activity was measured according to Sun et al. [14]. Estimates were based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which react with nitroblue tetrazolium (NBT) to form formazan dye. When xanthine is converted into uric acid by xanthine oxidase, SOD forms. If nitro blue tetrazolium (NBT) is added to this reaction, SOD reacts with NBT and a purple-colored formazan dye occurs. The sample was weighed and homogenized in 2 ml of 20 mM phosphate buffer containing 10 mM EDTA at pH 7.8. The sample was centrifuged at 6000 rpm for 10 min and then the brilliant supernatant was used as assay sample. The measurement mixture containing 2450 µl measurement mixture (0.3 mM xanthine,

0.6 mM EDTA, 150 μM NBT, 0.4 M Na_2CO_3, 1 g/l bovine serum albumin), 500 μl supernatant and 50 μl xanthine oxidase (167 U/l) was vortexed. Then it was incubated for 10 min. At the end of the reaction, formazan occurs. The absorbance of the purple-colored formazan was measured at 560 nm. As more of the enzyme exists, the least O_2^- radical that reacts with NBT occurs. SOD activity is expressed as U/g protein.

Malondialdehyde Analysis

The concentrations of liver tissue lipid peroxidation were determined by estimating malondialdehyde (MDA) using the thio barbituric acid test [15]. The rat liver tissue was promptly excised and rinsed with cold saline. The liver tissue was scraped, weighed, and homogenized in 10 ml of 100 g/l KCl. The homogenate (0.5 ml) was added to a solution containing 0.2 ml of 80 g/l sodium lauryl sulfate, 1.5 ml of 200 g/l acetic acid, 1.5 ml of 8 g/l 2-thiobarbiturate, and 0.3 ml distilled water. The mixture was incubated at 98°C for 1 h. Upon cooling, 5 ml of n-butanol: pyridine (15 : l) was added. The mixture was vortexed for 1 min and centrifuged for 30 min at 4000 rpm. The absorbance of the supernatant was measured at 532 nm. The standard curve was obtained by using 1, 1, 3, 3-tetramethoxypropane. MDA levels are expressed as μmol/g protein.

Glutathione Peroxidase Analysis

Glutathione peroxidase (GPx) activity was determined according to the method of Lawrence and Burk [16]. The absorbance at 340 nm was recorded for 5 min, and the activity was defined as the rate of NADPH oxidation. Results were expressed as U/g protein.

Glutathione S-Transferases Analysis

The supernatant's glutathione S-transferases (GST) activity was measured by using 1-chloro-2, 4-dinitrobenzene (CDNB) and GSH as described in Habig et al. [17]. Briefly, the enzyme activity was assayed spectrophotometrically at 340 nm in a 4 ml cuvette containing 0.1M PBS (pH 6.5), 30 mM glutathione, 30 mM 1-chloro-2,6-dinitrobenzene and tissue homogenate. Results were expressed as U/g protein.

Total Glutathione Analysis

The amount of (total glutathione) (GSH) in the liver tissue was measured according to the method described by Sedlak and Lindsay [18]. The liver tissue was collected by scraping, weighed, and then homogenized in 2 mL 50 mM Tris-HCl buffer containing 20 mM EDTA and 0.2 mM sucrose, pH 7.5. The homogenate was immediately precipitated with 0.1 mL of 25% trichloroacetic acid, and the precipitate was removed by centrifugation at 4200 rpm for 40 min at 4°C. The supernatant was used to determine GSH using 5,5'-dithiobis (2-nitrobenzoic acid). The absorbance was measured at 412 nm using a spectrophotometer. The GSH levels in the liver tissue are expressed as nmol/g protein.

Catalase Analysis

Decomposition of H_2O_2 in the presence of catalase (CAT) was measured at 240 nm [19]. CAT activity was defined as the amount of enzyme required to process 1 nanomole of H_2O_2 per min at 26°C and pH 7.8. Results are expressed as U/g protein.

