

## **MCT1 and MCT4 kinetic of mRNA expression in different tissues after aerobic exercise at Maximal Lactate Steady State workload**

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## SUMMARY

1. We evaluate the mRNA expression of monocarboxylate transporters 1 and 4 (MCT1 and MCT4) in skeletal muscle (soleus, red and white gastrocnemius), heart and liver tissues in mice submitted to a single bout of swimming exercise at the maximal lactate steady state workload (MLSSw).
2. After 72 hours of MLSS test, the animals were submitted to a swimming exercise session for 25 minutes at individual MLSSw. Tissues and muscle samples were obtained at rest (control,  $n=5$ ), immediately ( $n=5$ ), 5 hours ( $n=5$ ) and 10 hours ( $n=5$ ) after exercise for determination of the MCT1 and MCT4 mRNA expression (RT-PCR).
3. The MCT1 mRNA expression in liver increased after 10 hours in relation to the control, immediate and 5 hours groups, but the MCT4 remained unchanged. The MCT1 mRNA expression in heart increased by 31% after 10 hours when compared to immediate, but no differences were observed in relation to the control group. No significant differences were observed for red gastrocnemius in MCT1 and MCT4 mRNA expression. However, white gastrocnemius increased MCT1 mRNA expression immediately when compared to rest, 5 and 10 hours test groups. In soleus muscle, the MCT1 mRNA expression increased immediately, 5 and 10 hours after exercise when compared to the control. In relation to MCT4 mRNA expression, the soleus increased immediately and 10 hours after acute exercise when compared to the control group.
4. The soleus, liver and heart were the main tissues that showed improved the MCT1 mRNA expression, indicating its important role in controlling MLSS concentration in mice.

*Key Words:* GENE EXPRESSION, SWIMMING, MICE, LACTATE.

## INTRODUCTION

The sarcolemmal lactate/proton cotransporter is controlled by monocarboxylate transporters (MCT) 1 and 4. MCT1 is mainly present in oxidative muscle fibers and has been associated with lactate/proton influx and intracellular oxidation (Thomas *et al.* 2004; Thomas *et al.* 2005). Although high  $K_m$ , MCT4 is not correlated with fast fiber type and is characterized by large inter-individual variation (Dimmer *et al.* 2000; Thomas *et al.* 2012). Studies indicate that MCT1 (but not MCT4) contributes to increased metabolic use of lactate during and after muscle activity (Bonen *et al.* 1998; Dubouchaud *et al.* 2000). However, the roles of both MCT1 and MCT4 intracellular signals in endurance intensity are still uncertain, since most studies about these transporters have been conducted after high intensity exercise and associated high lactate production (Bickhan *et al.* 2006; Burgomaster *et al.* 2007; Bishop *et al.* 2007; Bishop *et al.* 2008).

The Maximal Lactate Steady State (MLSS) is defined as the highest blood lactate concentration and workload that can be maintained over time without continual blood lactate accumulation. MLSS workload is considered the 'gold standard' marker of aerobic capacity (Gobatto *et al.* 2001; Ferreira *et al.* 2007). Among the methods used for the development of aerobic adaptations, training prescription at MLSS workload is one of the most adequate (deAraujo *et al.* 2007). The lactate equilibrium (between 2-8 mmol/l) during exercise at MLSS workload allows a maximum reaction rate ( $K_m$ ) of MCT1 (Billat *et al.* 2003). When oxidative capacity of muscles is increased by endurance exercise, the mRNA expression of MCT1 is also increased (Bonen *et al.*, 2000). However, the contribution of MCT1 and MCT4 on different tissues and muscles on lactate equilibrium in MLSS workload has not yet been demonstrated.

Although there has been increasing interest in the scientific basis of intensity determination using the MLSS protocol, we still lack the necessary degree of MCTs specificity to regulate the lactate steady-state. Furthermore, it is not clear what are the main tissues and muscles involved in lactatemia equilibrium as well as participation of MCT1 and MCT4 at MLSS workload. Since most of the training sessions for laboratory rats and human beings (i.e., trained and untrained) are

performed near the MLSS intensity (Gobatto *et al.* 2001; Ferreira *et al.* 2007; Seiler and Kjerland 2006), a better understanding MCT1 and MCT4 responses at MLSS workload may provide valuable insights for several areas of research.

In order to investigate the kinetics of MCT1 and MCT4 mRNA expression in different tissues in response to endurance exercise, we examined the effect of a single bout of swimming exercise at MLSS workload on the gene expression in skeletal muscles (soleus, red and white gastrocnemius), heart and liver of laboratory mice. Specifically, we hypothesized that single effort at MLSS workload increases the kinetic MCT1 (but not MCT4) mRNA expression in oxidative tissues and muscles in order to regulate the MLSS.

