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Circulating and muscle glutathione turnover in human endotoxemia

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Abstract

Objective: Patients with septic shock have high plasma glutathione concentration, while intracellular concentrations in erythrocytes and muscle are low. Here we investigated the temporal pattern of glutathione status and glutathione kinetics in healthy volunteers during the initial phase of sepsis using a human endotoxin model.

Design and setting: Descriptive pilot study in healthy male volunteers (n = 8) before and after an endotoxin challenge.

Measurements and results: The glutathione status was determined in plasma and whole blood at baseline and hourly for 4 hours after intravenous endotoxin injection and in skeletal muscle at baseline and at 2 and 4 hours after endotoxin injection.

In plasma the concentration of total glutathione decreased by 24 % ($p < 0.05$) at 3 hours after endotoxin injection and by 32 % ($p < 0.001$) at 4 hours. In whole blood and skeletal muscle, the concentrations of both reduced and total glutathione as well as the redox status remained unaltered during the initial 4 hours after an endotoxin challenge.

The fractional synthesis rate (FSR) of glutathione in whole blood was 38 ± 20 (%/day) before and 59 ± 22 %/day 4 after the endotoxin challenge ($p = 0.088$) and in skeletal muscle 41 ± 25 and 46 ± 18 %/day ($p = 0.68$) respectively.

Conclusions: During the initial phase of sepsis as represented by an intravenous endotoxin challenge to healthy volunteers, plasma concentration of total glutathione decreased, while glutathione status and synthesis rate in skeletal muscle and whole blood remained unaltered. However, due to the variation in the synthesis measures, larger studies are needed to confirm these findings.

Introduction

Glutathione is a tripeptide with the constituent amino acids glutamate, cysteine and glycine. It is present at high concentration in all human cells and has many protective and metabolic functions [1]. In man, glutathione is quantitatively the most important antioxidant, protecting the cells from the toxic effects of reactive oxygen species. Other important functions of glutathione are the capacities to detoxify electrophilic metabolites including several drugs, being a cofactor of the enzyme glutathione transferase and to promote lymphocyte proliferation as well as antibody dependent and cell-mediated cytotoxicity [2, 3].

During severe sepsis and septic shock a depletion of glutathione is seen in both skeletal muscle and in whole blood, fully established within the initial 24 hours after admission to the ICU [4, 5]. The glutathione depletion seen in muscle in the initial phase of septic shock correlates with mortality [6]. During the multiple organ failure phase of sepsis, the temporal changes in glutathione status vary between skeletal muscle and whole blood. In muscle the glutathione concentration is normalized back to reference values within a week, whereas in whole blood the glutathione concentration remains at a low level [4, 5]. On the other hand, plasma glutathione in critically ill patients is increased compared to healthy controls within 24 hours and remains at a high concentration. Still the temporal pattern of changes occurring in glutathione status during the initial phase of sepsis is not well characterized. In a rat model of sepsis, whole blood glutathione concentration decreases by 50 %, while muscle glutathione concentration increases by 40 % 48 hours after a live E Coli challenge as compared to pair fed controls. This is accompanied by an unaltered synthesis rate in whole blood glutathione, while the de novo synthesis rate of glutathione in muscle increases by 70 % [7].

To elucidate the glutathione status in man during the early septic phase, a human model where healthy volunteers are exposed to endotoxin was used. Endotoxin is a major component of the outer membrane of gram-negative bacteria that initiates the immune response clinically manifested as the systemic inflammatory response syndrome (SIRS) [8, 9]. The hemodynamic response following endotoxin administration mimics changes seen in early sepsis, although less pronounced. Still increases in heart rate, cardiac output and decreases in systemic vascular resistance and mean arterial pressure are seen. The cytokine response shows a typical pattern with an early rise of the proinflammatory mediators TNF- α , followed by IL-1, IL-6 and IL-8, similar to what is seen during early sepsis [8, 9].

The primary aim of this study was to characterize glutathione status in skeletal muscle, whole blood and in plasma during the initial 4 hours after an endotoxin challenge in healthy volunteers. In addition, the synthesis rate of glutathione in skeletal muscle and whole blood was determined to elucidate whether a change in de novo glutathione synthesis was initiated.

