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Short communication

Simulated microgravity induce glutathione antioxidant pathway in *Xenopus laevis* embryos

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Abstract

Space flights cause a number of patho-physiological changes. Oxidative damage has been demonstrated in astronauts after space flights. Oxidative stress is due to an imbalance between production of oxidant and antioxidative defence. In embryos of *Xenopus laevis*, the glutathione system is an inducible antioxidant defence. For this reason, we investigated the effect of gravity deprivation on endogenous antioxidant enzymes in *X. laevis* embryos developed for 6 days in a Random Positioning Machine. The results show that glutathione content and the activity of antioxidant enzymes increase in RPM embryos, suggesting the presence of a protective mechanism. An induction of antioxidant defence might play an important role for animals to adapt to micro-gravitational stress, possibly during actual space flights.

Keywords: Simulated microgravity; *Xenopus laevis* embryos; Glutathione; Oxidative stress; Random positioning machine

1. Introduction

Oxygen dependent metabolism produces reactive oxygen species (ROS). Low levels of ROS play a beneficial role in processes such as defence against microorganisms and intracellular signalling. Nonetheless, ROS are a potential threat to important bio-molecules and metabolic functions. Indeed oxidative stress has been related to ageing and to a number of chronic and acute diseases (for a review see Valko et al., 2007). In aerobic organisms, enzymatic and non-enzymatic activities provide a defence against the accumulation of ROS. The health and survival of organisms is dependent on the regulation and balance of antioxidant activities.

The antioxidant defence of cells consists of enzymatic and non-enzymatic antioxidants that operate together with repairing mechanisms (Thorpe et al., 2004; Hayes and Mc Lellan, 1999; Yu, 1994). The enzymatic antioxidant system is composed mainly of peroxidases and catalases. A distinct type of antioxidant defence is represented by heat shock proteins (Anderson, 1996). Other non-enzymatic antioxidants are also available, such as vitamin E, Vitamin C, carotenoids, flavonoids, coenzyme Q_{10} and squalene.

Among endogenous antioxidants, reduced glutathione (GSH) plays a key role. GSH is an intracellular tripeptide that reacts with ROS, reduces hydro-peroxides and breaks disulfide bridges formed by oxidants; its antioxidant action produces glutathione disulfide (GSSG) that can be either exported from the cell or reduced back to GSH by GSH reductase and NADPH. GSH also produces antioxidant molecules regenerating ascorbate from dehydroascorbate and α-tocopherol from tocopheroxyl radical (Franco et al., 2007).

Exposure to microgravity and radiations during space flights could be responsible for a number of changes in living organisms resembling modifications that occur in ageing. In fact human life in space and ageing are both characterized by increased stress hormones, insulin resistance, anorexia, altered musculoskeletal system structure and function, and altered inflammatory response and mitochondrial function with

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increased oxidative stress (Stein, 2002; Finkel and Holbrook, 2000; Stein and Leskiw, 2000). Of note, increased oxidative stress and sensitivity to oxidants are two main aspects shared by ageing and space environment, and several studies on different species are consistent with a causal connection between increased stress resistance and extended life span (Orr and Sohal, 1994).

Oxidative stress can be caused either by a genetically determined ''weakness'' of the antioxidant defence, or by an increase of the random damage caused by an over exposure to reactive oxygen metabolites (ROS). Since many types of ionizing radiations that are encountered on Earth, and more in space, are capable of generating ROS and free radicals (Guan et al., 2006; Hollander et al., 1998; Lyng et al., 2001), radiation is a potential threat for long-term outer-space missions. Nonetheless many metabolic processes must be taken in account since the oxidative stress can be due to a number of co-existing factors.

The role of microgravity on oxidative stress during space flights has not been resolved. Some authors have suggested that microgravity might enhance pro-oxidant processes, while others did not record synergic effects. Although there are many discrepancies among the results, presumably due to the variety of organisms that have been tested, the relevance of such studies lies on the presumption that results could suggest strategies to protect living organisms, perhaps also humans, when exposed to the space environment (Manti, 2006).

