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Research Article

Biological Variation of Oxidative Stress Biomarkers and Lactic Dehydrogenase in Mice

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Abstract

This is the first report, we aimed to determine the biological variations, analytical quality specifications of oxidative stress biomarkers such as glucose-6-phosphate dehydrogenase (G6PD), catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), reduced glutathione (GSH), malondialdehyde (MDA), and also lactic dehydrogenase (LDH) in mice. In the our study, *Mus musculus* albino mice, forty-one adult (female 20, male 21), weighing between 23 and 40 g were enrolled for biological variation study during 30-day period. Samples were stored and then tested at the same time. Results were assessed in duplicate and coefficients of variation for each analyte which were isolated to distinguish variation such as within-, and between-individual variations. From these results, an index of individuality were determined for each analyte. The analytical, within-individual, between-individual variations were assessed in apparently healthy mice and were found to be; 1.19%, 22.51% and 7.38% for G6PD, 14.09%, 23.03% and 29.42% for CAT, 6.18%, 11.12% and 21.14% for SOD, 9.13%, 12.00% and 12.58% for GST, 4.12%, 23.40% and 11.20% for GSH, 2.11%, 16.45% and 11.27% for LDH, 4.75%, 9.03% and 31.55% for MDA, respectively. As a result, while population-based reference intervals for G6PD, GSH and LDH were appropriate, Subject-based reference intervals for SOD and MDA were more appropriate. CAT and GST had intermediate individuality so population-based reference intervals should be used with caution.

Keywords: *Biological variation, lactic dehydrogenase, mice, oxidative stress biomarkers*

Farelerde Oksidatif Stres Biyobelirteçlerinin ve Laktik Dehidrogenazın Biyolojik Varyasyonu

Özet

Bu çalışma, glukoz-6-fosfat dehidrogenaz (G6PD), katalaz (CAT), süperoksit dismutaz (SOD), glutatyon S-transferaz (GST), indirgenmiş glutatyon (GSH), malondialdehit (MDA) gibi oksidatif stres biyobelirteçlerinin ve laktik dehidrogenaz (LDH)'ın biyolojik varyasyonlarını, analitik kalite spesifikasyonlarını ve referans aralıklarını saptamak amacıyla yapılan ilk araştırmadır. Bu çalışmada, 41 erişkin, 23-40 g ağırlığındaki *Mus musculus* albino fare (20 dişi, 21 erkek), 30 gün boyunca biyolojik varyasyon ve referans aralıkları çalışmaları için kullanıldı. Sağlıklı görünen farelerde analitik, kişisel ve kişiler-arası varyasyonlar değerlendirildi ve sırasıyla G6PD: %1.19 %22.51 ve %7.38; CAT: %14.09, %23.03 ve %29.42; SOD: %18, %11.12 ve %21.14; GST: %9.13, %12.00 ve %12.58; GSH: %4.12, %23.40 ve %11.20; LDH: %2.11, %16.45 ve %11.27; MDA: %4.75, %9.03 ve %31.55 olarak bulundu. Bu bulgular G6PD, GSH ve LDH'ın referans aralıkları, toplum tabanlı referans aralıklara uygun iken SOD ve MDA'nın kişisel-tabanlı referans aralıklara daha uygun olduğunu gösterdi. CAT ve GST'nin toplum tabanlı referans aralıklarda dikkatli kullanılması gerektiği bulundu.

Anahtar sözcükler: *Biyolojik varyasyon, Laktik dehidrogenaz, Oksidatif stres biyobelirteçleri, Fare*

INTRODUCTION

Reactive oxygen species (ROS) are produced during normal metabolism and the biological effects of ROS are controlled *in vivo* by a variety of oxidative stress biomarkers such as glucose-6-phosphate dehydrogenase (G6PD), catalase (CAT), superoxide dismutase (SOD), and also glutathione-S-transferase (GST). They are inducible enzymes which react with activated oxygen species, both in the cytosol and in subcellular organelles, and function in decreasing the damaging effects of these molecules [1,2]. In addition to these enzymatic components of the oxidant defence system there are also non-enzymatic "radical scavengers" such as alpha-tocopherol, reduced glutathione

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(GSH) and ascorbic acid. Lipid peroxidation is a normal phenomenon that occurs continuously at low levels in all living cells. These peroxidation reactions are in part toxic to cells and cell membranes; however, they are normally controlled by countervailing biologic mechanisms [1]. Lactic dehydrogenase (LDH), a glycolytic enzyme, is present in various tissues and neoplasms of the mammalian body in multiple forms. Doherty et al.[3] reported that there was a relation between neoplasia and LDH activity.