Methods

Subjects and study protocol (Figure 1)

Healthy male volunteers (n=8), age 26 ± 3 year, weight 74 ± 9 kg, height 182 ± 5 cm and body mass index (BMI) 22 ± 2 kg/m² (mean \pm s.d.) participated in the study. All subjects were metabolically and otherwise healthy, as determined by medical history, physical examination, analyses of cell blood count and biochemical profile. The study started in the morning after an overnight fast at 0800 and lasted for eight hours. At 4 hours, all subjects received an intravenous injection of US Standard Reference *Escherichia Coli* endotoxin of 4 ng/kg (Lot EC-6, US Pharmacopeia, Rockville, MD, USA). The concentrations of cysteine, γ -glutamylcysteine and glutathione were measured before endotoxin injection and then hourly for four hours in both plasma and whole blood. The skeletal muscle concentration of cysteine, γ -glutamylcysteine and glutathione was measured 2 hours before and just before endotoxin injection and then at 2 and 4 hours after endotoxin injection. Glutathione synthesis rate in whole blood and in muscle was determined at baseline during the last 2 hours and following the endotoxin injection (2-4 h).

[¹³C₁] glycine (99.5 % atom percent excess (APE)) were obtained from Cambridge Isotope Laboratories (Andover MA, USA). Before use the tracer solution was confirmed to be sterile and pyrogen free and thereafter prepared for the study. After injection of a priming dose of 1.2 mg/kg, [¹³C₁] glycine was infused continuously for 8 hours at $1.2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$. Arterial blood samples for analyses of whole blood glutathione concentration and enrichment were taken before the infusion was started and then at 1 hour intervals throughout the study. Blood samples for determination of free glycine isotopic enrichment were taken before tracer infusion and then every 30 minutes throughout the study. Glutathione synthesis in whole blood and skeletal muscle was calculated from blood samples or biopsies taken at 2 and 4 hours before and 2 and 4 hours after the endotoxin challenge. The study protocol is depicted in (Figure 1).

Throughout the study, heart rate, electrocardiogram, oxygen saturation and blood pressure were continuously monitored, using Datex-Engstrom light monitor. Temperature was measured in the outer ear at hourly intervals.

The purpose of the study and potential risks involved were fully explained to all subjects before obtaining their written consent. The study protocol was approved by the Ethics Committee of Karolinska Institute, Stockholm, Sweden. All subjects received economical compensation for their participation in the study.

Muscle biopsies and blood sampling

Percutaneous muscle biopsies were taken from the vastus lateralis muscle. The skin and fascia were anaesthetised by local anaesthesia with lidocaine (1% solution). The biopsies at the later occasions were taken at least 4 cm distant from the former ones. Both legs were used. The muscle tissue specimen was carefully dissected to remove all visible fat and connective tissue and thereafter divided into separate portions to allow for the determination of cysteine, γ -glutamylcysteine and glutathione concentration and glutathione synthesis (30 – 40 mg wet weight). The separate portions were weighed on an automatic electro balance within 3

minutes. Biopsies were then immediately frozen in liquid nitrogen and stored at -80°C , pending analysis.

A 500 μl aliquot of blood was immediately added to 500 μl 2 mmol phenanthroline in 14 % perchloric acid, mixed, frozen in liquid nitrogen and stored at -80°C .

Blood samples for plasma were centrifuged at 4000g for 10 minutes at 4°C . EDTA was used as anticoagulant. The plasma obtained was then immediately frozen in liquid nitrogen and then stored at -80°C pending analyses.

Sample handling of muscle and blood

Frozen muscle biopsy specimens were homogenized using a mini beat beater (Biospec Products, Bartlesville, OK, USA) in 6.5% sulphosalicylic acid (SSA) solution. Samples were subsequently centrifuged at 3,000 x g for 15 minutes at 4°C , resulting in precipitation of proteins. Part of the supernatant was used for analyses of glycine enrichment and the other part was used for determination of glutathione concentration and enrichment.