## **MATERIALS AND METHODS**

### *Animals*

All experiments involving the animals were performed in accordance to the specific Brazilian resolutions on the Bioethics in Experiments with animals (no 93/08, approved in September 9<sup>th</sup>, 2008), that is in agreement to the guidelines of the Guide to the Care and Use of Experimental Animals for research involving animals.

Male C57BL/6J mice were used in all experiments (n=20). The animals were housed in a room with a light cycle from 06:00 am to 6:00 pm, at  $22\pm 2$  °C and it were fed a commercial rodent chow and water *ad libitum*. All experiments were preceded by 2 weeks of individual adaptation to the deep water ( $31\pm 1$  °C) environment consisting of 5 minutes water exposure daily, 5 days a week, in cylindrical tanks (80 cm diameter×120 cm depth), subdivided into cylindrical compartments of 30 cm diameter×120 cm depth for individual swimming (de Araujo *et al.* 2007).

### *Maximal lactate steady state (MLSS)*

For MLSS determination, 20 mice were submitted to 25 minutes of swimming, performed continuously with loads equivalent to 3; 4; 5; 6 and 7% of the body weight and blood samples (25 µl collected by the tail) collection each 5 minutes. This procedure occurred in alternated days and the loads were applied randomly. The MLSS was assumed as the highest exercise intensity in which

blood lactate elevation did not exceed 1 mmol/l between the 10<sup>th</sup> and 25<sup>th</sup> minute of exercise (Gobatto *et al.* 2001; Billat *et al.* 2003; Manchado *et al.* 2005).

#### *Blood Samples and Analysis*

During the tests, blood samples (25 µl) were placed in tubes with capacity to 1.5 ml containing 50 µl of sodium fluoride (1%). Blood lactate concentrations were determined in a lactate analyzer (YSI 1500 Sport, Yellow Springs, USA).

#### *Acute exercise at MLSS workload (MLSSw) and sample tissues*

After 72 hours of MLSS determination, 15 mice were selected randomly to swim 25 minutes continuously at individual MLSSw. The animals were randomly assigned in 3 groups (5 mice for groups) and euthanized at different times to obtain the muscles and tissues samples: immediately (I); 5 hours and 10 hours after acute exercise at MLSSw. The control group (C) was composed by 5 mice that were submitted to MLSS protocol but not performed the acute exercise in order to be sacrificed in rest.

The liver, heart, soleus, red gastrocnemius and white gastrocnemius samples were collected for measurement of MCT1 and MCT4 mRNA expression. The animals were euthanized with 20% chloralhydrate (0.3 ml x 100 g<sup>-1</sup> animal weight) for tissues excision (liver, soleus, heart, red and white gastrocnemius). The tissues were carefully dissected in sterilized place and then placed into autoclaved tubes. As a consequence, the tubes were inserted immediately in liquid nitrogen (- 190 °C).

#### *MCT1 and MCT4 mRNA expression*

The gene expression was determinate by semi-quantitative analysis using RT-PCR. Total RNA was extracted using TRIZOL (Invitrogen Life Technologies, Carlsbad, CA) as described by manufacturer instructions. Briefly, tissue was homogenized (Polytron, PT-MR 2100, Luzernerstrasse, Switzerland) with 1 ml Trizol reagent (Life Technology, Rodckville, MD, USA). After 5 minutes of incubation at room temperature, 200 µl chloroform were added to the tubes and centrifuged at 12.000 x g. The aqueous phase was transferred to another tube and the RNA was

pelleted by centrifugation (12.000 x g) with cold ethanol and dried in air. RNA pellets were diluted in RNase-free water and stored at -70°C until the time of the experiment. The RNA was quantified by measuring absorbance at 260 nm. The purity of the RNAs was assessed by the 260/280 nm ratio and on a 1% agarose gel stained with ethidium bromide at 0.5 µg/ml (Sambrook and Russell 2001). These samples were used for RT-PCR analysis.

### *RT-PCR*

The sequences of the primers were designed using information contained public database in GeneBank of the National Center for Biotechnology Information (NCBI Reference Sequence: NM\_009196.4 for MCT1 and NM\_030696.3 for MCT4). The RT-PCR was performed using parameters described by Verlengia *et al.* (2004). The number of cycles used was selected to allow quantitative comparison of the samples in a linear way (Verlengia *et al.* 2004). The reaction conditions of PCR annealing temperature and PCR fragment lengths for each gene and tissues are shown in the Table 1. For the semi-quantitative analysis, the housekeeping  $\beta$ -actin gene was used as reference (Rafalski *et al.* 2007). Published guidelines were followed to guard against bacterial and nucleic acid contamination (Kwok and Higuch 1989).

