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

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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, 2004). 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, 2004).

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 has been shown to induce apoptosis in 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 by

gravity 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). At present, however, 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 & Procedures/Best Practices for Laboratory Animal Care by the Stanford University School of Medicine (http://med.stanford.edu/compmed/animal_care/amphibians.html).

Xenopus 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) 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 forty 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℃ fluorescent lightening 12 hour day and night cycle. Eggs and embryos were examined daily and removed from the experiment when signs of abnormal development appeared. Treatment was terminated when controls, raised in normal gravity under the same light and temperature conditions as the high gravity groups, developed into hatching embryos.

2.3. In situ hybridization

Whole mount *in situ* hybridization was performed as depicted by Harland (1991) with the following modifications: (1) Bleached embryos were used for *in situ* hybridization instead of albino embryos, and NBT/BCIP was used instead of BM purple for staining; (2) Digoxigenin-labeled antisense RNA probes were generated from plasmids containing *Xag1* (Sive et al., 1989) or *Otx2* (Blitz and Cho, 1995). Quantitative analysis of *Xag1* expression was performed using NIH Image J (http://rsb.info.nih.gov/nih-image/Default.html). (3) Images were obtained using MZFLIII (Leica) and digital camera (Olympus DP70).

2.4. TUNEL analysis of brains and eyes

Apoptotic brain and eye cells were detected by the TdT-mediated dUTP digoxigenin nick end labeling (TUNEL) technique (Hensey and Gautier, 1998), and were quantified by microscopical examination of serial cross sections (10 μm) of paraffin-embedded TUNEL-stained embryos. Total fore- and midbrain area, as well as total retina and optic stalk area, examined in the serial cross sections was computed using NIH Image J. The number of TUNEL-positive cells (dead or dying apoptotic

cells) was counted for each cross section and the average number of TUNEL-positive cells/μm^2 was plotted for hypergravity groups and untreated controls.

2.5. Statistics

Survival rate was analysed using the Chi-square test. Comparisons between groups were performed by Student's *t* test. All results are expressed as mean +/- S.E.M. *P* values below 0.05 are considered significant.

3. Results and discussion

3.1. Effect on survival

We first investigated the effects of centrifically produced hypergravity environments on development. Fig. 1 shows survival rates of eggs and embryos exposed to 2G and 5G compared to normal gravity controls. Under 2G conditions there were no marked differences between hatching embryos developed from treated eggs (n = 480), 2 cell stage embryos (n = 480) and gastrulae (n = 480) compared with controls (n = 480) with respect to development and growth. Under 5G conditions, on the other hand, effects on development became severe, with a great number of embryos displaying such abnormalities as microcephaly, microphthalmia, smaller cement glands, and reduced size. Normal development decreased significantly in hatching embryos developed from 5G-treated eggs (n = 480) and 2 cell stage embryos (n = 480) ($P < 0.01$), while no significant differences were observed between hatching embryos developed from 5G-treated gastrulae (n = 480) and controls (n = 480).

Neff et al. (1990) reported that centrifugation at 15G and 30G for 4 min during fertilization (time 0.4; 40% of the first cleavage interval) results in the production of *X. laevis* embryos with abnormal cement glands and eyes, and tadpoles with two heads. Tadpoles with two heads were also found to develop from *X. laevis* eggs centrifuged at 10-30G before the first cleavage (time 0.40 or 0.60) (Black , 1990; Black and Gerhart, 1986). The results of these other investigations are in agreement with the results obtained in the present study and in our previous study of *Rana rugosa* (Kashiwagi et

al., 2003) where exposure of fertilized eggs and embryos to 2G and 5G resulted in two heads, microcephally and microphthalmia. Cross section analysis of these abnormal heads showed a high occurrence of diminutive brains and retinas (data not shown).