Just before analysis, whole blood samples were treated with 3 freeze-thaw cycles before centrifugation at 12500 x g for 15 min at 4°C . Part of the supernatant was then used for determination of free glycine enrichment and concentration and the other part for glutathione enrichment and concentration.

Analyses

Both reduced and total (after reduction with dithiothreitol) glutathione, cysteine and γ -glutamylcysteine concentrations were analysed in muscle, whole blood and plasma using a HPLC (high-performance liquid chromatography) technique as described before [10]. Briefly, the pH of the supernatant was adjusted with excess sodium bicarbonate powder, and part of the sample was derivatized directly for measurement of reduced glutathione, γ -glutamyl cysteine and cysteine. Total glutathione, total γ -glutamyl cysteine and total cysteine were measured after a reduction with dithiothreitol. Briefly, 100 μL portion of neutralised muscle supernatant was treated with 10 μL 50mmol/L dithiothreitol mixed and allowed to stand at room temperature for 30 minutes. Samples and standard were derivatized using monobromobimane (Cal-biocem, La Jolla, CA, USA) in sodium N-ethylmorpholine. The concentration of the oxidized form of glutathione (GSSG), which is a dimer of the reduced form of glutathione, was calculated as the difference between total and reduced glutathione divided by 2. The redox status of glutathione is expressed as the ratio between reduced and total glutathione.

Since plasma samples were not immediately treated with phenanthroline and precipitated with acid, only total concentrations of cysteine, γ -glutamyl cysteine and glutathione were analysed in plasma samples.

The glutathione synthesis rate in whole blood and skeletal muscle was calculated from the incorporation rate of [$^{13}\text{C}_1$] glycine into glutathione during a continuous infusion of [$^{13}\text{C}_1$] glycine. The isotopic enrichment of glycine in whole blood represented the precursor pool for whole blood glutathione synthesis. In muscle, free intracellular glycine represented the precursor pool.

The same HPLC procedure was used to isolate glutathione. The reduced form of glutathione was isolated and then collected using a fraction collector. The isolated glutathione was then hydrolyzed to its constituent amino acids at 110°C using 0.05 M HCl for 24 hours in the presence of ion exchange resin (AG-50 resin, Bio-Rad, Sundbyberg, Sweden). The amino acids were then eluted from the ion exchange resin with 4 M NH_3OH and dried. Thereafter the amino acids were derivatized with N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA). The tert-butyldimethylsilyl (tBDMS) derivative of glycine

was then analyzed on a gas chromatograph-mass spectrometer (GCMS) (Agilent 5973N, Kista, Sweden) detecting masses m/z 246/247.

Calculations

The synthesis rate of glutathione in both whole blood and skeletal muscle is based upon the gradual increment of isotopic enrichment of glutathione bound [$^{13}\text{C}_1$] glycine over time. In whole blood the incorporation was measured at 3 time points during 2 hours, immediately before endotoxin injection and between 2 and 4 hours after injection. The incorporation rate of glutathione in whole blood was then derived from a calculated regression line of the 3 measured time points. The precursor pool of whole blood and skeletal muscle is whole blood and free intracellular glycine respectively. The fractional synthesis rate (FSR) of whole blood and skeletal muscle is calculated according to the following equation:

$$FSR_{GSH} = \frac{E_{GSHt2} - E_{GSHt1}}{E_{Gly}} \times \frac{100}{t2 - t1} \times 12 \text{ [%/d]}$$

using the approach of Jahoor et al [11], where $E_{GSHt2} - E_{GSHt1}$ is the increase in isotopic enrichment of glutathione bound labelled glycine over the period $t2-t1$ of infusion, i.e. between 2 and 4 hours and between 6 and 8 hours in the present study. Since the time interval is 2 hours, the calculated value must be multiplied by 12 to yield %/d. E_{Gly} is the plateau isotopic enrichment of free glycine in whole blood or muscle tissue.