### **TABLE 1**

#### *Analysis of the PCR products*

The analysis of PCR amplification products was performed in 1.5% agarose gel containing 0.5 µg/ml ethidium bromide and electrophoresed for 1 hour at 100 V. The gels were visualized using an ultraviolet light in Chemi System (UVP Bioluminescence imaging systems, Uppsala, Sweden) and photographed using a Kodak<sup>®</sup> Digital Science DC120 Zoom Digital Camera (Gibco-BRL, Life Technologies, Inc., Gaithersburg, MD, EUA). The images were processed and analyzed in the software Kodak Digital Science 1 D Image Analysis (Gibco-BRL, Life Technologies, Inc., Gaithersburg, MD, EUA). PCR band intensities were expressed as OD normalized for  $\beta$ -actin expression. The data are presented as the ratio with respective controls, which received an arbitrary value of 1 in each experiment.

### *Statistical analysis*

The results are presented as mean  $\pm$  standard error (SEM). One-way ANOVA was used to compare the MCT1 and MCT4 mRNA expression values obtained immediately, 5 and 10 hours after acute exercise at MLSSw. When necessary, Tukey post-hoc test was applied. Individual relationships between variables were studied by means of linear regressions using Excel<sup>®</sup> software. Effect sizes were determined by the formula:  $[\text{mean}_1 - \text{mean}_2]/\text{pooled SD}$  for the differences of variables. In all statistical analysis the significant level was set at  $P < 0.05$  (Statistica 7.0<sup>®</sup>).

## **RESULTS**

### *Maximal Lactate Steady State*

The mice had MLSS concentrations of 5.00 to 6.00 mmol/l at a workload between 3-5% of body weight (Figure 1).

### **FIGURE 1**

#### *MCT1 mRNA expression in different tissues after acute swimming exercise in the MLSSw*

MCT1 mRNA expression in heart, liver, red gastrocnemius, white gastrocnemius and soleus are presented in Figure 2 and Figure 3. The MCT1 mRNA expression in heart tissue increased 31% after 10 hours when compared to immediate (I) period (effect size= 1.71), but no differences were observed in relation to the control (C) group (effect size= 0.56) (Figure 2). In liver tissue, MCT1 mRNA expression after 10 hours was 39, 35 and 37% higher than the C, I and 5 hours groups, respectively (Figure 2).

### **FIGURE 2**

No differences in MCT1 were observed for red gastrocnemius muscle after acute exercise at MLSSw in relation to the Control (Figure 3). However, a significant increase in MCT1 mRNA expression was observed in white gastrocnemius muscle immediately after exercise in comparison to control (62%, effect size= 2.08), 5 hours (61%, effect size= 3.49) and 10 hours (57%, effect size= 3.35) test groups (Figure 3). MCT1 mRNA expression in soleus muscle increased in the immediate

(202%), 5 hours (227%) and 10 hours (230%) group when compared to the control group (Figure 3).

### **FIGURE 3**

*MCT4 mRNA expression in different tissues after acute swimming exercise in the MLSSw*

MCT4 mRNA expressions in heart, liver red gastrocnemius, white gastrocnemius and soleus are presented in Figure 4 and Figure 5. MCT4 mRNA expression in heart tissue reduced 28% (immediately); 35% (after 5 hours) and 38% (after 10 hours) compared with control group (Figure 4). Conversely, there were no observed differences in MCT4 mRNA expression between treatments and control in liver tissue (Figure 4).

### **FIGURE 4**

Likewise, no differences were observed between treatment and control groups in red gastrocnemius and white gastrocnemius muscles (Figure 5). However, in soleus muscle there was an immediate increase of 82% and a 56% increase 10 hours after acute exercise in comparison to the control group (Figure 5).

### **FIGURE 5**

*Relationships between variables*

In heart tissue, MCT1 mRNA expression was positively correlated with the MLSS workload ( $r=0.89$ ,  $n=5$ ) 10 hours after acute exercise. The MLSS concentration was positively correlated with MCT1 10 hours after acute exercise in white gastrocnemius muscle ( $r=0.91$ ,  $n=5$ ). There were no significant correlations observed for the other tissues and muscles used in the study.

## **DISCUSSION**

To the best of our knowledge, this is the first study to investigate MCT1 and MCT4 mRNA expression in numerous tissues and muscles under conditions of MLSS workload. MLSS has been considered the gold standard protocol to evaluate the aerobic capacity (Beneke 2000; Billat *et al.* 2003). Billat *et al.* (2003) defined MLSS as the highest blood lactate concentration and workload that is maintained over time without continual blood lactate accumulation. Thus, the investigation



of MCT1 and MCT4 mRNA expression during a MLSS test provides clear insights for understanding the mechanisms of lactate equilibrium.