Nitric Oxide Analysis

Tissue nitric oxide (NO) levels were measured as total nitrite + nitrate levels with the use of the Griess reagent as previously described [20]. The Griess reagent consists of sulfanilamide and *N*-(1-napthyl)-ethylenediamine. The method is based on a two-step process. The first step is the conversion of nitrate into nitrite using a nitrate reductase. The second step is the addition of the Griess reagent, which converts nitrite into a deep purple azo compound; photometric measurement of absorbance at 540 nm is due to the fact that this azo chromophore accurately determines nitrite concentration. NO levels were expressed as μmol/g protein.

The supernatant's protein concentration was measured by the method of Bradford [21].

Statistical Methods

Data analysis was performed by using SPSS for Windows, version 18.0. All data was analyzed by an application of one-way ANOVA. Differences among groups were attained using the LSD option. The degrees of associations between oxidant and antioxidant variables were calculated by Pearson's "r" correlation coefficient. The authors performed a linear regression analysis in order to search for factors predicting time of death. Following variables included in the model: dependent variable (time of death) and independent variables (SOD, MDA, GPx, GST, GSH, CAT, and NO). Coefficient of regression and 95% confidence intervals for each variable were also calculated. The significance was declared at $p < 0.05$. Results are means ± SEM.

Table 1. The level of analyzed oxidants and antioxidants in rat liver tissue depending on the time of extraction. P was found comparing the values from 1 to 6 hour after death in comparison with 0–1 hour

Tabela 1. Stężenie analizowanych utleniaczy i przeciwutleniaczy w tkance wątroby szczurów w zależności od czasu ekstrakcji. P otrzymano przez porównanie wartości od 1 do 6 godzin po śmierci, w porównaniu z pierwszą godziną

Time of death – hours (Czas zgonu – godziny)	SOD (U/g protein)	MDA (μmol/g protein)	GPx (U/g protein)	GST (U/g protein)	GSH (nmol/g protein)	CAT (U/g protein)	NO (μmol/g protein)
0–1	15 ± 3.8	7 ± 1.2	87 ± 0.8	136 ± 23	81 ± 16	48 ± 10	8 ± 1.8
1–2	13 ± 2	4 ± 0.14	38 ± 6.8	66 ± 15	107 ± 22	9 ± 0.8	9 ± 2
	ns.	ns.	P < 0.05	P < 0.001	ns.	P < 0.001	ns.
2–3	7 ± 051	10 ± 2.2	52 ± 7	75 ± 16	87 ± 9.8	15 ± 1.8	12 ± 1.2
	P < 0.01	ns.	P < 0.05	P < 0.01	ns.	P < 0.01	ns.
3–4	6 ± 0.8	14 ± 4	31 ± 7	21 ± 5	23 ± 5.8	13 ± 2	22 ± 5
	P < 0.01	P < 0.01	P < 0.01	P < 0.0001	P < 0.001	P < 0.001	P < 0.01
4–5	1.4 ± 0.2	11 ± 1.6	29 ± 5.5	29 ± 4.6	47 ± 10	12 ± 1.3	32 ± 3.6
	P < 0.0001	ns.	P < 0.001	P < 0.0001	P < 0.05	P < 0.001	P < 0.0001
5–6	1.2 ± 0.22	15 ± 2.3	33 ± 7.3	31 ± 9	25 ± 7.6	11 ± 1.8	38 ± 5.6
	P < 0.0001	P < 0.01	P < 0.01	P < 0.0001	P < 0.001	P < 0.001	P < 0.0001

Results

In Table 1, the levels of some oxidant and antioxidant parameters were shown in rat liver tissue in the EPI. The authors found a new formulation for the results for estimating the EPI. It was calculated using the linear regression formula given below, and the authors applied the linear regression formula to the present cases, which resulted with a good ratio of prediction (Fig. 1). This model resulted with a high predictive value (adjusted R square = 94.0%).

