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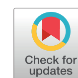
# LC-MS analysis of key components of the glutathione cycle in tissues and body fluids from mice with myocardial infarction



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ABSTRACT

Oxidative stress is suggested to play an important role in several pathophysiological conditions. A recent study showed that decreasing 5-oxoproline (pyroglutamate) concentration, an important mediator of oxidative stress, by over-expressing 5-oxoprolinase, improves cardiac function post-myocardial infarction in mice. The aim of the current study is to gain a better understanding of the role of the glutathione cycle in a mouse model of myocardial infarction by establishing quantitative relationships between key components of this cycle. We developed and validated an LC–MS method to quantify 5-oxoproline, L-glutamate, reduced glutathione (GSH) and oxidized GSH (GSSG) in different biological samples (heart, kidney, liver, plasma, and urine) of mice with and without myocardial infarction. 5-oxoproline concentration was elevated in all biological samples from mice with myocardial infarction. The ratio of GSH/GSSG was significantly decreased in cardiac tissue, but not in the other tissues/body fluids. This emphasizes the role of 5-oxoproline as an inducer of oxidative stress related to myocardial infarction and as a possible biomarker. An increase in the level of 5-oxoproline is associated with a decrease in the GSH/GSSG ratio, a well-established marker for oxidative stress, in cardiac tissue post-myocardial infarction. This suggests that 5-oxoproline may serve as an easily measurable marker for oxidative stress resulting from cardiac injury. Our findings show further that liver and kidneys have more capacity to cope with oxidative stress conditions in comparison to the heart, since the GSH/GSSG ratio is not affected in these organs despite a significant increase in 5-oxoproline.

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## 1. Introduction

Oxidative stress is defined as the imbalance between the production of reactive oxygen species (ROS), and the capacity of the endogenous antioxidant defense system to deal with ROS [1]. Under physiological conditions, small quantities of ROS, which function in cell signaling, can be readily neutralized by the antioxidant defense system. However, under pathophysiological conditions, ROS production may exceed the buffering capacity of the antioxidant defense system, resulting in cell damage and ultimately cell death. This imbalance in redox state is implicated in the onset and progression of several diseases, including cardiovascular disease [1,2].

The major source of antioxidants in mammalian cells is glutathione (GSH), which is formed by the glutathione cycle, also formerly known as γ-glutamyl cycle (Fig. 1) [3]. Although the enzymes and metabolites of the glutathione cycle have been characterized extensively, only recently have they been associated with heart failure [4]. One such enzyme, 5-oxoprolinase (OPLAH), is responsible for converting 5-oxoproline, both a degradation product and an intermediate of *de novo* GSH synthesis, into L-glutamate [3,5]. 5-Oxoproline has been shown to induce oxidative stress in brain tissue and cardiomyocytes [4,6]. Furthermore, decreasing the level of 5-oxoproline by over-expressing OPLAH in mice, improves cardiac function post cardiac injury [4]. These observations suggest a major role of the glutathione cycle in heart failure.

To obtain a better understanding of the involvement of the glutathione cycle in heart failure, it is essential to decipher how key components change under physiological and pathophysiological conditions. Numerous analytical methods have been established to quantify 5-oxoproline, L-glutamate, GSH and GSSG [7–12]. Here we

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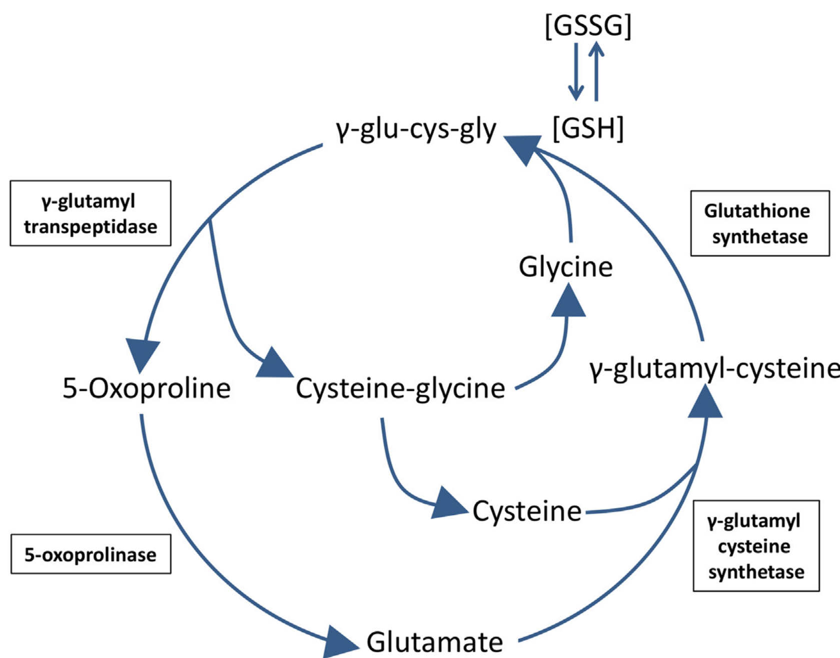