Absolute synthesis rate of whole blood glutathione is calculated from the glutathione concentration and the FSR giving the following equation:

$$ASR = C_{GSH} \times FSR_{GSH}$$

The units of ASR are expressed as $\mu\text{mol} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$ for whole blood and $\text{mmol} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for skeletal muscle.

Statistics

All results are expressed as means \pm SD. The temporal changes of the concentrations of thiols in whole blood and skeletal muscle were assessed by One-way ANOVA for repeated measurements followed by Scheffé F-test. (Statistica, Statsoft Inc, Tulsa, OK, USA). A paired t test was used to compare the glutathione synthesis rate before and after endotoxin injection. All data were normally distributed according to the Kolmogorov-Smirnov test (Statistica).

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Results

All healthy volunteers developed similar symptoms starting approximately 1 hour after administration of endotoxin. The signs and symptoms consisted of shivering, headache, muscle pain, malaise and in some volunteers also nausea. A low grade fever occurred after 90 minutes and remained during the study period. The intensity of symptoms varied to some extent between the subjects, but was less pronounced during the last hour of the study.

Concentrations of glutathione in plasma and whole blood (Table 1 & 2)

Samples for plasma and whole blood glutathione analyses were taken before and then hourly for 4 hours following endotoxin injection. See study protocol in Figure 1. The plasma concentration of total glutathione decreased by 24 % ($p < 0.05$) at 3 hours after endotoxin injection and by 32 % ($p < 0.001$) at 4 hours, while the precursors total cysteine and total γ -glutamylcysteine stayed unaltered.

In whole blood, the concentration of both reduced and total glutathione, as well as the precursors, total cysteine and total γ -glutamylcysteine remained unaltered during the study period. In addition, the redox status of glutathione in whole blood remained unaltered.

Concentrations of glutathione in skeletal muscle (Table 3)

Muscle concentrations of reduced and total glutathione were analyzed in specimens taken at 2 and 4 hours for baseline measurement and then at 2 and 4 hours after endotoxin injection. See study protocol in Figure 1. During the study period the concentration of both reduced and total glutathione, as well as the precursors total cysteine and total γ -glutamylcysteine remained unaltered. Also the redox status of skeletal muscle glutathione stayed unaltered.

Glutathione kinetics in whole blood and skeletal muscle

In whole blood, the fractional synthesis rates (FSR) of glutathione was 38 ± 20 %/day (mean \pm SD) before and 59 ± 22 %/day after the endotoxin challenge ($p = 0.088$). The individual values are given in Figure 2. The absolute synthesis (ASR) were 360 ± 177 $\mu\text{mol} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$ (mean \pm SD) before and 522 ± 326 $\mu\text{mol} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$ after endotoxin challenge ($p = 0.10$).

In skeletal muscle the fractional synthesis rate of glutathione was 41 ± 25 and 46 ± 18 %/day before and after the endotoxin challenge ($p=0.68$). The ASR were 614 ± 410 and 688 ± 289 $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ before and after the endotoxin respectively ($p=0.68$)

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Discussion

For the first time, glutathione status and kinetics were characterized during the initial phase of sepsis in man, using an endotoxin model. The results showed a decrease of total glutathione concentration in plasma, while it stayed unaltered in whole blood and muscle. This decrease in plasma glutathione concentration contrasts to what is seen in septic patients with multiple organ failure, where the plasma concentration of total glutathione is high as compared to healthy volunteers and patients with end stage chronic obstructive pulmonary disease [4]. Concerns may be raised, that the decreased concentration of plasma glutathione seen in the present study was an effect of continued fasting. However, in a recent study conducted by our research group, the concentration of plasma glutathione was shown to stay unaltered during 3 days of starvation [12]. Therefore it is unlikely that prolonged fasting did influence the decrease in plasma glutathione.

The decrease may be a consequence of an increased demand of cysteine in the activated immune system. Lymphocytes are depending upon plasma glutathione for their uptake of cysteine [13]. In addition, the low plasma glutathione concentration could also reflect uptake of glutathione, or the precursors of glutathione via γ -glutamyl transpeptidase, to maintain the intracellular glutathione pool in various tissues. This phenomenon is seen during strenuous physical exercise [14]. The high concentration of total glutathione in plasma in patients with septic shock later in the course of their disease is likely to be explained by leakage of oxidized glutathione from erythrocytes and probably also from other tissues via the endothelium [15].