Laboratory mouse is an animal most commonly used in mammalian biological studies and in the human disease modeling. In spite of numerous experiments conducted on laboratory mice, their biochemical phenotype still remains not fully discovered. Only limited data on the clinical biochemistry of mice are available from studies of various authors [4]. Currently, the biochemistry results of laboratory animal are assessed in relation to population-based reference intervals [5]. However, it is considered more appropriate to use 'subject-based' reference values to assess analytes that have a high degree of between-individual variation because many unhealthy individuals may have values that significantly differ from their regular analyte determination, but fall within population-based reference intervals [6,7].

Determination of 'subject-based' reference values requires knowledge of inherent physiological variation of analytes which is referred to as biological variation. Suitability of an analyte to be assessed in relation to population- or subject-based reference intervals is determined by that analyte's index of individuality (II), a ratio of within-individual biological variation (CV_I) to between-individual (CV_G) biological variation. Although biological variation data have been generated for several blood components in various veterinary animals (dog, cat and horse), there are no biological variation data for mice blood component published in the peer-reviewed literature. Therefore, in the present study, we aimed to determine biological variation data of erythrocyte oxidative stress biomarkers and LDH in mice. These data will be used to set analytical quality specifications, as well as to select those biomarkers most appropriate, for consideration in a larger observational study investigating on oxidative stress biomarkers and LDH in mice.

MATERIAL and METHODS

All chemicals used in the levels of oxidative stress biomarkers and LDH activity assays were analytical grade and were from the Sigma Chemical Company (St. Louis, MO, USA).

Animals

Mus musculus albino mice, forty-one adult (female 20, male 21), weighing between 25 and 35 g were obtained from the Experimental Research Center of the University of Sutcu Imam. They were fed with a standard laboratory diet and tap water. Illumination was 12 h light/dark cycle and room temperature was 22-24ºC. They were enrolled for biological variation study during 30 days. This study was approved by the local ethics board in Kahramanmaras Sutcu Imam University Faculty of Medicine, Experimental Animals Laboratory (KSU Ethics Committee Number: 2000-18).

The Blood Samples

The blood samples were taken into EDTA tubes from tail vein from each individual (between 08:00 and 09:00 a.m.) on the zero, 1st, 3rd, 5th, 7th, 15th and 30th days. All the blood samples were centrifuged and the plasma was removed. The erythrocytes were then washed three times in 0.9% saline solution. All the oxidative stress biomarkers and LDH in erythrocyte were measured as spectrophotometric. Furthermore, erythrocyte samples previously collected from a group of eight healthy mice as described in this study and stored in 250 µL aliquots directly at -80ºC, were used as internal control material for the oxidative stress biomarkers (G6PD, CAT, SOD and MDA) and LDH. All analyses were carried out in duplicate, in randomized order on separate tubes of EDTA, during the same day, using one batch of reagents and one calibration of the analyzers.

Determination of Oxidative Stress Biomarkers and LDH

Assay of G6PD Activity: G6PD activity was determined at 37ºC in the erythrocyte according to Beutler [8]. The reaction mixture contained 1M Tris-HCl pH 8.0, 6 mM G6P Na, 2 mM NADP, 0.1 M $MgCl_2$ and hemolysate in a total volume of 3.0 mL. One unit of enzyme activity was the amount of enzyme catalyzing the reduction of 1mM of NADP per minute. G6PD activity was expressed as U/g Hb.

Assay of CAT Activity: CAT activity was determined by measuring the decrease in hydrogen peroxide concentration at 230 nm by the method of Beutler [8]. Assay medium consisted 1 M Tris HCl, 5 mM Na_2EDTA buffer solution (pH 8.0), 10 mM H_2O_2 and haemolysate in a final volume of 1.0 mL. CAT activity was expressed as U/g Hb.

Assay of SOD Activity: SOD activity was measured according to the method described by Fridovich [9]. This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with p-iodonitrotetrazolium violet (INT) to form a red formazan dye which was measured at 505 nm. Assay medium consisted of the 0.01 M phosphate buffer, CAPS (3-cyclohexilamino-1-propanesulfonicacid) buffer solution (50 mM CAPS, 0.94 mM EDTA, saturated NaOH) with pH 10.2, solution of substrate (0.05 mM xanthine, 0.025 mM INT) and 80 U/L xanthine oxidase. SOD activity was expressed as U/g Hb.

