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Effects of hypergravity environments on amphibian development, gene expression and apoptosis

Satomi Kawakami [a], Keiko Kashiwagi [a], Nobuaki Furuno [a], Masamichi Yamashita [b], Akihiko Kashiwagi [a,*]

[a] *Institute for Amphibian Biology, Graduate School of Science, Hiroshima University, Higashihiroshima 739-8526, Japan*
[b] *Institute of Space and Astronautical Science (ISAS), Japan Aerospace Exploration Agency (JAXA), Yoshinodai, Sagamihara, Kanagawa 229-8510, Japan*

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Abstract

This study investigates how rearing under conditions of hypergravity affects amphibian development, *Xotx2* and *Xag1* gene expression and apoptosis. Uncleaved *Xenopus laevis* eggs 20 min after insemination, 2 cell stage embryos, and gastrula stage embryos were raised at 2G and 5G, while controls were raised in normal gravity. Apoptosis in brain and eye inner structures of hatching embryos was scored using the TUNEL staining method, and gene expression in tail-bud embryos was analyzed by whole-mount *in situ* hybridization. Results showed that: (1) 5G retarded the development of eggs and embryos and induced microcephaly and microphthalmia. (2) 5G suppressed the expression of the two genes, *Xotx2* (involved in fore- and midbrain and eye development) and *Xag1* (regulating cement gland formation). (3) Eggs and 2 cell stage embryos raised at 5G showed a greater extent of brain and eye apoptosis compared with controls, while those raised at 2G showed no significant difference. These findings suggest that high gravity suppresses certain gene functions and induces abnormal apoptosis in brain and eyes, resulting in developmental retardation and various morphological abnormalities.

Keywords: Space environments; Hypergravity; *Xenopus laevis*; Amphibian development; Gene expression; Apoptosis; Developmental retardation; Morphological abnormalities

1. Introduction

Amphibians have proved to be suitable model organisms for space environment studies (Snetkova et al., 1995; Gualandris-Parisot et al., 1996, 2001, 2002; Neubert et al., 1998; Yamashita et al., 1999; Wassersug and Yamashita, 2000; Husson et al., 2001; Horn et al., in press). Experiments have shown that amphibian embryos are sensitive to changes in gravity environment in the following manner: (1) Real microgravity during parabolic or orbital space flights resulted in a thickening of the blastocoel roof in the gastrula (Ubbels et al., 1995; De Mazière et al., 1996; Wassersug, 2001) and neural retina regeneration (Grigoryan et al., 1998, 2002); (2) Clinostating-simulated microgravity increased the animal vegetal cleavage ratio (AVCR), while centrifuge-induced hypergravity decreased AVCR (Yokota et al., 1992); (3) Hypergravity affected embryonic axis formation (Black, 1990), and brain metabolism during development (Slenzka et al., 1993); (4) Hypergravity retarded the development of eggs and embryos and induced various abnormalities (Neff et al., 1990; Kashiwagi et al., 2003). Other investigations on the other hand have shown developing embryos and tadpoles to be remarkably flexible when exposed to space flight or artificially altered gravitational environments (Neff et al., 1993; Souza et al., 1995; Black et al., 1996; Ubbels, 1997; Dournon et al., 2001; Dournon, 2003; Horn et al., in press).

Apoptosis plays a major role in development and tissue homeostasis (Kerr et al., 1972; Ellis et al., 1991; Vaux et al., 1994; Jacobson et al., 1997; Mignotte and Vayssiere, 1998), but too much or too little apoptosis causes a number of functional disorders in humans (Thompson, 1995). Real and simulated microgravity have been shown to induce apoptosis in

* Corresponding author. Tel./fax: +81 82 424 7485.
E-mail address: akashiwa@hiroshima-u.ac.jp (A. Kashiwagi).

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osteoblasts (Nakamura et al., 2003; Bucaro et al., 2004), thyroid cells (Schonberger et al., 2000; Kossmehl et al., 2003), lymphocytes (Lewis et al., 1998; Bakos et al., 2001; Schatten et al., 2001), endothelial cells (Morbidelli et al., 2005; Infanger et al., 2006) and glial cells (Uva et al., 2002).

The mechanism by which altered gravity induces apoptosis is not well understood. According to Lalani et al. (2000), exposure to spaceflight upregulates negative modulators of skeletal muscle mass, including myostatin, and downregulates positive modulators of muscle differentiation and growth, including insulin-like growth factor-II (IGF-II), causing an increase in apoptosis, eventually resulting in loss of skeletal muscle mass and function. Nomura et al. (2002) reported that the stresses imposed bygravity changes during free fall induce apoptosis in human cells, marked by the accumulation of apoptosis-inducing p53 and pro-apoptotic Bax. Hypergravity has also been found to induce phosphorylation of p53 (Okaichi et al., 2004) and expression of cyclooxygenase-2 (Oshima et al., 2005).