In whole blood as well as in skeletal muscle on the other hand, the concentrations of both reduced and total glutathione remained unaltered the initial 4 hours after the intravenous endotoxin challenge. This contrasts to septic ICU patients 24 hours after admission to the ICU, when a glutathione depletion by approximately 40 % is seen in both these tissues [5, 16]. Also the redox status and the concentration of the oxidized fraction of glutathione remained unaltered in both skeletal muscle and whole blood after the endotoxin challenge. This also contrasts to septic ICU patients where a more oxidized glutathione status is seen [4, 5, 16].

There are two possible explanations for the difference with ICU patients. Either the metabolic response to endotoxin is less severe compared to septic patients to result in a glutathione depletion, or more likely, it is due to a time effect, meaning that a longer time period of sepsis or inflammation is needed for glutathione to be affected. The latter explanation is supported by animal data showing similar results after exposure to either live bacteria or endotoxin. Both studies show that glutathione depletion in erythrocytes does not occur until 6 hours after exposure [17, 18]. This suggests that glutathione depletion in erythrocytes is a slow phenomenon.

The fractional synthesis rate (FSR) and the absolute synthesis rate (ASR) of whole blood glutathione stayed unaltered immediately following the endotoxin injection. However, FSR and ASR increased in 6 out of 8 subjects, (Figure 2). The variation of the method turned out to be rather large, meaning that with 8 subjects a power of only 0.5 was obtained. To achieve a power 0.8 at least 19 subject would be needed. In contrast, pediatric patients with sepsis studied 48 hours after admission to the ICU show low values of both FSR and ASR, which might be due to a more severe insult and also due to that these septic patients were studied at a later time point [19].

For muscle tissue, the initial time pattern of glutathione changes is less well characterized. In patients with septic shock the depletion and the oxidized state is well established 24 hours after ICU admission, but no information regarding the temporal development is at hand from animal studies. Following moderate size surgery, the concentrations of total and reduced glutathione concentration decrease in muscle to the same degree as in ICU patients with sepsis (40 %) at 24 hours postoperatively, but without any effect upon redox status [20]. In contrast, at 6 hours postoperatively, no significant changes in muscle are seen [20]. In the present study, glutathione in muscle was unaltered the initial 4 hours following endotoxin injection, although an increased efflux of glutamine and a decreased uptake of glutamate across the leg are seen within the same time frame [21]. Also the synthesis rates of glutathione in skeletal muscle were not changed by the endotoxin challenge. However, we show here for the first time that the fractional synthesis rate of glutathione in human skeletal muscle is about 40 %/day which is of the same magnitude as in whole blood.

The concentrations of cysteine and γ -glutamyl cysteine were also determined as they are direct precursors for glutathione synthesis. Both remained unchanged during the study period, indicating that substrate supply of cysteine for glutathione synthesis was sufficient. The utilization of glutathione increases in septic ICU patients as evidenced by the low concentrations seen within the first days of sepsis in both whole blood and muscle as well as a more oxidized redox status of glutathione [4, 5, 19]. This also indicates the possibility that the glutathione de novo synthesis capacity is insufficient in this situation.

In summary, a decrease of plasma total glutathione concentration was seen in healthy subjects exposed to intravenous endotoxin, although the concentrations of both reduced and total glutathione as well as the redox status of glutathione in erythrocytes and muscle were unaltered. No changes in in vivo synthesis rates of glutathione were observed. However, the variation in these measurements is rather large. Therefore a larger study population is needed to confirm this finding. The study suggests that very early in the course of sepsis, as represented by an endotoxin model, the erythrocytes and muscle tissue are still able to maintain glutathione status.

Acknowledgements

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Figure legends

Figure 1. Schematic presentation of the study protocol in healthy volunteers ($n = 8$), indicating the times for plasma and blood collection and muscle biopsies, as well as the time of endotoxin injection and tracer infusion.