The first step of this study was to determine MLSS in mice using a swimming exercise. Despite using an adapted protocol (Gobatto *et al.* 2001; Ferreira *et al.* 2007), the observed values of MLSS concentration were similar to those found in humans (Beneke 1995; Billat *et al.* 2003) and running rats (Manchado *et al.* 2005). Specifically, an MLSS concentration of ~4.00 mmol/l has been recorded in both humans and running Wistar rats (Pilis *et al.* 1993; Manchado *et al.* 2005; Faude *et al.* 2009). In Wistar rats submitted to swimming, the MLSS concentration was ~5.50 mmol/l (Pilis *et al.* 1993; Gobatto *et al.* 2001; deAraujo *et al.* 2007). However, during running the lactate MLSS concentration was lower in mice (3.00 mmol/l) than Wistar rats submitted to the same protocol (Ferreira *et al.* 2007). These results indicate that MLSS concentration is both ergometer and species dependent. Thus, measurement of the MCT kinetic of mRNA expression in other studies could be an effective means to elucidate these differences among mice, rats, humans and ergometers.

As hypothesized, our results indicate significantly higher MCT1 mRNA expression in liver, heart and soleus muscle as compared to MCT4 mRNA expression 10 hours after endurance exercise (at MLSS<sub>w</sub>). The metabolic state of the tissues or the availability of substrate as metabolic fuel seems influence the level of mRNA and protein expression, and may involve both transcriptional and post-transcriptional mechanisms (Enerson and Drewes, 2003; Philp *et al.* 2005).

Previous studies have indicated that MCT1 (but not MCT4) protein expression is related to the increase the aerobic capacity and uptake of lactate in skeletal muscle after endurance interventions (Bonen *et al.* 2000; Benton *et al.* 2008). Benton *et al.* (2008) applied a chronic electric stimulation (12 Hz, 24h/day, 7 days) in different muscle tissues (i.e., soleus, red and white gastrocnemius) to simulate an endurance muscle contraction, recording a positive relationship between PGC-1alpha protein and MCT1 protein expression as well as an increase the rate of lactate uptake into muscle. This data suggests that PGC-1alpha is a key co-activator of selected

transcription factors that induce an oxidative phenotype in skeletal muscles. It is important to note that none of these experiments detected an association between MCT4 expression and PGC-1alpha.

The control mechanisms of MCT4 expression are still poorly understood. It is thought that the hypoxia-inducible factor -1 (HIF-1) contributes to MCT4 up-regulation by hypoxia as observed in other components of the glycolytic metabolism (Ullah *et al.* 2006). Lindholm *et al.* (2014) reported a significant negative regulation of HIF-1 in elite cyclists and triathletes in relation to moderately active men after 6 weeks of an endurance training program (four 45 minutes sessions per week at 70% of VO<sub>2</sub> peak). HIF activity influences muscle metabolism and leads to increased lactate accumulation and reduced muscle pH in response to exercise. The results of these studies suggest that negative regulation of HIF-1 mediates the attenuation of PDK-1 and contributes to skeletal muscle aerobic adaptation to endurance exercise. Moreover, RIP140 could be a likely candidate for regulating MCT4 based on its inverse relationship with the oxidative capacity (Seth *et al.* 2007). In the context of our study, this may mean a predominance of aerobic metabolism during MLSS workload. Probably, the lower lactate/proton efflux at MLSS intensity in comparison to high intensity exercise is not sufficient to signal the MCT4 in oxidative muscle (Pilegaard *et al.* 1999; Bonen *et al.* 2000; Yoshida *et al.* 2004).

Our results indicate that liver tissue and soleus muscle experienced an increase in MCT1 mRNA expression 10 hours after acute exercise at MLSS workload in relation to the control group, indicating its role in controlling MLSS concentration (Coles *et al.* 2004). Billat *et al.* (2003) argues that lactate clearance is primarily through oxidation in active muscle tissue. Thus, the MCT1 mRNA expression in soleus muscle indicates that an active slow-twitch oxidative fiber may be important for lactate oxidation and, by extension, control of MLSS concentration (Roy *et al.* 1991; Bonen *et al.* 2000; Dubouchaud *et al.* 2000; Bonen, 2001).