Assay of GST Activity: GST activity was measured by the method described by Mannervik and Guthenberg [10]. 1mM 1-chloro-2.4-dinitrobenzene (CDNB) was used as

the substrate. The reaction mixture contained phosphate buffer (pH 6.5), 20 mM GSH and 20 mM CDNB. Increase in optical density was followed at 340 nm and GST activity was expressed as U/g Hb.

Assay of LDH Activity: In the assay the oxidation of NADH was followed spectrophotometrically at 340 nm by the method of Beutler [8]. Assay medium consisted of the 1M Tris-HCl and 5mM EDTA solution with pH 8.0, 2mM NADH, hemolysate and 10 mM sodium pyruvate in a final volume of 1 mL. LDH activity was expressed as U/g Hb.

Measurement of GSH: GSH levels were determined by measuring a highly coloured yellow anion formed by the reduction of DTNB [5,5′-Dithiobis (2-nitrobenzoic acid)] with nonprotein sulfhydryl compounds of erythrocytes by the method of Beutler [8]. The optical density of yellow anion was measured at 412 nm within the first 10 minutes of colour development. Entire procedure was carried out at room temperature. The level of GSH was calculated as µmol/g Hb.

Measurement of MDA: Malondialdehyde (MDA) levels, as an indicator of lipid peroxidation. It was measured in erythrocyte according to procedure of Ohkawa et al.[11]. The reaction mixture contained 0.1 mL of sample, 0.2 mL of 8.1% sodium dodecyl sulphate (SDS), 1.5 mL of 20% acetic acid and 1.5 mL of 0.8% aqueous solution of TBA. The mixture pH was adjusted to 3.5 and volume was finally made up to 4.0 mL with distilled water and 5.0 mL of the mixture of n-butanol and pyridine (15:1, *v/v*) were added. The mixture was shaken vigorously. After centrifugation at 4.000 rpm for 10 min, the absorbance of the organic layer was measured at 532 nm.

The hemoglobin concentration of the hemolysate was measured in digital Spectronic-20 spectrophotometer by the method of Beutler [8].

Statistical Analysis

Seven blood samples were taken from each individual on the zero, 1st, 3rd, 5th, 7th, 15 and 30th days. General statistical analyses were carried out with SPSS 15.0 for Windows. Outliers were determined according to Fraser and Harris. We looked for outliers in the sets of duplicate results. When one outlying variance (an unexpected large difference in the duplicates) was detected, both data points were rejected [12]. As a result of these steps the assay results of 4 subjects were identified as outliers for GST and were excluded from the estimations. After exclusion of any outliers, the data of 37 individuals for oxidative stress biomarkers and LDH were evaluated to estimate components of biological variation. Each sample from one individual was assayed in duplicate and, the average value of the individual's duplicate measurements was used for the statistical procedures. Analytical variance (SD_A^2) was calculated from the difference between each pair of duplicates according to the formula [12,13]:

$SD_A^2 = (\Sigma d^2/2n)$ and $CV_A = (SD_A/Mean) \times 100$ as a percentage.

CV_I was calculated from the total within subject variance (CV_{TI}) minus the analytical variance according to the formula described by Fraser and Harris as $CV_I = (CV_{TI}^2-CV_A^2)^{1/2}$. CV_{TI} includes both analytical and biological variation. To determine between-individual variance (SD_G^2), the total variance (SD_T^2) was calculated by use of all of the individual data sets and transformed to relative SD (CV_T) by use of the overall mean. CV_G was calculated by means of the following formula as $CV_G = (CV_T^2-CV_I^2-CV_A^2)^{1/2}$. Index individuality (II) was calculated as CV_I/CV_G [12-15]. The reference change value (RCV) or critical differences, which is the difference required for two serial measurements of the oxidative stress biomarkers to have significantly changed at $P<0.05$, was calculated as $RCV = 2^{1/2} \times 1.96 (CV_I^2+CV_G^2)^{1/2} = 2.77(CV_{TI})$. The objective analytical performance standards for imprecision $CV_{max} = 0.5CV_I$, inaccuracy $B_{max} = 0.25(CV_I^2+CV_G^2)^{1/2}$ and total error $TE = [1.65(CV_{max}) + B_{max}]$ were also calculated [16].