Different batches of *Xenopus* eggs exposed to hypergravity produced embryos displaying great variation in survival and two headedness (Neff et al., 1990). Similar results were seen in our experiments. In our previous investigation (2003) we found that under 2G conditions many *Xenopus* eggs showed arrested development and became edematous. In the present study, however, most 2G treated eggs developed normally. According to Neff et al. (1990), eggs with higher cytoplasmic immobility (CIM) values showed higher survival rates and lower frequency of two headedness after centrifugation, suggesting that cytoplasmic rigidity may help to mitigate the detrimental effect of hypergravity. Several other investigators speculated on the cause of developmental abnormalities in amphibian embryos kept in hypergravity environments. *Xenopus* embryos exposed to hypergravity from the time of fertilization to 8 cell stage were found to have the first horizontal cleavage furrow situated closer to animal pole, which corresponded to strikingly reduced survival (Yokota et al., 1992). The authors speculated that this change in the position of cleavage furrow is probably due to the rearrangement of yolk platelets. In another investigation Aimar et al. (2000) reported that microvillus elevation was increased in *Pleurodeles waltl* fertilized eggs exposed to hypergravity, with no abnormalities being produced in later developmental stages. Black (1990) found that hypergravity disrupted the normal process of *Xenopus* embryonic axis formation, which is established before the first cleavage, leading to an increased rate of two headedness twinning.

3.2. Effect of Xotx2 *and* Xag1 *expression*

The molecular mechanism by which brain and eye size is reduced by hypergravity remains poorly understood. Complete elimination of *Otx2* function in knockout mice (*Otx2*-/- mutants) results in the absence of forebrain and midbrain regions (Acampora et al., 1995), therefore demonstrating that *Otx2* is essential for normal brain development. *Otx2* heterozygous mice (*Otx2*+/- mutants) show severe eye defects, including loss of lens, cornea and iris, and display anophthalmia or microphthalmia (Matsuo et al., 1995), which demonstrates the relevance of *Otx2* to normal eye development. *Xotx2* expression

in frogs is initially located in cells of Spemenn's organizer at the beginning of gastrulation, and, from the subsequent neurula stage on, is seen in forebrain and midbrain regions as well as developing eye anlage (Kablar et al., 1996; Blitz and Cho, 1995; Pannese et al., 1995). In the present study, *Xotx2* expression was examined in all animals when untreated controls developed to stage 26 tail bud embryos, and it was found that *Xotx2* was expressed in a broad anterior region including the forebrain and midbrain region and eyes in controls and 2 G-treated eggs, 2 cell stage embryos and gastrulae. At 5G *Xotx2* expression was markedly reduced in eggs and 2 cell stage embryos, but showed normal expression in embryos developed from gastrulae (Fig. 2). Expression of the cement gland-specific gene *Xag1* remains largely unchanged at 2G, but was very sensitive to 5G, decreasing by 90% in embryos developed from treated eggs and 80% in embryos developed from 2 cell stage, while showing no reduction in embryos developed from gastrulae (Fig. 3). Investigations of *X. laevis* have shown that *Xotx2*-dependent pathways are involved in the activation of *Xag1* expression (Blitz and Cho, 1995; Pannese et al., 1995). In the present study we also found that the expression of both *Xotx2* and *Xag1* is sensitive to high gravity-related stimuli, suggesting *Xag1* activation in response to *Xotx2*.

3.3. Apoptosis in brain and eye

A study of chimeric mouse embryos containing both normal embryonic stem (ES) cells and ES cells lacking *Otx2* show an increased number of apoptotic cells in the forebrain, suggesting that *Otx2* plays some role in protecting the forebrain against apoptosis (Rhinn et al., 1999). According to Di et al. (2005), all-*trans* retinoic acid (ATRA) represses *Otx2* expression, causing apoptosis, which results in inhibition of *Otx2*-expressing medulloblastoma cell growth. In order to clarify the relationship between apoptosis and *Xotx2* downregulation caused by hypergravity, we examined TUNEL reactivity in the brain and eyes of 2G and 5G-treated hatching embryos and untreated controls at stage 35, at which time apoptosis has been shown to play a role in the morphogenesis of the developing *Xenopus laevis* brain (Hensey and Gautier, 1998). The number of TUNEL- positive cells in the fore- and midbrain region of embryos developed from 2G-treated eggs, 2 cell stage embryos, and gastrulae was not significantly different from that found in controls (Fig. 4). Under 5G treatment

however, the number of TUNEL- positive cells increased 4.5-fold in embryos developed from eggs 20 min after insemination and 3-fold in embryos developed from 2 cell stage embryos, compared to control counterparts. No differences were observed between embryos developed from gastrulae and controls. The same results were seen for the eyes. The number of TUNEL-positive cells in the retina and optic stalk of embryos developed from 2G- treated eggs, 2 cell stage embryos and gastrulae was not significantly different from that of the controls (Fig. 5). In contrast, the eyes of embryos developed from 5G-treated eggs and 2 cell stage embryos showed a 6-fold and 3-fold increase in the number of TUNEL-positive cells, while embryos developed from 5G-treated gastrulae showed no increase in the number of apoptosis cells (Fig. 5). Histological sections show that embryos developed from 5G-treated eggs and 2 cell stage embryos usually present fore- and midbrain abnormalities as well as eye malformations, including the absence or loss of the lens and a strong reduction of the retina (Fig. 5). Therefore, it seems likely that such abnormalities seen in the brain and eyes are due to excessive apoptosis.