In relation to liver tissue, MLSS workload was important to increase the MCT1 mRNA expression 10 hours after exercise. It is well known that hepatocytes are important for gluconeogenesis in order to control lactate influx during moderate intensity exercise (Messonnier *et*

*al.* 2007). Our results support this interpretation, indicating that the main function of hepatocytes during MLSS is to improve the lactate influx and consequently glucose efflux (Halestrap *et al.* 1997; Halestrap and Meredith 2004; Messonnier *et al.* 2007). However, this process appears to be more related to MCT1 than MCT4 mRNA expression. Thus, the liver is important for lactate clearance during MLSS as indicated by the increase in MCT1 mRNA expression 10 hours after exercise (Billat *et al.* 2003).

MCT1 mRNA expression in heart tissue was not altered after exercise when compared with control. This result may be attributed to high signaling and endogenous levels of MCT1 protein in the myocardium because it is a specialized organ for lactate oxidation (Bonen *et al.* 2006). The high correlation between MLSS workload and MCT1 mRNA expression in heart 10 hours after exercise ( $r=0.89$ ) is interesting, and may indicate a predisposition to aerobic capacity in mice with enhanced MCT1 expression in heart tissue. The MCT4 mRNA expression decreased in heart tissue 10 hours after exercise. Differences in abundance and sub-cellular distribution of MCT1 and MCT4 in rat heart tissue were described by Bonen *et al.* (2000), who reported that MCT4 mRNA expression is barely detectable and MCT4 protein appears to be absent (Bonen *et al.* 2000). In contrast, both MCT4 mRNA and MCT4 protein are detectable in human heart tissue (Price *et al.* 1998; Wilson *et al.* 1998). This indicates abundant species level variation in MCT4 expression, though there is no comparable data on MCT4 mRNA expression in mice.

Contrary to our hypothesis, MCT1 gene expression in white gastrocnemius muscle increased immediately after the endurance exercise. Considering that MCT1 mRNA expression is highly controlled by acidosis level, the lactate concentration in MLSS (5.50 mmol/l) may indicate adaptations in gene expression independent of fiber type due to  $K_m$  between 3.00-5.00 mmol/l (Tonouchi *et al.* 2002). Our data support the existence of such adaptations since the MLSS concentration was correlated with MCT1 10 hours after exercise in white gastrocnemius muscle ( $r=0.91$ ). Also, these data agree with the results of Coles *et al.* (2004) using a treadmill, which demonstrated that the muscles with the lowest MCT1 content (i.e., white gastrocnemius) and MCT4

content (i.e., soleus) have the greatest relative signaling of MCT1 and MCT4, respectively. This response is related with the principle of expression-rapid induction (c.f. Hildebrand *et al.* 2003).

In comparison to the control group, acute exercise did not induce adaptations in red gastrocnemius, since MCT1 and MCT4 remained unchanged after acute exercise. However, the absence of changes in red gastrocnemius in relation control group does not indicate that the tissue is not important to MLSS concentration (Bonen *et al.* 2003) and further studies are clearly needed to better understand the intracellular signaling in muscle.

Our results have implications for experimental research that uses physical exercise to understand the metabolism/substrate regulation with different interventions (i.e., pathologies, nutrition, drugs and other) and, more specifically, for studies with human beings that aim to establish reference values in relation to MCTs kinetic of mRNA expression during endurance exercise. It appears that MCT1 mRNA expression is regulated by MLSS concentration in predominantly oxidative tissues such as the soleus muscle, liver and heart. In white gastrocnemius muscle, the MLSS workload increased MCT1 mRNA expression immediately after exercise. However, exercise at MLSS workload did not increase MCT4 mRNA expression in oxidative tissues (liver and heart) and soleus muscle. Further studies are now required to investigate protein concentrations in tissues since there seems to be no direct correlation between protein and mRNA levels in mammalian cells (Pradet-Balade *et al.* 2001).

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## LEGENDS

**FIGURE 1** – Maximal lactate steady state (MLSS) determination in sedentary mice. The MLSS determination used randomized loads of 3.00; 4.00; 5.00; 6.00 and 7.00% of body weight. The MLSS was considered as the maximal overload that shows lactate stabilization between the 10<sup>th</sup> and 25<sup>th</sup> minute (min) of exercise. The results are presented as mean± SEM.

**FIGURE 2** - Effect of the swimming exercise in the MLSS intensity (25 minutes) on the gene expression of the MCT1 in heart and liver obtained immediately (I), 5 and 10 hours (h) after the exercise. Data are reported as the mean ± SEM of 6 experiments performed in duplicate. # Significantly different in relation to immediately (I); \* Significantly different in relation all the groups.

**FIGURE 3** - Effect of the swimming exercise in the MLSS intensity (25 minutes) on the gene expression of the MCT1 in red gastrocnemius, white gastrocnemius and soleus obtained immediately (I), 5 and 10 hours (h) after the exercise. Data are reported as the mean ± SEM of 6 experiments performed in duplicate. \* Significantly different in relation all the groups.