**Fig. 1.** Schematic representation of the glutathione cycle. 5-oxoproline, both a degradation product and an intermediate of *de novo* glutathione (GSH) synthesis, is transformed into L-glutamate via 5-oxoprolinase (OPLAH) activity. GSH can then be utilized as an antioxidant, producing oxidized glutathione (GSSG) in the process.

report the development and validation of an LC–MS method for the simultaneous quantitation of 5-oxoproline, L-glutamate, GSH and GSSG in different biological samples (heart, kidney, liver, plasma and urine) of mice with and without myocardial infarction (MI). From a methodological point of view, we show that certain matrices may lead to interferences. From a disease mechanism point of view, we show that the failing heart has limited anti-oxidant capacity compared to the kidneys and liver, making it particularly vulnerable to ROS.

## 2. Materials and methods

### 2.1. Solvents, chemicals and standards

All chemicals used had the highest purity commercially available. Methanol (MeOH, HPLC SupraGradient grade) was purchased from Biosolve (Valkenswaard, The Netherlands). Phosphate buffered saline (PBS), bovine serum albumin (BSA), formic acid (LC–MS grade), N-ethylmaleimide (NEM) and all standard compounds ($^{13}$C-labeled L-glutamic acid, $^{13}$C, $^{15}$N-labeled GSH and non-labeled 5-oxoproline, L-glutamic acid, GSH and GSSG) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Ultrapure water was obtained from a Milli-Q Advantage A10 water purification system at a resistivity of 18.2 MΩ cm (Millipore SAS, Molsheim, France).

### 2.2. Permanent myocardial infarction in wild-type mice

The animal protocol was approved by the Animal Ethical Committee of the University of Groningen (permit number: DEC6632), and performed conform the ARRIVE guidelines [13]. A total of 24 C57BL/6 J mice were included in the MI study. All mice were 14–20 weeks of age and 35–40 g of body weight. The mice were randomized into the SHAM-operated group and the MI group. Animals were anesthetized with isoflurane and medical oxygen, followed by the administration of 5 mg/kg of carprofen. The MI group (n = 13) underwent permanent ligation of the left anterior descending branch (LAD) of the left coronary artery. The ligation of the LAD was placed to achieve a ±30% area at risk of the left ventricle. The SHAM operated group (n = 11) underwent the same procedure without ligation of the LAD. After 4 weeks, animals were sacrificed and blood, urine, and organs were collected, immediately placed in liquid nitrogen, and stored for further sample preparation and subsequent LC—MS analysis.

### 2.3. Production of isotopically-labeled internal standards (IS)

5-Oxoproline internal standard (IS) was prepared from $^{13}$C-labeled L-glutamic acid. Briefly, L-glutamic acid (250 mg) was dissolved in 0.1 M HCl and heated at 80 °C for 72 h, to convert $^{13}$C-L-glutamic acid into $^{13}$C-5-oxoproline, as previously described [12]. Later, the solution was dried under a stream of nitrogen and re-dissolved in 50 mL water.

GSSG (IS) was prepared by a controlled oxidation of $^{13}$C, $^{15}$N-labeled GSH. Briefly, 10 mg of $^{13}$C, $^{15}$N-labeled GSH were dissolved in 1 mL water. Half of the solution (0.5 mL) was mixed with of 0.5 mg NaI (final concentration 6.7 mM) and 1 μL 30% $H_2O_2$. The mixture was heated at 25 °C for 60 min to allow oxidation. Excess $H_2O_2$ was eliminated by increasing the temperature of the mixture to 65 °C for 5 min as previously described [14].

Both solutions (one containing $^{13}$C-5-oxoproline and $^{13}$C-L-glutamic acid, and the other containing $^{13}$C, $^{15}$N-labeled GSH and $^{13}$C, $^{15}$N-GSSG) were mixed and the solvent was evaporated. Finally, the mixture was resuspended in 1 mL water and used as IS for further experimental work. The final ratio of the components in the IS solution was 1:1,5:6:12 for $^{13}$C-L-glutamic acid, $^{13}$C-5-oxoproline, $^{13}$C, $^{15}$N-GSSG and $^{13}$C, $^{15}$N-labeled GSH, respectively.

### 2.4. Sample preparation

Murine plasma and urine were prepared by adding 200 μL of cold (−20 °C) extraction solution (0.5 μL isotopically-labeled IS and 1.25 mg of NEM in 75% methanol) to 25 μL of sample. Snap frozen murine tissues (heart, kidney, and liver) were powdered using a mortar and pestle and ± 1 mg of powdered tissue was mixed with 200 μL of cold (−20 °C) extraction solution. Plasma and urine sam-

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