4. Concluding remarks

Results obtained in the present study of *Xenopus laevis* development under artificial hypergravity suggest that early (20 min after insemination through 2 cell stage) treatment in a high gravity (5G) environment induces abnormal *Xotx2* expression, causing abnormal *Xag1* expression, which results in an over-induction of apoptosis, leading to developmental arrest.

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Legends to figures

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 Cell = 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.

Fig. 2. Effect of 5G on *Xotx2* expression at stage 26. Dorsoanterior (A), dorsal (B) and lateral (C) views of normal expression in untreated embryos. Dorsoanterior (D), dorsal (E) and lateral (F) views of embryos developed from treated eggs 20 min after insemination. Dorsoanterior (G), dorsal (H) and lateral (I) views of embryos developed from treated 2 cell stage embryos. Dorsoanterior (J), dorsal (K) and lateral (L) views of embryos developed from treated gastrulae. Each experiment was carried out using 6 individual embryos. Scale bars = 200 μm.

Fig. 3. Effect of 5G on *Xag1* expression at stage 26. *Xag1* expression in untreated embryos (A), and embryos developed from treated eggs (B), 2 cell stage embryos (C) and gastrulae (D). Each experiment was carried out using 6 individual embryos. Scale

bars = 200 μm. (E) Changes in *Xag1* expressed area. Embryos developed from treated eggs (■), 2 cell stage embryos (▨) and gastrulae (▨). Values represent mean values (+/- S.E.M.) for 6 embryos. *Significantly less ($P < 0.01$) than the corresponding untreated control value.

Fig. 4. TUNEL-stained cross sections of control and 5G stage 33 ~34 embryo fore- (A - E) and midbrains (F - J). Arrowheads indicate TUNEL-positive cells. A, F = untreated controls; B, G = embryos developed from eggs beginning treatment 20 min after insemination; C, H = embryos developed from 2 cell stage embryos; D, I = embryos developed from gastrulae, and E, J = negative controls showing no TUNEL-labelling. Each experiment was performed on five individual embryos. Scale bars =100 μm. (K) Number of TUNEL- posotive cells. Embryos developed from treated eggs (■), 2 cell stage embryos (▨) and gastrulae (▨). Values represent mean values (+/- S.E.M.) for 5 embryos. *Significantly less ($P < 0.05$) than the corresponding untreated control value.

Fig. 5. TUNEL-stained cross sections of control and 5G stage 33 ~34 embryo retinas (A - E) and optic stalks (F - J). Arrowheads indicate TUNEL-positive cells. A, F = untreated controls; B, G = embryos developed from eggs beginning treatment 20 min after insemination; C, H = embryos developed from 2 cell stage embryos; D, I = embryos developed from gastrulae, and E, J = negative controls showing no TUNEL labelling. Each evaluation was carried out on 5 individual embryos. R, Retina. L, Lens. OP, Optic stalk. Scale bars = 100 μm. (K) Number of TUNEL- positive cells. Embryos developed from treated eggs (■), 2 cell stage embryos (▨) and gastrulae (▨). Values represent mean values (+/- S.E.M.) for 5 embryos. *Significantly less ($P < 0.05$) than the corresponding untreated control value.