**FIGURE 4** - Effect of the swimming exercise in the MLSS intensity (25 minutes) on the gene expression of the MCT4 in heart and liver obtained immediately (I), 5 and 10 hours (h) after the exercise. Data are reported as the mean ± SEM of 6 experiments performed in duplicate. \* Significantly different in relation all the groups.

**FIGURE 5** - Effect of the swimming exercise in the MLSS intensity (25 minutes) on the gene expression of the MCT4 in red gastrocnemius, white gastrocnemius and soleus obtained immediately (I), 5 and 10 hours (h) after the exercise. Data are reported as the mean ± SEM of 6 experiments performed in duplicate. # Significantly different in relation to control (C); \* Significantly different in relation all the groups.

**TABLE 1**

**TABLE 1.** The standardized conditions for RT-PCR analysis. The sequences of the primers, the PCR fragment lengths, the temperature and the number of cycles are shown for each gene and tissues (H=heart; L=liver; RG=red gastrocnemius; WG= white gastrocnemius; S=soleus) under study.

<i>Gene</i>	Sense primer	Antisense primer	Anneling Temperature (°C)	PCR fragment lengths (bp)	Tissues	Amplification Cycles
					H	34
	5'-GTG ACC	5'-GTC TCC TTT			L	32
<b>MCT1</b>	ATT GTG GAA	GGC TTC TCG	57.1	254	RG	34
	TGC TGC-3'	TCG-3'			WG	35
					S	37
					H	34
	5'-TGC CAT	5'-TCT GCC TTC			L	37
<b>MCT4</b>	TGG TCT CGT	AGG AAG TGC	58.2	291	RG	34
	GCT G-3'	TCC-3'			WG	34
					S	37
					H	30
	5'-ACA GGC	5'-TGT CAC CGA			L	34
<b>β-actin</b>	ATT GTC ATG	TTT CCC TCT C-	58.2	205	RG	32
	GAC TCC G-3'	3'			WG	32
					S	34