Linear regression analysis was used to look for significant trends in values for the levels of oxidative stress biomarkers and LDH and to investigate the time dependence of the within-individual variations. Also, comparisons according to days were performed using the Wilcoxon test. A $P<0.05$ was considered to be statistically significant.

RESULTS

Of the 2009 data points of 41 subjects, 4 subjects were classified as outliers, but as the duplicate results were similar, these results were considered to be physiological variation and maintained. Also shown in *Table 1* are the overall means, ranges of each oxidative stress biomarker and LDH activity. There was no significant differences for all parameters between sexes ($P>0.05$). Therefore only the total results for all parameters are presented.

Table 2 shows, the day-to-day changes as within-subject CVs for all analytes in mice. There were no significant differences between values of each day and initial day by Wilcoxon test. The criterion for comparing variables with the initial day was chosen at $P<0.05$.

Table 3 shows, CV_I, CV_G, II and categories of II, which are derived from the data on biological variation. Because there were no significant differences in the CV_I between the sexes (*F*-test) for the analytes investigated in this study, only the totals were presented. The CV_I values of G6PD, LDH and GSH were larger than CV_G values ($CV_I>CV_G$). However, CV_I values of CAT, SOD and MDA were smaller than CV_G values ($CV_I<CV_G$). CV_I and CV_G values of GST analyte were found as similar to each other. Furthermore, While G6PD, LDH and GSH showed high index of individuality (II>1.4), SOD and MDA showed low index of individuality (II<0.6). CAT and GST showed intermediate individuality

Table 1. *The levels of oxidative stress biomarkers and LDH in mice.*
Tablo 1. *Farelerde oksidatif stres biyobelirteçlerinin ve LDH'ın düzeyleri*

Parameters	Mean	SD	Median	Min-Max	95% Confidence Interval	*Values Reference Pool
G6PD	19.31	4.92	18.00	10.40-30.50	9.47-29.15	17.00
CAT	2.31	1.04	2.30	0.80-4.20	0.23-4.39	2.03
SOD	1620.80	409.40	1520	1000-2500	802.00-2439.60	1632.42
GST	8054.31	1403.65	8255	5000-10300	5247.12-10861.52	7245.03
LDH	277.60	44.53	285	187-390	188.60-366.60	254.01
GSH	4.17	0.88	3.95	3.00-6.30	2.41-5.93	3.87
MDA	680.70	217.49	710	350-1020	245.72-1115.68	584.02

*The activities of G6PDH, CAT, SOD, GST and LDH were expressed as U/g Hb; The levels of GSH and MDA were expressed as μmol/g Hb and nmol/g Hb, respectively; * Values reference pool were given as mean, which consisted of eight blood samples*

Table 2. *The within-individual variations in oxidative stress biomarkers and LDH for 30 days duration*
Tablo 2. *Oksidatif stres biyobelirteçlerinin ve LDH'ın 30 günlük sürede ki birey-içi varyasyonları*

Days	Body wg	G6PD	CAT	SOD	GST	LDH	GSH	MDA
Zero-day	31.8±4.4 (13.9)	19.3±4.6 (23.8)	2.2±1.0 (45.4)	1555±364 (23.3)	7702±1404 (18.2)	279±46 (16.3)	4.3±0.8 (18.6)	681±218 (31.9)
First day	32.1±4.4 (13.8) [1]p:0.776	18.8±5.4 (28.7) [1]p:0.776	2.3±1.1 (47.8) [1]p:0.776	1641±394 (23.9) [1]p:0.293	8202±1553 (18.9) [1]p:0.118	300±69 (23.0) [1]p:0.079	4.0±1.7 (42.5) [1]p:0.690	695±231 (33.2) [1]p:0.826
Third day	32.4±4.5 (13.9) [2]p:0.660	18.9±5.3 (28.0) [2]p:0.820	2.3±0.9 (39.1) [2]p:0.753	1614.3±485 (30.0) [2]p:0.433	7779±1785 (22.9) [2]p:0.551	280±52 (18.5) [2]p:0.315	4.0±1.5 (37.5) [2]p:0.432	674±229 (34.0) [2]p:0.421
Fifth day	33.0±4.4 (13.4) [3]p:0.967	17.6±3.5 (19.8) [3]p:0.410	2.2±0.8 (36.3) [3]p:0.875	1563±432 (27.6) [3]p:0.887	8190±1557 (19.0) [3]p:0.321	284±45 (19.2) [3]p:0.182	4.4±0.8 (18.1) [3]p:0.330	683±231 (33.8) [3]p:0.593
Seventh day	33.2±4.6 (13.9) [4]p:0.835	18.7±4.3 (22.9) [4]p:0.732	2.6±0.9 (34.6) [4]p:0.735	1730±434 (25.1) [4]p:0.240	7860±1718 (21.8) [4]p:0.513	278±53 (19.2) [4]p:0.561	3.8±1.4 (36.8) [4]p:0.095	676±214 (31.6) [4]p:0.417
Fifteenth day	33.8±4.4 (13.0) [5]p:0.505	16.4±3.4 (20.7) [5]p:0.235	2.3±0.1 (4.34) [5]p:0.318	1621±398 (24.5) [5]p:0.397	7869±1573 (19.9) [5]p:0.495	276±59 (21.6) [5]p:0.535	4.3±0.7 (16.2) [5]p:0.197	683±219 (32.0) [5]p:0.557
Thirtieth day	34.0±4.6 (13.7) [6]p:0.337	18.8±3.3 (17.5) [6]p:0.734	2.5±0.8 (32.0) [6]p:0.826	1666±342 (20.5) [6]p:0.253	8200±1576 (19.2) [6]p:0.096	306±55 (18.0) [6]p:0.087	4.5±1.1 (24.4) [6]p:0.123	759±286 (37.6) [6]p:0.176