Figure 2. The fractional synthesis rate (FSR) of whole blood glutathione(A) and skeletal muscle (B) in healthy volunteers ($n = 8$) before and after endotoxin challenge. The graphs represent the individual subjects. The bold line represents the mean values.

Tables

Table 1. The concentration of thiols in plasma ($\mu\text{mol/l}$) of total glutathione (tGSH), total cysteine (tCYS) and total γ -glutamyl-cysteine (tGC). Values are presented as means \pm SD. Significantly different from baseline ** ($p < 0.01$).

Plasma concentration

	Baseline	60 min	120 min	180 min	240 min
tCYS	73 \pm 16	68 \pm 10	67 \pm 8	66 \pm 5	67 \pm 9
tGC	2.3 \pm 0.3	2.3 \pm 0.5	2.3 \pm 0.5	2.0 \pm 0.4	1.9 \pm 0.4
tGSH	1.9 \pm 0.9	1.7 \pm 0.7	1.6 \pm 0.7	1.4 \pm 0.5**	1.3 \pm 0.6 **

Table 2. The concentration of thiols in whole blood ($\mu\text{mol/l}$) of reduced glutathione (GSH), total glutathione (tGSH), oxidized glutathione (GSSG), redox ratio (GSH/tGSH), total cysteine (tCYS) and total γ -glutamyl-cysteine (tGC). Values are presented as means \pm SD.

Whole blood concentration

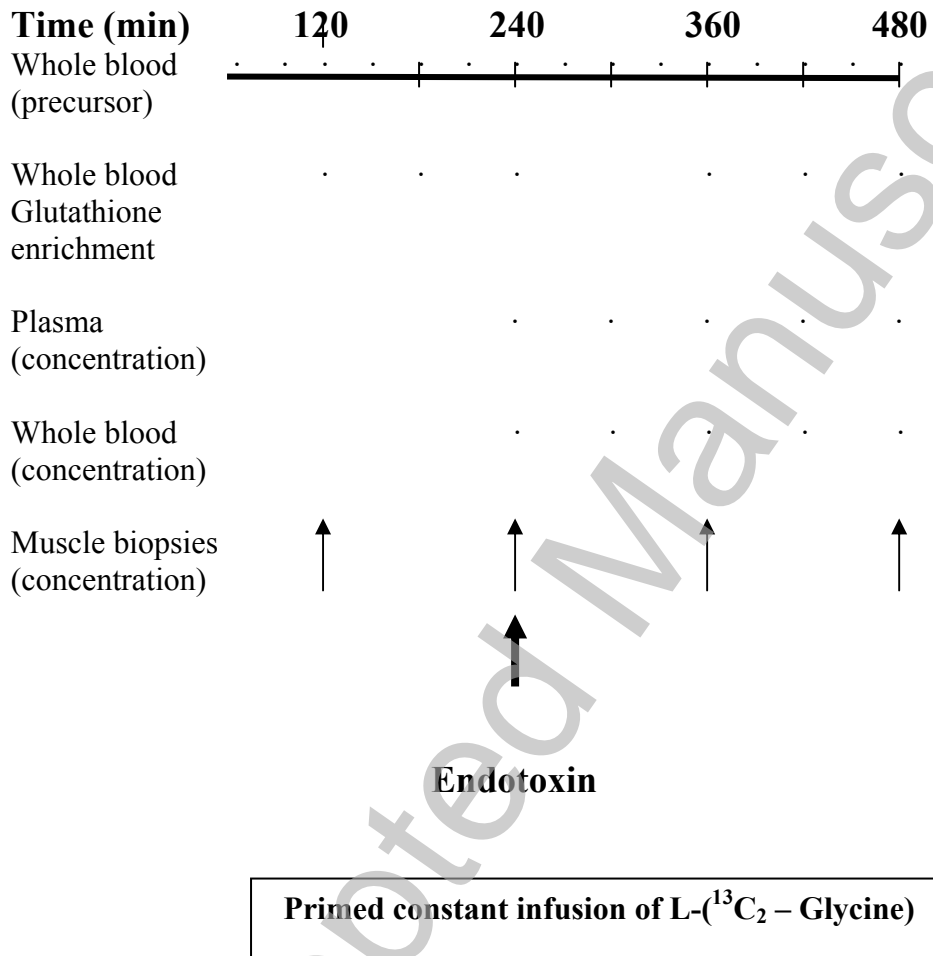
	Baseline	60 min	120 min	180 min	240 min
tCYS	76 \pm 8	74 \pm 10	74 \pm 7	71 \pm 6	79 \pm 17
tGC	2.4 \pm 0.3	2.3 \pm 0.4	2.8 \pm 1.8	2.1 \pm 0.4	2.7 \pm 1.8
GSH	896 \pm 153	904 \pm 144	879 \pm 151	922 \pm 191	889 \pm 175
tGSH	1032 \pm 159	1012 \pm 167	1023 \pm 162	1036 \pm 182	1004 \pm 176
GSSG	67.9 \pm 30.5	53.4 \pm 28.5	72.1 \pm 35.8	57.2 \pm 26.6	57.5 \pm 24.2
Redox	0.87 \pm 0.05	0.89 \pm 0.05	0.86 \pm 0.07	0.89 \pm 0.06	0.88 \pm 0.05

