Novel harmful effects of [60]fullerene on mouse embryos in vitro and in vivo

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Abstract [60]Fullerene (C60) was solubilized with poly(vinylpyrrolidone) (PVP) in water, and the aqueous solution was applied to a mouse midbrain cell differentiation system. On incubation of C60 with various concentrations of PVP, cell differentiation and proliferation were potently inhibited, although weaker than the vehicle controls. C60 was clearly distributed into the yolk sac and embryos by intraperitoneal administration to pregnant mice at 50 mg/kg and had a harmful effect on both conceptuses by microscopical evaluation. This in vivo and in vitro action on embryogenesis is a novel and seriously harmful activity of C60.

Key words: Fullerene; Midbrain; Mouse; Developmental toxicity; Yolk sac; Cell differentiation

1. Introduction

The fullerenes are condensed aromatic compounds with extended η-systems and have unique cage structures. Since their discovery [1], unique interactions with biomolecules have been expected and also anticipated from the toxological point of view, because fullerenes have recently been synthesized on a large scale and many researchers have been exposed to them, mainly in the electrochemical field. There are a few interesting studies concerning the biological activities of water-soluble [60]fullerene (C60) derivatives such as DNA-cleaving activity [2] and HIV-1 inhibition activity [3,4]. As for unfunctional fullerenes, owing to their low solubility in water, it is hard to examine the biological activities of fullerenes alone especially in in vitro systems. Recently, Yamakoshi et al. [5] succeeded in solubilizing C60 in water with poly(vinylpyrrolidone) (PVP), and applied it to a hemolysis test [5]. Further, we clarified the promoting action of unfunctionalized C60 on the chondrogenesis of the rat limb bud (LB) cells [6]. C60 was recently found to produce active oxygen species in the mouse LB cells by us [7], and active oxygen species are known to have toxic effects on the embryonic development of neurons, especially during the early to middle stages of embryogenesis. Furthermore, it is important to clarify whether C60 can be distributed and whether or not it affects the embryos after the administration to pregnant mice.

In this paper, we examine the novel harmful effects of C60 on embryos under in vitro and in vivo experimental conditions. These effects represent the first reported dangerous biological activity of C60 itself.

2. Materials and methods

C60 (Terms Co., purity >99.9%) with PVP was solubilized into media according to the method of Yamakoshi et al. [5]. Catalase and superoxide dismutase (SOD) were obtained from Sigma Chemical Co., USA. In vivo exposure experiments were carried out as follows: two pregnant SLC-mice (day 10, plug = day 0) were intraperitoneally administered with C60 (25–137 mg/kg), PVP (3.3–18 g/kg) or distilled water (25 ml/kg: control) in each group. Embryos (day 11) were examined at 18 h after administration.

Midbrain (MB) micromass culture was carried out according to a procedure reported previously [8].

MB tissues were removed from the embryos of pregnant SLC-ICR mice on day 11 of gestation. MB tissues were dissociated into individual cells by successive washing in calcium- and magnesium-free balanced salt solution (CMF) and by trypsin (0.25% in CMF) digestion (15 min, 37°C). Cell suspensions were prepared in culture medium consisting of Ham's F12 plus 10% fetal calf serum (F12-10% FCS) and were adjusted to give 5×10^6 MB cells/ml. A 20 μl aliquot of each cell suspension was delivered to a well of 24-well tissue culture plates. The next day, C60 or PVP solution in the medium was incorporated into the MB culture plates, then further cultured for another 6 days. MB cells grew and differentiated in vitro. The extent of neural cell differentiation was assessed by counting the differentiated foci under a dissecting microscope [8].

Cell proliferation was determined by the neutral red staining method. Cell cultures were fixed in 1% glutaraldehyde for 20 min, washed and stained with 0.1% neutral red solution for 90 min at room temperature. For the spectrophotometric measurement of cell proliferation, the bound dye neutral red was extracted with 0.5% acetic acid in 50% ethanol, and the optical density at 550 nm was determined. The procedures have been described in detail by Tsuchiya et al [8].

For experiments in which the effects of the active oxygen species, were investigated, catalase and SOD were added 24 and 96 h after the start of culture, each at a final concentration of 50 U/ml.