Xenopus laevis has been one of the first organisms used in space and stratospheric balloon flights to study fertilization and embryogenesis processes (Rizzo et al., 2007a,b). Our aim was to investigate whether microgravity exposure of *X. laevis* embryos might influence antioxidant defences of the animal contributing to enhance oxidative stress.

2. Materials and methods

2.1. Substrates and reactive for enzyme determinations

NAD(P)H, DTNB, GSH, GSSG, glutathione reductase and tert-butyl hydroperoxide were purchased from Sigma–Aldrich (St. Louis, Missouri, USA).

2.2. Embryo culture

Embryos of *X. laevis* were obtained by *in vitro* fertilization (Rizzo et al., 1994). Briefly, *Xenopus*, (Rettili, Varese, Italy), were maintained in aquaria (Tecniplast, Varese, Italy). Females were injected with 700–800 I.U. of human chorionic gonadotropin in the evening, and the next day they were made to lay eggs in 90 mm plastic Petri dishes. Eggs were immediately inseminated with sperm suspension obtained by mincing testes in 1–2 mL of cold DBT solution. Eggs were selected, and the embryos with deformations were eliminated. Embryos were maintained in a thermostatic chamber at 23 ± 0.5 °C for 1 day, and collected with large bore pipettes.

2.3. Microgravity simulation

Simulation of microgravity was achieved with a Random Positioning Machine (RPM, Dutch, Space, Leiden, The Netherlands). It is essentially a 3-axis clinostat, in which the weight vector is continually reoriented as in traditional clinorotation, but with increased directional randomization (van Loon, 2007).

In the RPM, probes are fixed as close as possible to the centre of 2 frames rotating one inside the other, driven by separate motors. The rotation of each frame is random and autonomous under computer control. The low g conditions are modelled by averaging the gravity vector via the independent rotation of the 2 frames. The outer frame rotated perpendicular to the inner frame, which caused the samples to move randomly in 3 axes.

The speed of rotation was 60 °/s (about $10^{-3} \times$ g), and direction and interval were set at random. The RPM was accommodated in a temperature-controlled incubator at 23 °C. In the experiments, both control and microgravity embryos (ageing 1 day) were housed in T25 vented cell culture flask full of Fetax medium (Rizzo et al., 1994). After 3 days of incubation, the instrument was stopped for few minutes to allow medium changes. Control samples ($1 \times$ g) were placed on the fixed base of the RPM, facing the same vibrations as the rotating ones. At day 3 and 6, RPM and control embryos were sampled, frozen at −80 °C for enzymatic assays, or immediately homogenized for total glutathione determination.

2.4. Enzymes assay

Embryos were homogenized on ice in H_2O or 100 mM, pH 7.0 K-phosphate buffer and centrifuged at 1000 ×g for 10 min at 4 °C.; supernatants were assayed for protein content according to Peterson (1977).

2.4.1. Superoxide dismutase (SOD) activity assay

Enzyme activity was assayed using the method based on NAD(P)H oxidation inhibition, according to Paoletti and Mocali (1990). Briefly, the inhibition of NADPH oxidation by chemically generated superoxide was measured at 340 nm for 20 min, in the presence of tissue extracts. The incubation mixture included: 800 µl of TDB (triethanolamine/diethanolamine 100 mM, pH 7.4), 40 µl NADPH 7.5 mM, 25 µl EDTA–$MnCl_2$ (100 mM/50 mM), and 100 µl of sample or blank. One unit of SOD activity is defined as the amount of enzyme required to inhibit the rate of NADPH oxidation by 50%.

2.4.2. Catalase (CAT) activity assay

Supernatant enzyme activity was assayed by measuring the consumption of H_2O_2 according to Aebi (1984). The consumption of hydrogen peroxide by the tissue extracts was determined at 240 nm for 1 min at 30 °C. The incubation mixture included: 50 µl H_2O_2 200 mM, 100 µg of proteins of tissue extract, and Na-phosphate buffer (50 mM pH 7.0) to reach a final volume of 1 mL. One unit of CAT activity is