Figure 1

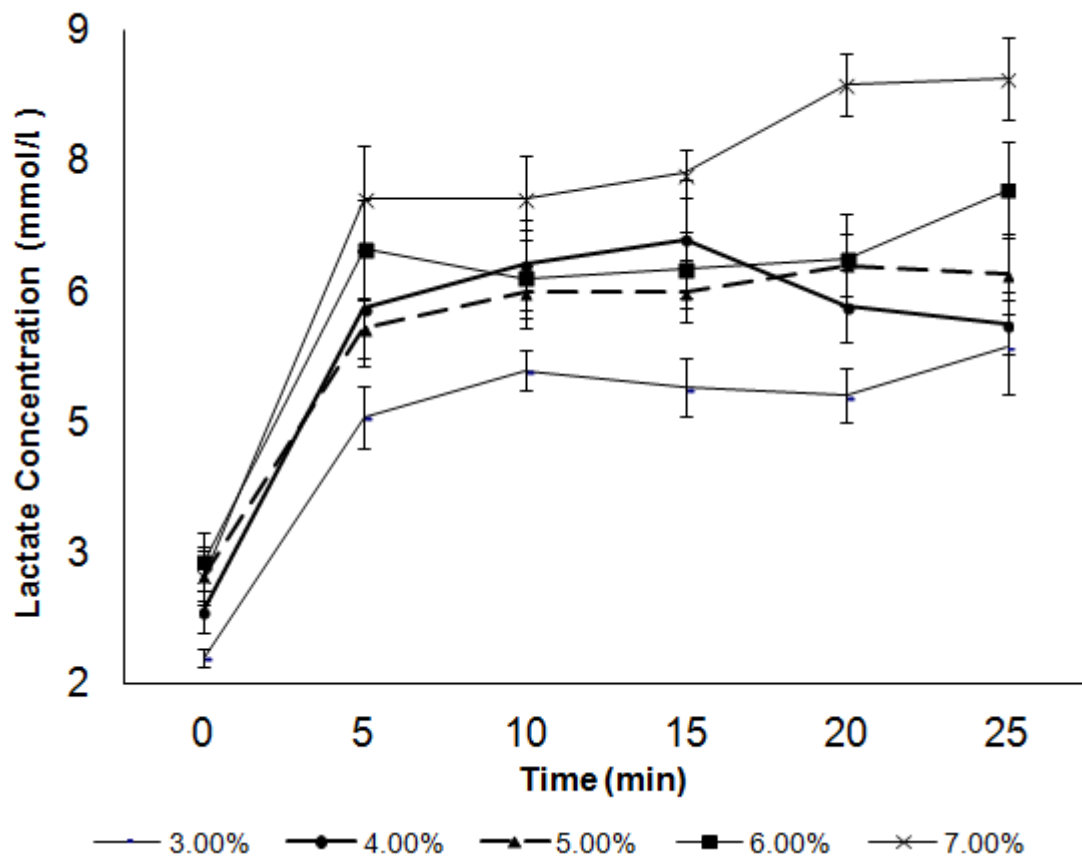


Figure 2

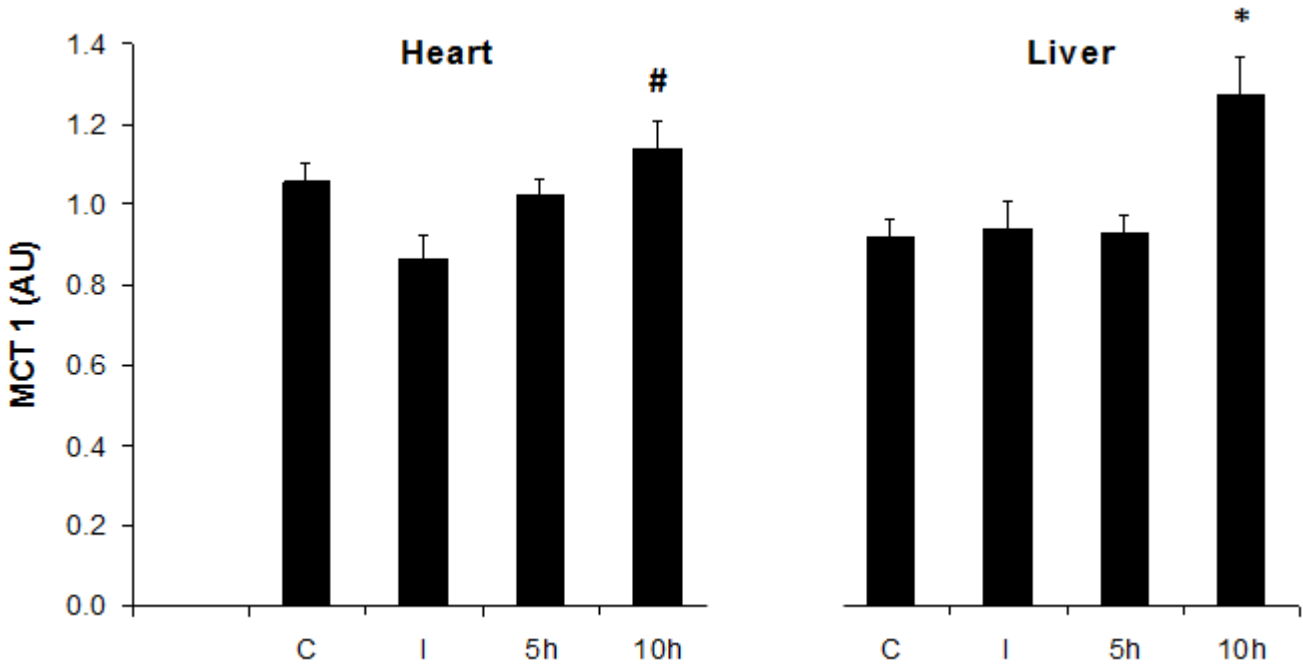