3. Results and discussion

Fig. 1 shows the differentiated foci stained with hematoxylin. In proportion to C60 concentrations, the number of differentiated foci decreased, although the extent of neuronal differentiation in C60-treated MB cells was higher than in each vehicle control (Fig. 1).

Fig. 2 shows the results of the quantitative determination of cell differentiation and cell proliferation. C60 with PVP and PVP alone inhibited cell differentiation and proliferation at a similar concentration. Thus, the IC_{50} values of C60 in cell differentiation and proliferation were 0.43 and 0.47 mg/ml, respectively (Fig. 2A), and those of PVP alone were 33 and 49 mg/ml, respectively (Fig. 2B). Differentiation was inhibited along with an increase in the cytotoxicities caused by these chemicals.

We investigate the effects of the addition of antioxidant enzymes such as catalase and SOD on midbrain cells cultured with C60 and PVP or with PVP alone (Fig. 3). As for C60 with PVP and PVP alone, there was almost no difference in cell differentiation between the presence and absence of these

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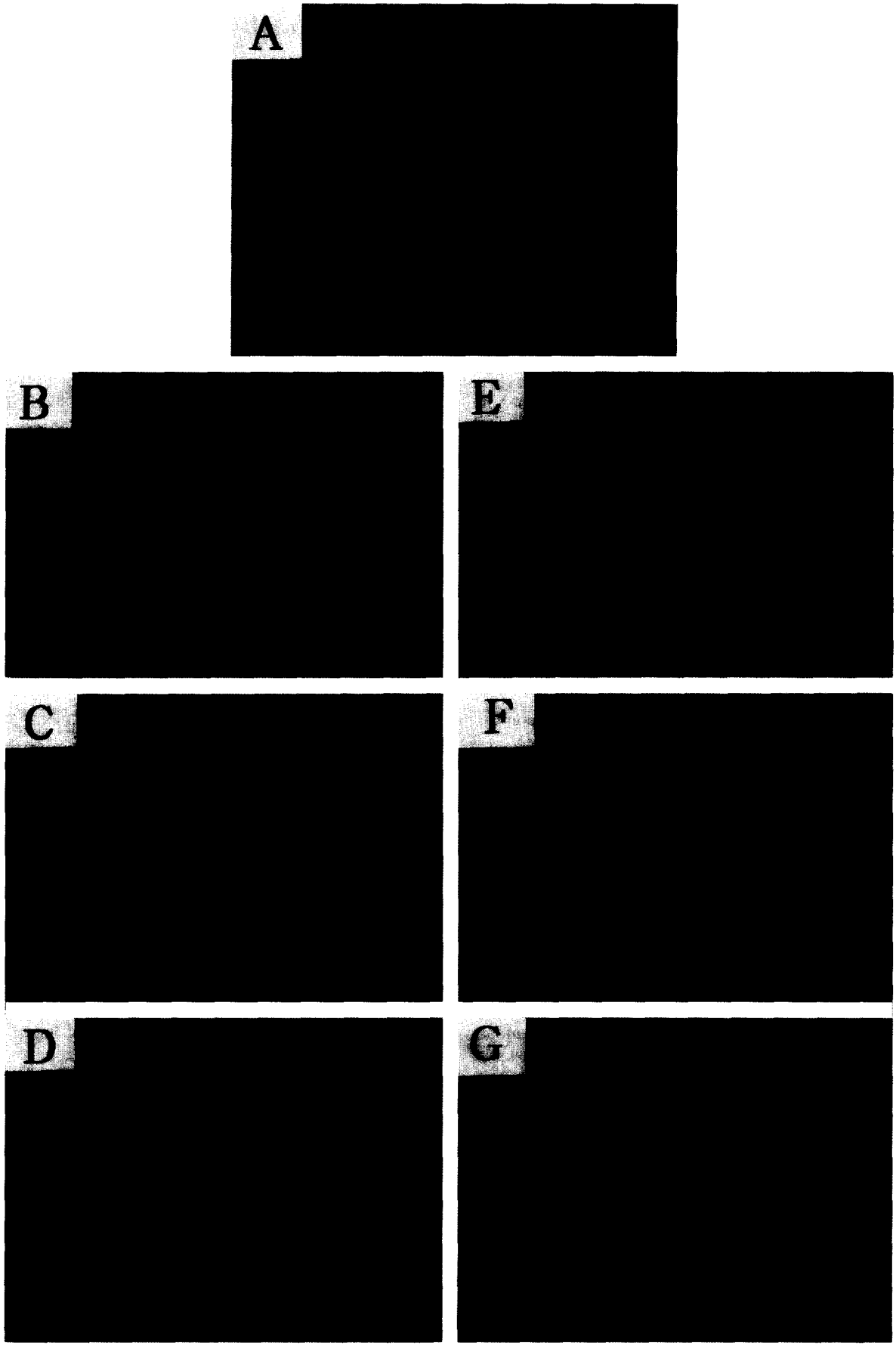
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Fig. 1. Appearance of hematoxylin staining cultures of mouse midbrain cells after incubation with or without C60 solubilized with PVP. (A) Control; (B) C60, 600 μg/ml+PVP, 80 mg/ml; (C) C60, 300 μg/ml+PVP, 40 mg/ml; (D) C60, 150 μg/ml+PVP, 20 mg/ml; (E) PVP, 80 mg/ml; (F) PVP 40 mg/ml; (G) PVP, 20 mg/ml (magnification ×7.5).
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antioxidant enzymes (data not shown). However, cell proliferation inhibited by C60 was partly restored by the addition of these enzymes (Fig. 3A), although there was no significant difference between the presence and absence of the antioxidant enzymes. In contrast, in the case of PVP alone, there was almost no change in the cell proliferation profile (Fig. 3B) between both conditions. Therefore, C60 was assumed to decrease cell proliferation via the active oxygen species.