The mouse homeobox gene *Otx2* is thought to be involved in brain patterning and morphogenesis (Rhinn et al., 1999; Crossley et al., 2001; Acampora et al., 1995), eye development (Acampora et al., 2005; Nishida et al., 2003) and prevention of apotosisis in the forebrain (Rhinn et al., 1999). The *Xenopus laevis Xotx2* gene–homologous to the murine *Otx2* gene–is expressed in the fore- and midbrain regions and the eyes (Kablar et al., 1996; Viczian et al., 2003). *Xotx2* in embryos activates expression of the cement gland marker gene *Xag1* (Blitz and Cho, 1995; Wardle et al., 2002). However, at present it is not known if *Xotx2* and *Xag1* are affected by changes in gravity environment.

The present study investigates the effects of hypergravity on apoptosis and *Xotx2* and *Xag1* expression.

2. Materials and methods

2.1. Animals

Animals were treated according to the basic principles expressed in International Guiding Principles for Biomedical Research Involving Animals (1985), as well as Policies and Procedures/Best Practices for Laboratory Animal Care by the Stanford University School of Medicine (http://med.stanford.edu/compmed/animal_care/amphibians.html).

X. laevis were derived from standard strains maintained by the Hiroshima University Institute for Amphibian Biology. Ovulation was induced in mature females by injecting 700 units of human chorionic gonadotropin (hCG; Sigma-Aldrich) into the dorsal lymph sac. Mature males received 125 units as a stimulant. Eggs were artificially fertilized and collected from females, with over 90% cleaving normally. Embryos were staged according to Nieuwkoop and Faber (1956).

2.2. Hypergravity experiments

Only eggs with the lighter animal pole facing upward and undamaged embryos of normal appearance were selected with the aid of a binocular dissecting microscope. Eggs 20 min after insemination and 2 cell stage and gastrula stage embryos were placed in polystyrene cups (W7 cm, H6.5 cm; Iwasaki Industry Co., Nara, Japan) containing 100 ml of Cl-free tap water at a ratio of 40 individuals per cup. Cups were placed in 12.5 cm × 12.5 cm × 20.0 cm polystyrene containers and subjected to 2G and 5G hypergravity treatment in a swing-bucket type centrifuge at 20 °C with fluorescent lighting and a 12 h day and night cycle. Eggs and embryos were examined daily and removed from the experiment when signs of abnormal

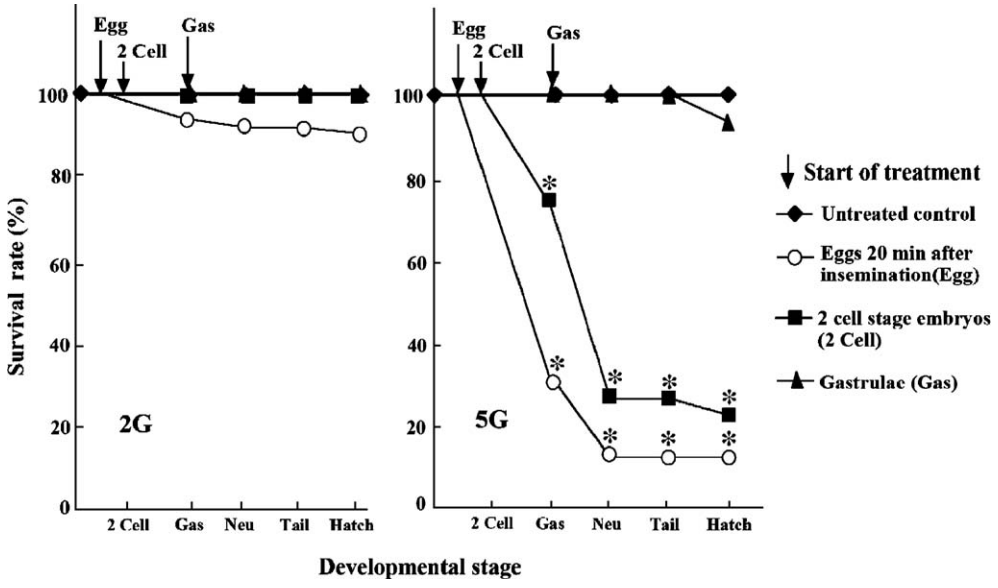

Fig. 1. Effect of hypergravity on egg and embryo survival. Survival rate: surviving embryos were those which neither died nor were removed from the experiment because of abnormal development. 2 Cells = 2 cell stage; Gas = Gastrula stage; Neu = Neurula stage; Tail = Tail-bud stage; Hatch = Hatching stage. *Significantly less ($P < 0.01$) than corresponding values for untreated control embryos.