Table 3. Thiol concentration in skeletal muscle ($\mu\text{mol/kg}$) of reduced glutathione (GSH), total glutathione (tGSH), oxidized glutathione (GSSG), redox ratio (GSH/tGSH), total cysteine (tCYS) and total γ -glutamyl-cysteine (tGC). Values are presented as means \pm SD.

	2h control	4h control	2h post LPS	4h post LPS
tCYS	33 \pm 7	33 \pm 6	30 \pm 5	30 \pm 5
tGC	2.2 \pm 0.8	2.3 \pm 0.8	2.1 \pm 0.6	2.1 \pm 0.6
GSH	1211 \pm 194	1194 \pm 175	1345 \pm 244	1250 \pm 194
tGSH	1439 \pm 210	1412 \pm 189	1564 \pm 286	1457 \pm 210
GSSG	114 \pm 26	109 \pm 20	110 \pm 25	103 \pm 23
Redox	0.84 \pm 0.03	0.84 \pm 0.03	0.86 \pm 0.02	0.86 \pm 0.03

Figures

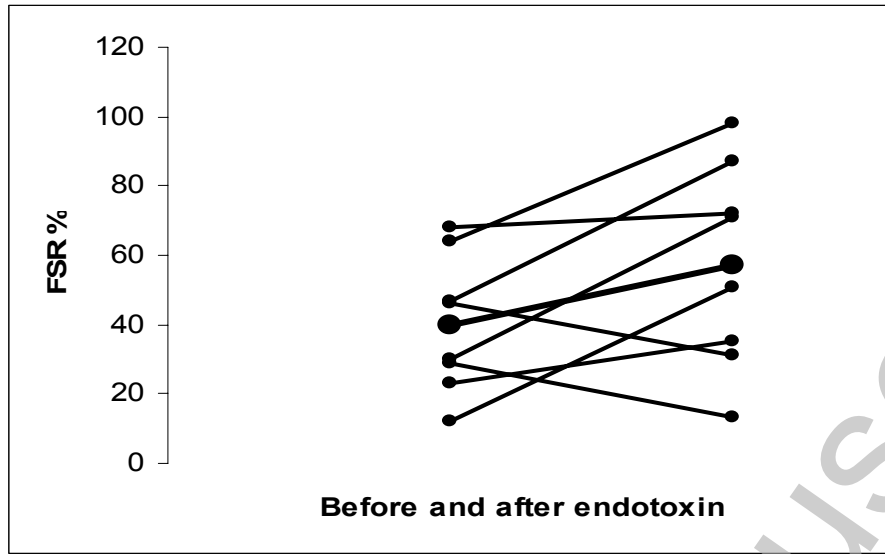
Figure 1.



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Figure 2.



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