Next, an in vivo exposure experiment was carried out to determine whether C60 also exerts a harmful effect on the embryos in vivo. Embryos were examined 18 h after intraperitoneal administration to pregnant mice at a C60 dose of 137 mg/kg (Fig. 4C). Control (distilled water 25 ml/kg)- and PVP (18 g/kg)-treated embryos appeared to be non-toxic under our experimental conditions (Fig. 4A,B); in contrast, C60-treated embryos all died and also showed severe abnormalities such as abnormal flexion (Fig. 4C). For determining the lowest effective dose, lower dosages were administered to two pregnant mice per experimental group. At 50 mg/kg, C60 was clearly distributed into the embryos based on the characteristic color development of C60, and caused abnormalities especially around the head region and tail (Fig. 5C). At 25 mg/kg, one embryo showed abnormal enlargement of the head (Fig. 5B). Each PVP vehicle control (Fig. 5D,E) resembled the normal control embryo (Fig. 5A). We also confirmed that cell functions such as proliferation and differentiation of MB cells were clearly damaged by in vivo exposure to a fullerene derivative (data not shown).

Fig. 6 shows the dorsal region of embryos at higher magnification. The appearance of the neural tube at the dorsal region of C60-treated embryos (Fig. 6B) was different from that of the controls (Fig. 6A) and appeared to have imperfect fusion and an irregular shape. 50% of the embryos were abnormal in shape following dosing of C60 at 50 mg/kg to two pregnant mice. In the case of a 25 mg/kg dose, one pregnant mouse had all normal embryos and the other had only one abnormal embryo (Fig. 5B). Moreover, there was no toxic effect in the cell function of differentiation and proliferation of the embryonic midbrain cells prepared from the pregnant rats pre-exposed at a dose of C60 of 16.7 mg/kg (data not shown). From these results, the lowest effective dose of C60 was considered to be around 25 mg/kg. We further observed the yolk sac removed from C60-treated pregnant mice (Fig. 7). At 50 mg/kg, there was a seriously harmful effect on the yolk sac such as a shrunken membrane and narrow blood vessels (Fig. 7D). Blood flow is important in the normal morphogenesis of embryos. Clearly, these destructive actions on the yolk sac affect the development of the whole embryo.

Scrivens et al. [9] reported that C60 became rapidly cell-associated though it did not affect the proliferation of human keratinocytes or human fibroblasts, indicating that the rapid accumulation of C60 in human cells does not result in acute toxicity [9].