The activities of G6PDH, CAT, SOD, GST and LDH expressed as U/g Hb. The levels of GSH and MDA were given as μmol/g Hb and nmol/g Hb, respectively; [1,2,3,4,5,6] *Comparison of the values with initial value was done by Wilcoxon test (P<0.05).*

Table 3. *Components of biological variation for oxidative stress biomarkers and LDH in mice*
Tablo 3. *Farelerde oksidatif stres biyobelirteçlerinin ve LDH'ın biyolojik varyasyon komponentleri*

Variables	[a]CV_A (%)	[a]CV_I (%)	[b]CV_G (%)	[d]RCV (%)	[e]II (Category[f])
G6PD (U/g Hb)	1.19	22.51	7.38	62.43	3.05 (High)
CAT (U/g Hb)	14.09	23.03	29.42	74.78	0.78 (intermediate)
SOD (U/g Hb)	6.18	11.12	21.14	35.23	0.52 (Low)
GST (U/g Hb)	9.13	12.00	12.58	41.76	0.95 (intermediate)
LDH (U/g Hb)	2.11	16.45	11.27	45.93	1.45 (intermediate)
GSH (μmol/g Hb)	4.12	23.40	11.20	65.81	2.08 (High)
MDA (nmol/g Hb)	4.75	9.03	31.55	28.26	0.28 (Low)

All samples analyzed in duplicate; [a]CV_I *within-subject coefficient of variation;* [b]V_G *between-subject coefficient of variation;* [d]*RCV, reference change value;* [e]*II, index of individuality;* [f]*Categories of II=High (>1.4), Low (<0.6), intermediate (0.6-1.4)*

(II, between 0.6 and 1.4). Furthermore, the observed CV_A of G6PD, LDH and GSH were lower than CVmax except for the CAT, SOD, GST and MDA ($CV_A<CV_{max}$) as shown in *Table 4*. The CV_A values which achieved desired performance of $0.5CV_I$ are marked with a superscript.

The regression analysis showed no trends for the changes in the levels of oxidative stress biomarkers and LDH activity during 30 days in mice.

Table 4. *Method imprecision and quality specifications for oxidative stress biomarkers and LDH measurements in mice*

Tablo 4. *Farelerde oksidatif stres biyobelirteçleri ve LDH ölçümleri için metot belirsizliği ve kalite özellikleri*

Parameters	CV_A[a] %	CV_{max}[b], %	B_{max}[c], %	TE_{max}[d], %
G6PD (U/g Hb)	1.19*	11.25	5.92	24.48
CAT (U/g Hb)	14.09	11.51	9.34	28.33
SOD (U/g Hb)	6.18	5.56	5.97	15.14
GST (U/g Hb)	9.13	6.00	4.34	14.24
LDH (U/g Hb)	2.11*	8.25	4.98	18.59
GSH (µmol/g Hb)	4.12*	5.60	6.48	15.72
MDA (nmol/g Hb)	4.75*	15.77	8.20	34.22