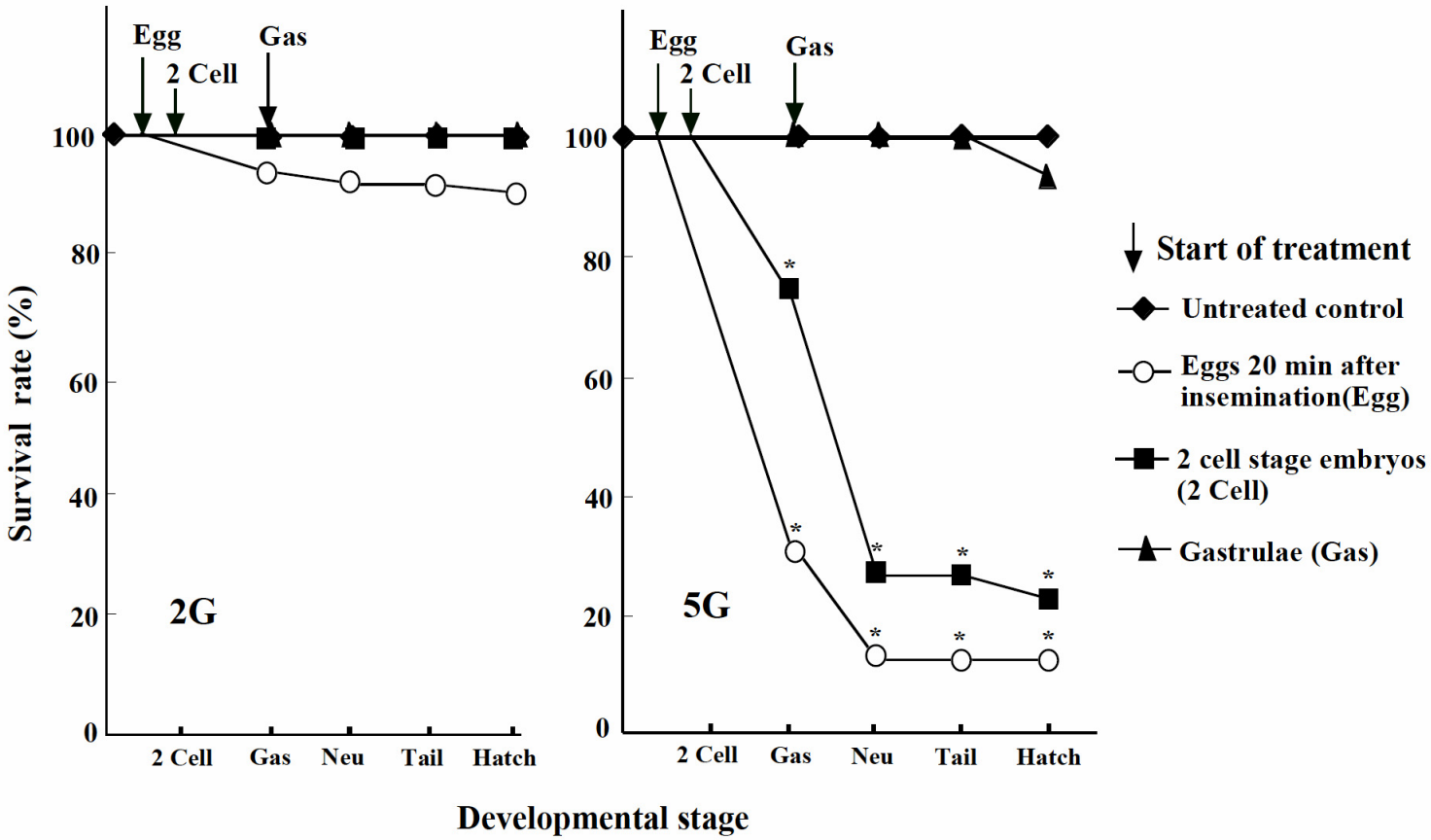
Egg
2 Cell
Gas
100
80
60
40
20
0
Survival rate (%)
2G
5G
2 Cell
Gas
Neu
Tail
Hatch
Developmental stage
Start of treatment
Untreated control
Eggs 20 min after insemination(Egg)
2 cell stage embryos (2 Cell)
Gastrulae (Gas)