Our in vivo exposure test indicated that C60 was incorporated into conceptus via the maternal blood flow or organ and severely disrupted the function of the yolk sac and embryonic morphogenesis. As for the in vitro results, midbrain cell differentiation and proliferation were dose-dependently inhibited by C60, and these harmful effects were partly associated with the toxicities of the active oxygen species produced by C60. Sinet et al. [10] reported that hydrogen peroxide can induce tissue damage, and such damage should be considered as a component in pathological processes such as the rapid brain ageing seen in Down's syndrome or Alzheimer's disease or in

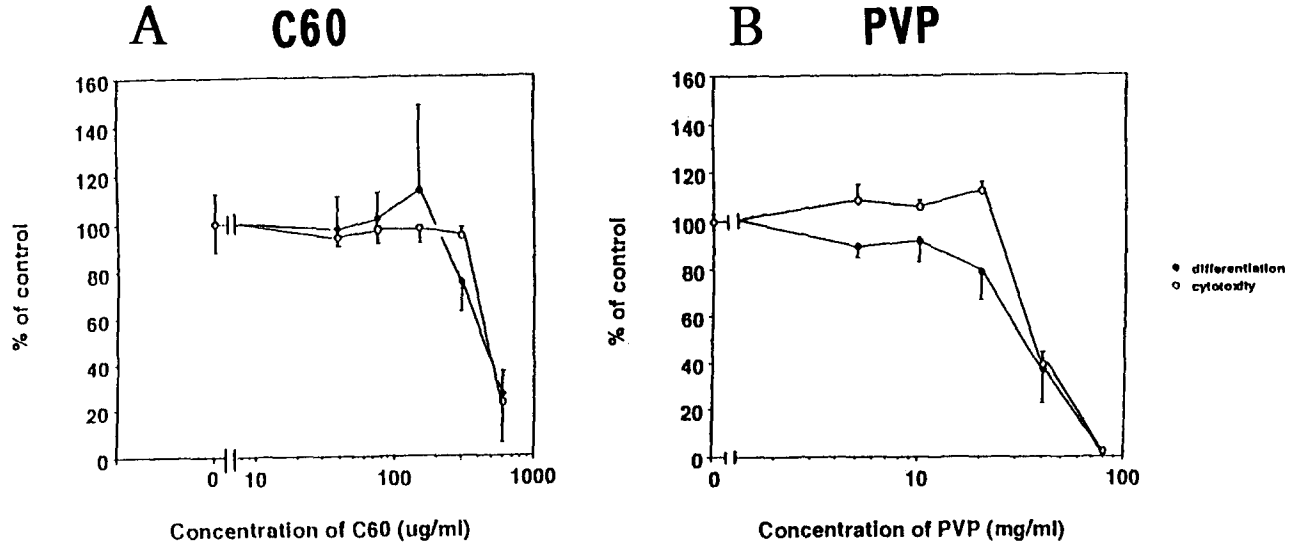

Fig. 2. Effect of C60 solubilized with PVP (A) or PVP (B) on cell differentiation (●) and proliferation (○) in the mouse midbrain micromass culture system. For the measurement of cell differentiation and proliferation, the degrees of staining were measured following the procedure described by Tsuchiya et al. [7]. Individual control neuronal cell foci counted by dissecting microscopy (differentiation) and the control optical density measured by neutral red staining method (cell proliferation) were regarded as 100%, respectively. Each value is the mean ± S.D.