*All samples analyzed in duplicate; [a]CV_A, analytical coefficient of variation; CV_{max}[b], objective analytical performance standard for imprecision; B_{max}[c], objective analytical performance for inaccuracy; TE_{max}[d], objective analytical performance standard for total error; *Desirable performance ($CV_A < CV_{max}$)*

DISCUSSION

To our knowledge, this is the first study to carry out the determination and application of data on biological variations of oxidative stress biomarkers and LDH in erythrocytes of mice. Limited studies have been conducted to address these methodological issues [17,18] and there is still controversy over which oxidative stress biomarkers and LDH to use. It has been suggested that oxidative stress biomarkers and LDH should be utilized for research purposes until more sensitive and specific assays are developed [19,20]. The current consensus in laboratory medicine is that quality specifications should be based on the parameters of biological variation [15,21]. In this study we generated data of biological variations for oxidative stress biomarkers and LDH that are commonly measured in the laboratory when assessing oxidative stress in mice. In the present study, we found no significant difference in the within-subject CVs of all analytes in mice, so these analytes can be used in deriving criteria for decision making. The CV_G values for CAT, SOD and MDA analytes in our study were found as generally larger than CV_I values. We thought that these analytes displaying small CV_I also allow more precise knowledge of the homeostatic set point and leave less margin for ambiguity in recognizing the patient's status. However, the average CV_G values of G6PD, GST, GSH and LDH analytes was smaller than CV_I values, probably because of the lack of homogeneity in the CV_I and perhaps due to problems of stability. The lack of homogeneity in the data of these analyte may cause erroneous calculations of biological variation and false interpretations of results, as such calculations involve an analysis of means and may incorporate individuals with CV_I values much larger than CV_G values.

For detection of disease, subject-based reference intervals are more sensitive than population-based reference intervals when CV_I values are less than CV_G values, because many individuals will have values that differ from their usual values, but which fall within population-based reference intervals [15,21,22]. For any individual, the homeostatic health setting for a given analyte may be near the limits or toward the center of a population-based reference interval, but maintains its relative position within the reference interval [20]. When CV_I is markedly lower than CV_G, any change must be considerable to be detected using a population-based reference intervals, especially if the individual's health setting is near the population's mean. To determine how much lower CV_I must be in order for subject-based reference intervals to be of use, the "individuality" of the analyte, namely the index of individuality, can be calculated. Using the criteria described by Fraser and Harris [14], and recently reviewed in the veterinary literature [15], an II <0.6 indicates that subject-based reference values are more appropriate to use; when the II >1.4, population-based reference intervals are more appropriate, and when between 0.6 and 1.4, population-based ranges should be used with caution [15]. In our study, SOD and MDA had low index of individuality and therefore subject-based reference intervals are more appropriate; G6PD, LDH and GSH had high index of individuality, indicating that population-based reference intervals are appropriate. CAT and GST had intermediate index of individuality so population-based reference intervals should be assessed in relation to subject based reference intervals. To our knowledge, there is no study on this subject, therefore we did not compare our results. We only compared our results with human blood for genetically close to each other. Covas et al.[23] reported that II for SOD and glutathione peroxidase in human blood was 0.45 (low individuality), suggesting that it has little value as a diagnostic or screening tool. The components of variation for MDA in human plasma reported in the literature [18,24] are similar to our values. The RCV is an important clinical tool for the assessment of changes in patient animal being monitored in pathologicalsituations [24,25]. In the present study, the RCVs were high for G6PD, CAT, LDH, GST and GSH due to their higher within-subject component of variation. Furthermore, RCV can be of diagnostic use in cases where the population-based reference range may be an insensitive interpretation criterion (i.e. low index of individuality). In such cases the RCV can be used to detect small, but statistically significant changes within the reference range between serial measurements in the same individual. For a change between serial measurements exceeding the RCV, and thus the limits of biological variation, could identify animal "at risk of disease" so that further diagnostic workup or clinical intervention can be instituted at an earlier stage of disease.