$$EPI = 3.7 - (0.089 \times SOD) - (0.008 \times GST) + (0.052 \times NO)$$

SOD, GST, CAT, GPx values were decreased after at 2nd hour of the EPI. MDA, NO values were increased after at 3rd hour. Correlations between oxidant and antioxidant variables were checked using Pearson's correlation analysis. There were significant correlations between all variables (Table 2). SOD, GST and NO were found to be significant factors independently effecting prediction of the EPI (Table 3)

Discussion

This study depicted that the Chemical parameters are known as oxidants and antioxidants

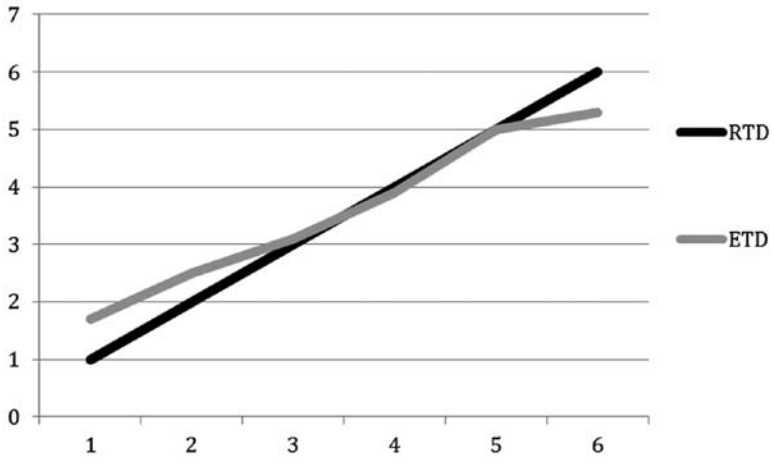

Fig. 1. The comparison between RTD and ETD with linear regression formula according to SOD, GST and NO parameters (RTD = Real Time of Death, ETD = Estimated Time of Death)

Ryc. 1. Porównanie RTD i ETD ze wzorem regresji liniowej według SOD, GST i NO (RTD = prawdziwy czas zgonu, ETD = szacowany czas zgonu)

Table 2. Evaluation of Pearson's correlation analysis relationship between oxidant and antioxidant variables

Tabela 2. Analizy korelacji Pearsona między zmiennymi oksydacyjnymi i antyoksydacyjnymi

	SOD	MDA	GPx	GST	GSH	CAT	NO
SOD		−0.547**	0.565**	0.566**	0.495**	0.468**	−0.754**
MDA	−0.547**		−0.333*	−0.505**	−0.661**	−0.24	0.624**
GPx	0.565**	−0.333*		0.763**	0.402*	0.811**	−0.538**
GST	0.566**	−0.505**	0.763**		0.468**	0.777**	−0.632**
GSH	0.495**	−0.661**	0.402*	0.468**		0.21	−0.715**
CAT	0.468**	−0.24	0.811**	0.777**	0.21		−0.407*
NO	−0.754**	0.624**	−0.538**	−0.632**	−0.715**	−0.407*	

*$p < 0.05$, **$p < 0.01$.

Table 3. Evaluation of linear regression analysis independent variables for predicting time of death

Tabela 3. Analizy liniowej regresji zmiennych niezależnych do przewidywania czasu zgonu

Variables (Zmienne)	Standardized Coefficients		t	p	95.0% CI	
	β	std. error			lower bound	upper bound
Constant	3.772	0.568	6.637	0	2.608	4.937
SOD	−0.307	0.02	−4.492	0	−0.129	−0.048
MDA	0.095	0.021	1.551	0.132	−0.01	0.074
GPx	−0.001	0.006	−0.007	0.995	−0.013	0.013
GST	−0.208	0.003	−2.463	0.02	−0.015	−0.001
GSH	−0.096	0.003	−1.425	0.165	−0.011	0.002
CAT	−0.094	0.01	−1.104	0.279	−0.031	0.009
NO	0.375	0.011	4.533	0	0.028	0.075

CI – Confidence Interval for B.

CI – przedział ufności dla B.