Figure 3

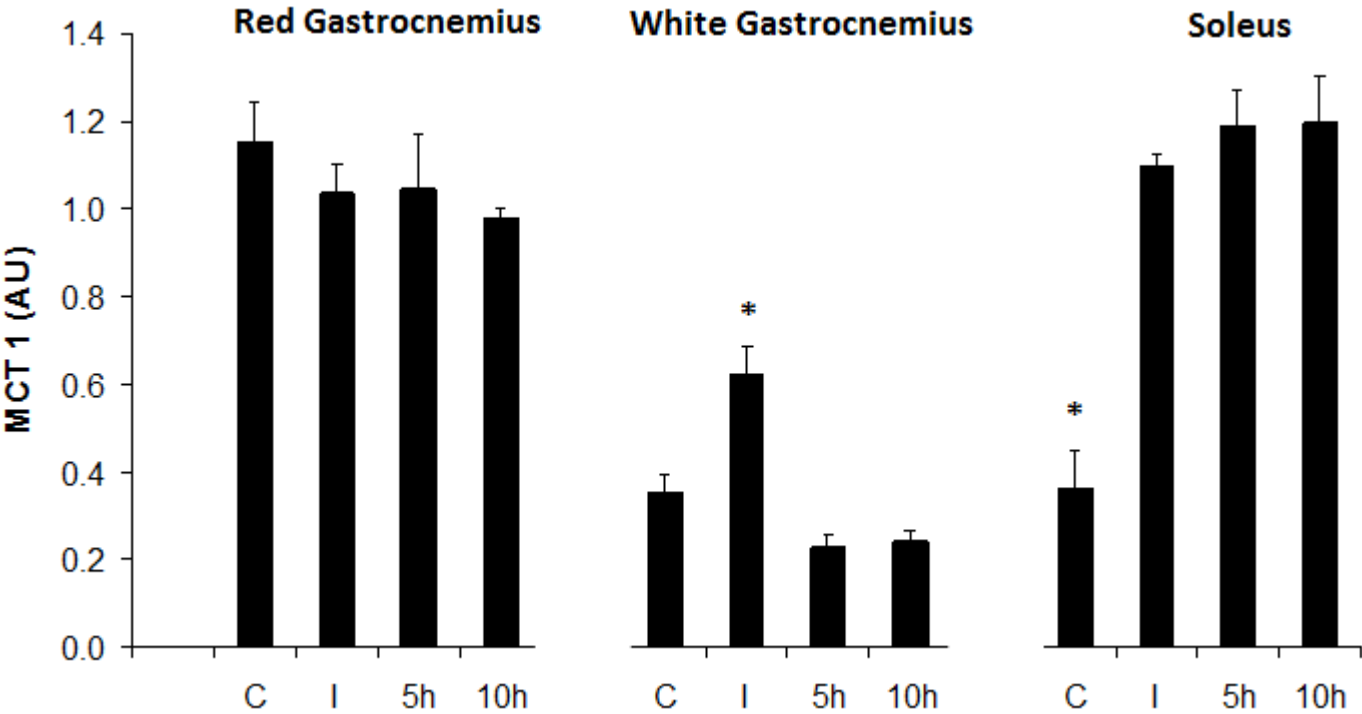


Figure 4

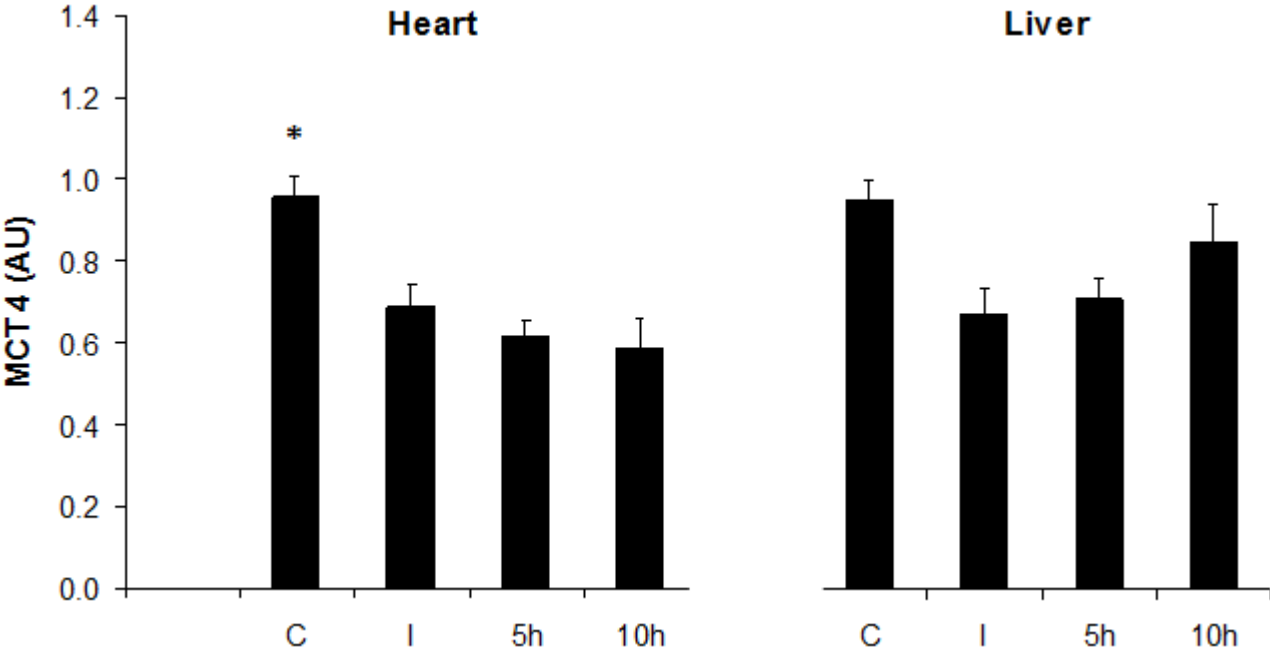


Figure 5