Every test result is subject to a number of sources of biological variation causing measurements in the same individual to change with time. Knowledge of these temporal changes is useful information when establishing whether the performance of a test is appropriate for

interpreting results and making a diagnosis. Desired analytical imprecision and within-subject biological variation are particularly important in this regard. The influence of both these parameters on the precision of single test results and on the number of samples required to make clinical decisions can be easily calculated using simple formulae. Furthermore the effect of performing replicate analyses of the same sample versus taking multiple samples can also be investigated. In the present study, we have determined desired analytical imprecision on the biological variation of oxidative stress biomarkers and LDH assays and furthermore assessed the utility of reference intervals for interpretation of results. The CVmax should be perceived as a desired minimum requirement for the level of imprecision [26]. However studies in humans have shown, that the desired analytical variation (CVmax) is difficult to achieve when working with oxidative stress biomarkers and LDH assays and often laboratories fail to accomplish this analytical goal [18]. Of these analytes, G6PD, LDH and GSH achieved a CV_A lower than desired [27]. However, CAT, SOD, GST and MDA achieved a CV_A higher than desired CV_A for this assay. The results indicated that for CAT, SOD, GST and MDA analysis in duplicate could be necessary, which would theoretically lower the CV_A and bring analytical variation below the desired level. There is no apparent analytical error, which can explain the observed high analytical variation of the MDA (34.0%), CAT (28.3%) and G6PD (24.4%) assays examined in this study. Internal controls of these tests using the pooled erythrocyte analyzed with each batch of samples was within a well defined control range, which indicates that there may be some pre-analytical variation causing the aberrant results of the samples from the eight mice examined. Oxidative stress biomarkers and LDH tests in laboratory medicine can be measured with a wide variety and combination of reagents and instruments, which can influence the results of the analysis. All seven measured erythrocyte oxidative stress biomarkers and LDH parameters met the objective analytical performance standard for imprecision except for the G6PD, LDH, GSH and MDA for which analysis in duplicate could also be necessary. An approach the estimate of within-subject variation would be confounded by between batch analytical variations. Thus an estimate of between-batch analytical variation must be obtained from assays of quality control materials, which are currently not available for these markers and LDH assays. Though oxidative stress biomarkers and LDH performed on erythrocyte samples might be as diagnostically relevant or informative of the patient animal. Therefore, it is hoped that these biological variation data may serve to apply this approach in the analysis of oxidative stress biomarkers and LDH in mice.

This study represents the first assessment of biological variation for oxidative stress and LDH in blood of mouse within the peer-reviewed literature. The results of this study showed that SOD and MDA had low index of individuality (<0.6), which may make the use of population-based reference ranges alone an insensitive interpretation criterion. However, G6PD, GSH and LDH had high index of individuality (>1.4), which may make the use of conventional population-based reference ranges is a sensitive interpretation criterion of these measurements. Another our results indicated that CAT and GST had intermediate index of individuality (between 0.6-1.4), population-based reference of these analytes values may be used, but with caution. Analytical quality specifications were derived from biological variation data, and imprecision goals can be reasonably achieved with current methods. Only three (G6PD, LDH and GSH) of the six traditionally used assays examined in this study achieved the analytical goal of $CV_A < CV_{max}$, indicating that the analytical variation of the four other assays (CAT, SOD, GST and MDA) was too high and that measures need to be instituted to address this. Here, we suggest that biological variations and quality specifications for analytical performance of a method should not be veterinary diagnostic ignored in assessing oxidative stress biomarkers and LDH obtained through the same method in the same laboratory or from laboratories applying different methods. There is no information regarding biological variation studies in mice so this paper may provide strong evidence for serial sampling in small animal practice.

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Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

REFERENCES

1. Kurutas EB, Doran F, Gumuşalan Y: The levels of oxidative stress biomarkers of liver and mammary tissues of apparently healthy mice. *Cukurova Med J,* 38, 7-14, 2013.

2. Belge Kurutas E, Tuncer I: A mouse model for evaluating the induction of liver glucose-6-phosphate dehydrogenase by halothane. *Turk J Vet Anim Sci*, 24, 511-515, 2000.

3. Doherty JR and Cleveland JL: Targeting lactate metabolism for cancer therapeutics. *J Clin Invest,* 123, 3685-3692, 2013. DOI: 10.1172/JCI69741

4. Wirth-Dzięciołowska E, Karaszewska J, Sadowski T, Pyśniak K, Gajewska M: Selected blood serum biochemical indicators in twelve inbred strains of laboratory mice. *Anim Sci Pap Rep,* 27, 159-167, 2009.

5. Geffré A1, Friedrichs K, Harr K, Concordet D, Trumel C, Braun JP: Reference values: A review. *Vet Clin Pathol,* 38, 288-298, 2009. DOI: 10.1111/j.1939-165X.2009.00179.x

6. Baral RM, Dhand NK, Freeman KP, Krockenberger MB, Govendir M: Biological variation and reference change values of feline plasma biochemistry analytes. *J Feline Med Surg,* 16, 317-325, 2014. DOI: 10.1177/1098612X13508770

7. Harris EK: Effects of intra-and inter-individual variation on the appropriate use of normal ranges. *Clin Chem*, 20, 1535-1542, 1974.