Fig. 1

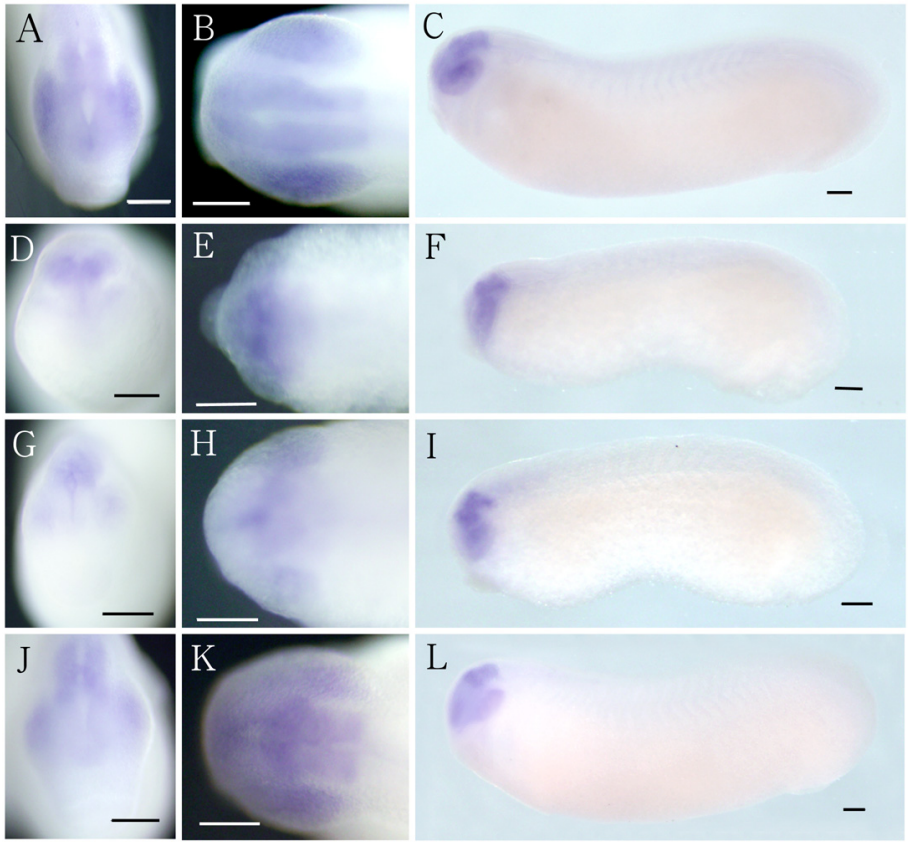

Fig. 2

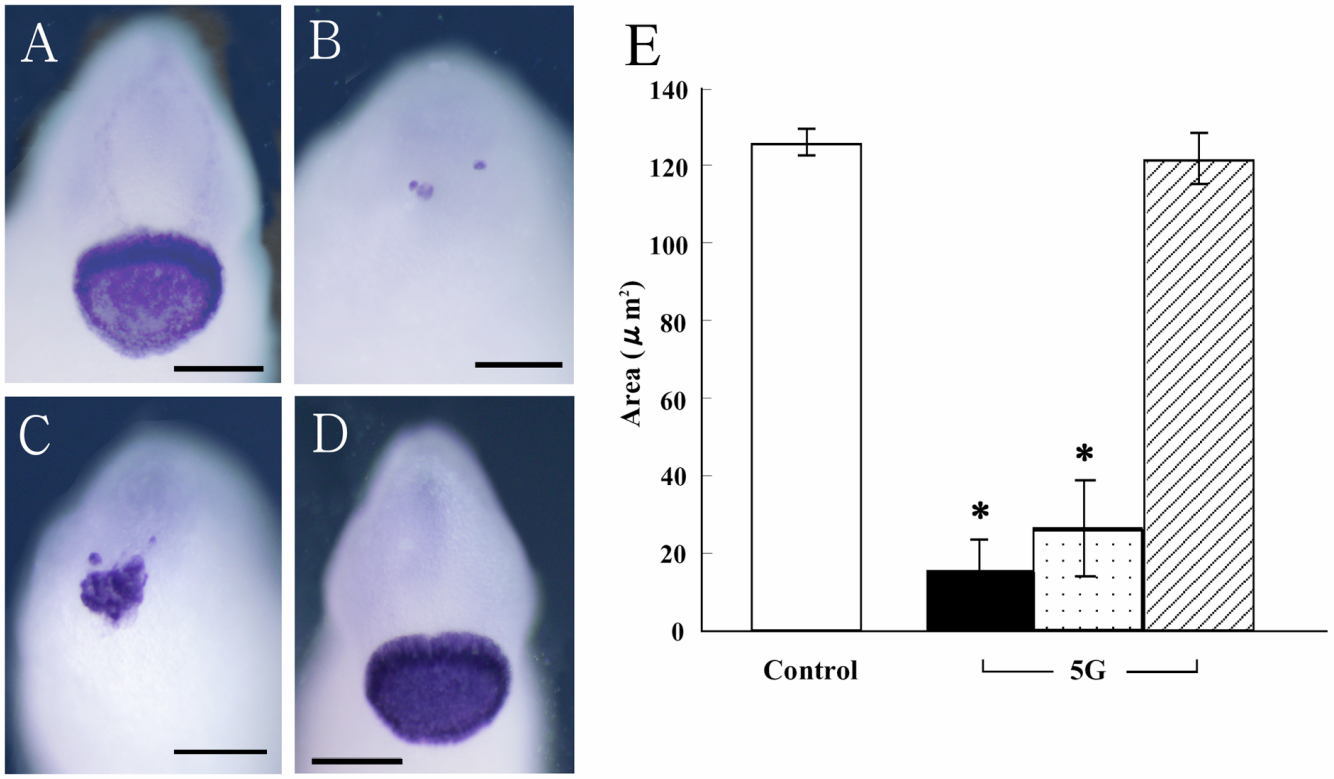

Fig. 3

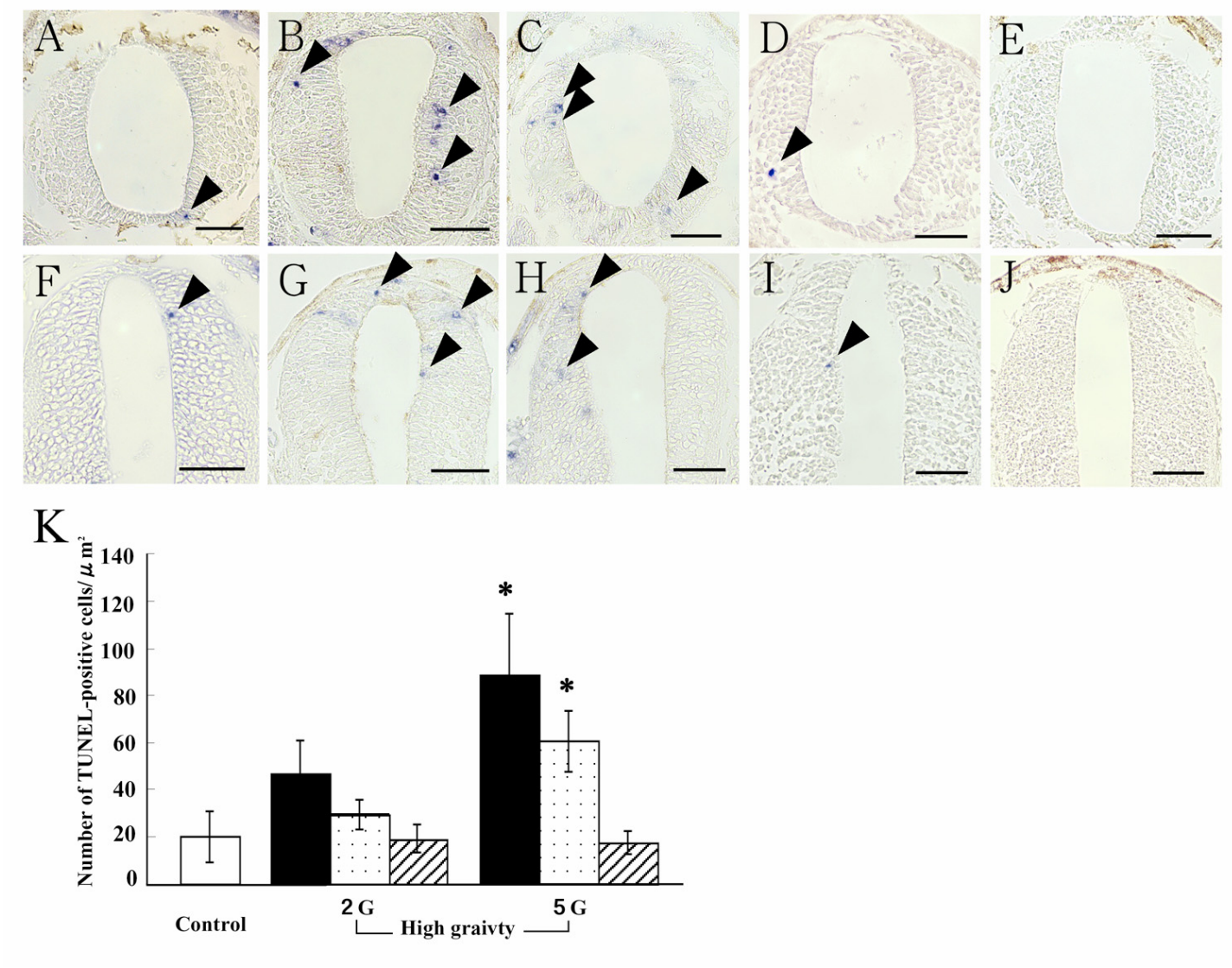
A
B
C
D
E
F
G
H
I
J
K
Number of TUNEL-positive cells/ μ m²
0
20
40
60
80
100
120
140
*
*
Control
2 G
5 G
High graivty