(SOD, MDA, GPx, GST, GSH, CAT and NO) play important role in determining the EPI. Under physiological conditions, the oxidant/antioxidant defense system in human body is in a state of continuous equilibrium; namely, equilibrium in aerobic metabolism (in alive tissue) is characterized by the formation of free radicals and their removal by means of antioxidant systems [13].

The oxidant/antioxidant equilibrium in injured tissue models produced in live animals is different. Increase in oxidant levels and a decrease in antioxidant levels are observed in the damaged tissues of any live animal [22, 23]. This event is a reaction induced against damage in a certain area of body [24]. Differences between biochemical parameters were observed, no matter how much viability the cells possessed in the early hours of death. Differences in postmortem period of oxidant and antioxidants parameters must be assessed as biochemical disturbances, as it cannot be proposed that the oxidant/antioxidant equilibrium be controlled sufficiently by the body, thus no matter how much viability the cells show in the early hours of death. Therefore, the sole balance between oxidant and antioxidant parameters does not suffice in determining the time of death.

In the present study, MDA amount, a lipid peroxidation product, in the 0–1 and 1–2 hours of the EPI was found to be lower than enzymatic and non-enzymatic antioxidant parameters like SOD, GPx, GST, GSH and CAT. It is seen in the present experiment that the oxidant/antioxidant balance in the 0–1 and 1–2 hours of death is in favor of antioxidants. Antioxidant superiority in the oxidant/antioxidant balance is still maintained under physiological conditions. The tradeoff between ox-

idants and antioxidants in favor of antioxidants in the 0–1 and 1–2 hours or probably death could be a continuing physiological reaction of the body.

Although SOD is found to be high in the (0–1 and 1–2 hours interval) hour of death, after this time SOD level was decreased. SOD protects cells from the toxic effects of superoxide radicals (O_2–) by exposing them to mutation [25]. The fact that SOD is high in the zero and first hour of death can lead one to think that an aerobic medium is sufficient for SOD to exert its antioxidant activity in these hours in the liver. While pancreatic and stomach mucosa face a more rapid enzymatic degradation by lytic enzymes, this event commences later in the liver [26]. Found to be higher in the 0–1 hour of death, GPx and GST activities showed a significant decrease at the end of the 1–2 hour of death. Contrary to GPx, the GSH level was significantly increased in the 1–2 hour of death. Decrease in GPx activity gives way to a decrease in total glutathione (GSH) and an increase in oxidized glutathione (GSSG). That is, the GSH/GSSG ratio decreases in oxidative damaging process [27]. However, an increase in GSH levels in the 1–2 hour of death might rely on the induction of ligase enzyme expression by 4-hydroxy-2-nonenal. Investigations have documented that 4-hydroxy-2-nonenal performs de novo synthesis of GSH from amino acids [28].

A moderately significant increase in the level of CAT observed in the 0–1 hour of death disappeared in the 1–2 hours. CAT catalyzes the reaction where hydrogen peroxide (H_2O_2) is degradated to oxygen and water and has no effect on lipid peroxides [29]. NO levels in the 1–2 hours interval of death were nearly the same. It was in the 2–3 hour of death when NO levels commenced to show a significant increase.

The authors performed a linear regression analysis in order to search for factors predicting the EPI. Time of death was taken as dependent variable with SOD, MDA, GPx, GST, GSH, CAT, and NO as independent variables. This model resulted with a high predictive value (adjusted R square = 94.0%). SOD, GST and NO were found to be significant factors independently effecting prediction of the EPI (Table 3). The authors applied the linear regression formula to the present cases, which resulted with a good ratio of prediction (Fig. 1). According to this formula SOD, GST and NO was significant informative in estimated the EPI.

In conclusion, the relationship between oxidant and antioxidant parameters is crucial in determining the EPI. This formulation can be helpful in detecting the EPI in forensic medicine. Postmortem autolytic process is related to factors, such as the cause and manner of death, temperature, air humidity and environmental conditions are not examined in this study. These results are preliminary, although significant results were found in this animal study. Therefore, further research on humans is needed.

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