8. Beutler E: Red Cell Metabolism. London: Grune and Stratton Inc., 1984.

9. Fridovich I: Superoxide dismutase. *Adv Enzymol,* 41, 35-97, 1974.

10. Mannervik B, Guthenberg C: Glutathione-S-transferase (human placenta). *Meth Enzymol,* 77, 231-235, 1981.

11. Ohkawa H, Ohishi N, Tagi K: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem,* 95, 351-358, 1979. DOI: 10.1016/0003-2697(79)90738-3

12. Fraser CG, Harris EK: Generation and application of data on biological variation in clinical chemistry. *Crit Rev Clin Lab Sci,* 27, 409-437, 1989.

13. Fraser CG: Biological variation: from principles to practice. Washington DC: AACC Press, 2001.

14. Fraser CG: Inherent biological variation and reference values. *Clin Chem Lab Med,* 42, 758-764, 2004. DOI: 10.1515/CCLM.2004.128

15. Walton RM: Subject-based reference values: Biological variation, individuality, and reference change values. *Vet Clin Pathol,* 41, 175-181, 2012. DOI: 10.1111/j.1939-165X.2012.00414.x

16. Wiinberg B, Jensen AL, Kjelgaard-Hansen M, Rojkjaer R, Johansson PI, Gade LP, Gram DX, Kristensen AT: Study on biological variation of haemostatic parameters in clinically healthy dogs. *Vet J,* 174, 62-68, 2007. DOI: 10.1016/j.tvjl.2006.05.003

17. Kato I1, Ren J, Heilbrun LK, Djuric Z: Intra- and inter-individual variability in measurements of biomarkers for oxidative damage *in vivo*: Nutrition and breast health study. *Biomarkers*, 11, 143-152, 2006.

18. Browne RW1, Bloom MS, Schisterman EF, Hovey K, Trevisan M, Wu C, Liu A, Wactawski-Wende J: Analytical and biological variation of biomarkers of oxidative stress during the menstrual cycle. *Biomarkers,* 13, 160-183, 2008. DOI: 10.1080/13547500701775563

19. Yapar K, Atakisi E, Uzlu E, Atakisi O, Citil M, Uzun M, Erdogan HM: The effect of different doses of metamisole sodium on serum enzyme activities and tissue oxidant levels in liver and kidney in mice. *Kafkas Univ Vet Fak Derg,* 13, 121-125, 2007. DOI: 10.9775/kvfd.2007.13-A

20. Belge Kurutaş E, Doran F, Çıralık H: The effect of endosulfan on lactic dehydrogenase enzyme system in liver of *Mus musculus:* A histochemical study. *Eur J Gen Med,* 3, 148-151, 2006.

21. Fraser CG: Making better use of differences in serial laboratory results. *Ann Clin Biochem,* 49, 1-3, 2012. DOI: 10.1258/acb.2011.011203

22. Solberg HE: Subject-based reference values. *Scand J Clin Lab Invest,* 222, 7-10, 1995.

23. Covas MI, Coca L, Ricós C, Marrugat J: Biological variation of superoxide dismutase in erythrocytes and glutathione peroxidase in whole blood. *Clin Chem,* 43, 1991-1993, 1997.

24. Nielsen F, Mikkelsen BB, Nielsen JB, Andersen HR, Grandjean P: Plasma malondialdehyde as biomarker for oxidative stress: reference interval and effects of life-style factors. *Clin Chem,* 43, 1209-1214, 1997.

25. Ricós C, Cava F, García-Lario JV, Hernández A, Iglesias N, Jiménez CV, Minchinela J, Perich C, Simón M, Domenech MV, Alvarez V: The reference change value: A proposal to interpret laboratory reports in serial testing based on biological variation. *Scan J Clin Lab Invest,* 64, 175-184, 2004.

26. Fraser CG: The application of theoretical goals based on biological variation data in proficiency testing. *Arch Pathol Lab Med,* 112, 404-415, 1988.

27. Fraser CG, Petersen PH, Ricos C, Haeckel R: Proposed quality specifications for the imprecision and inaccuracy of analytical systems for clinical chemistry. *European J Clin Chem Clin Biochem,* 30, 311-317, 1992.