Fig. 4

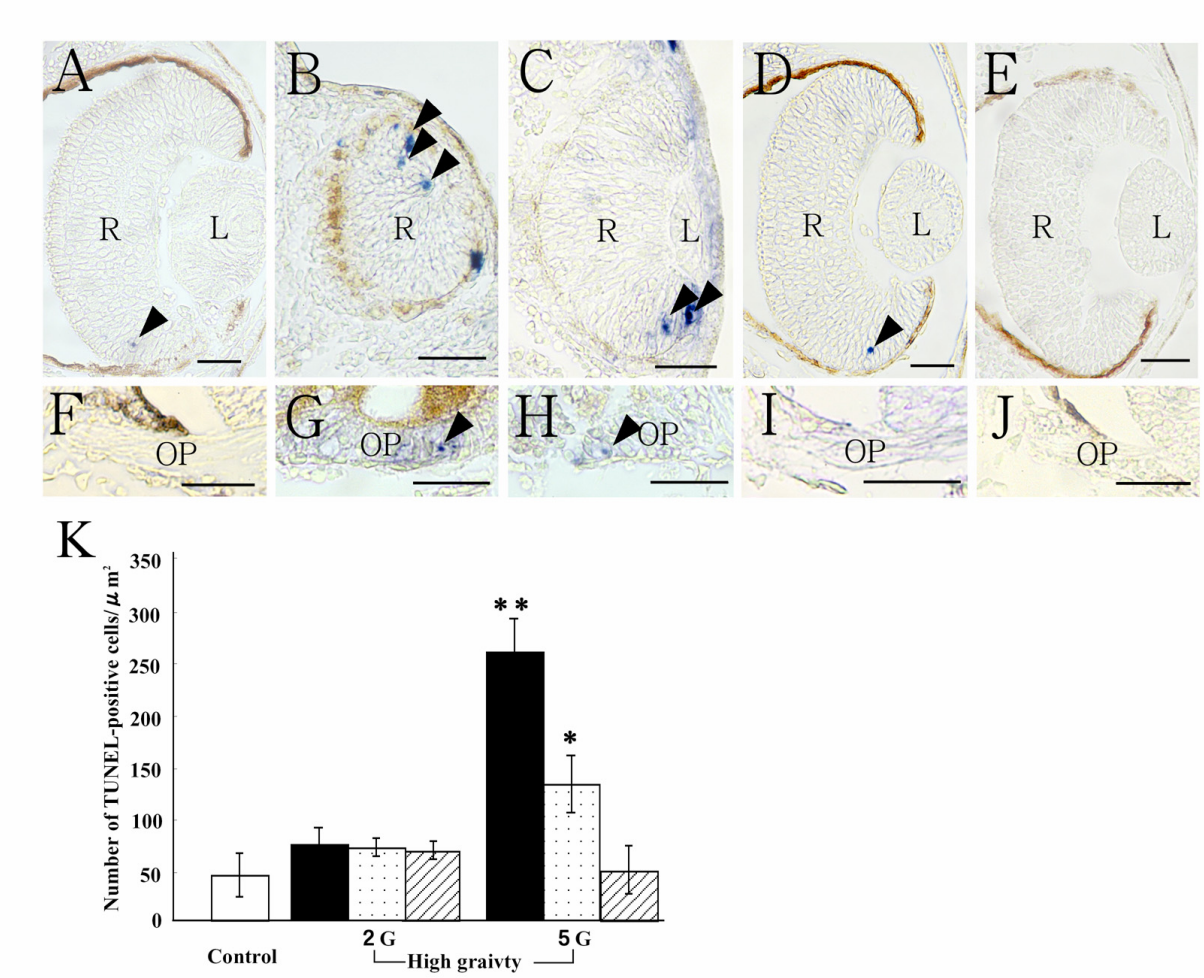
A
B
C
D
E
R
L
F
G
H
I
J
OP
K
Number of TUNEL-positive cells/μm²
350
300
250
200
150
100
50
0
**
*
Control
2 G
5 G
High graivty

Fig. 5