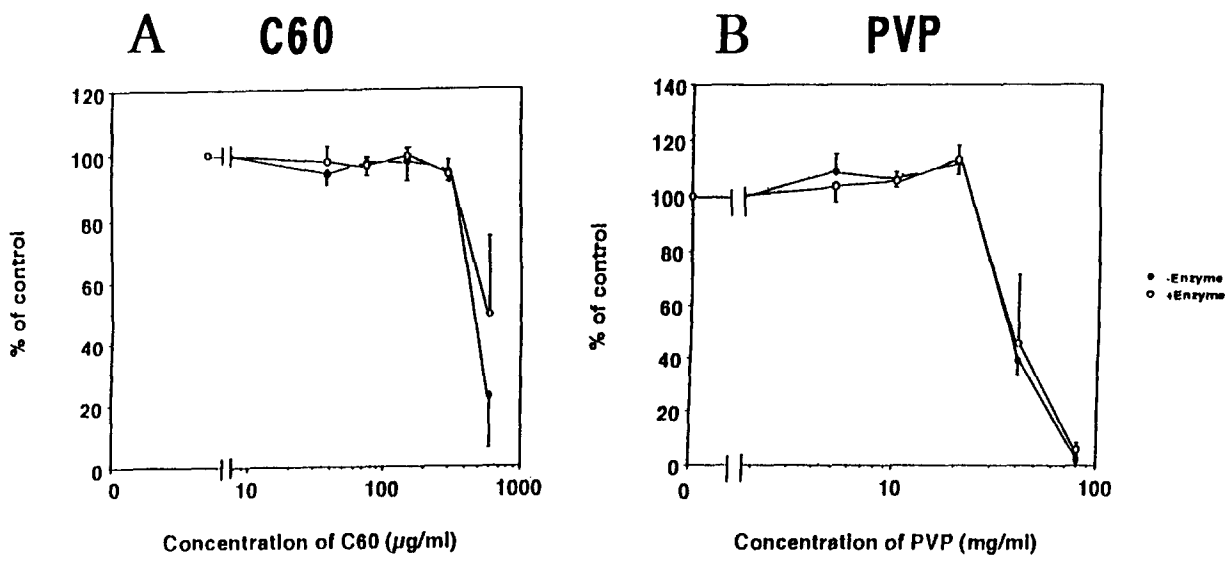

Fig. 3. Effect of C60 solubilized with PVP (A) or PVP (B) on the mouse midbrain cell proliferation in the absence (●) or presence (○) of superoxide dismutase and catalase. The effect of the addition of superoxide dismutase and catalase on cell proliferation was investigated using a neutral red staining method as described in Section 2. Each value is the mean ± S.D.

Fig. 4. Appearance of mouse embryos after in vivo exposure to C60 solubilized with PVP or PVP. C60 (137 mg/kg) in PVP (18 g/kg), PVP (18 g/kg) in distilled water or control vehicle (distilled water, 25 ml/kg) was intraperitoneally administered to the pregnant female mice on day 10 of gestation (day of discovery of copulatory plug = day 0). After 18 h, the embryos were removed from the uteri, and pooled from two test or control animals. (A) Control (distilled water, 25 ml/kg); (B) PVP (18 g/kg) in distilled water (25 ml/kg); (C) C60 solubilized with PVP (C60, 137 mg/kg+PVP, 18 g/kg+distilled water, 25 ml/kg). Arrow shows abnormal flexion (magnification ×7.5).

disorders of more focal neuronal degeneration, such as that seen in Parkinson's disease. Hydrogen peroxide and hydroxy radicals have been implicated in neuronal degeneration induced by monoamine neurotoxins such as 6-hydroxydopamine or 5,7-dihydroxytryptamine. Recently, Gurney et al. [11] reported that the transgenic mice that express wild-type or mutant forms of human SOD were produced and that mice expressing wild-type human SOD show no signs of neuron disease, but the transgenic mice expressing the largest amounts of mutant SOD in the brain caused motor neuron disease. These and our present results indicate that the neurons of embryos are sensitive to active oxygen species at the developmental stage.

The mechanism of the toxicities caused by C60 is suggested to be as follows: C60 is readily reduced via one electron by cells, and the one-electron reduced form of C60 radical anion generates the superoxide anion via transfer of the electron to O_2 and simultaneously returns to C60. The superoxide anions further change into hydrogen peroxide. These active oxygen species including superoxide anion, hydrogen peroxide, etc. cause cell damage such as cell lysis and cell growth inhibition.

Okuda et al. [12] reported that the growth inhibitory action of a water-soluble fullerene derivative on *Escherichia coli* is dependent on O_2. This recent result also supports our suggestion. Therefore, it is recommended that women avoid exposure to C60 especially during pregnancy, although its in vivo tumor-promoting results showed a negative response [13].

We clarified the promoting action of C60 on the chondrogenesis of the LB cells previously [6]. In contrast, the harmful action of C60 on embryonic midbrain cells is revealed by the present study. In vivo exposure experiments also showed abnormalities of embryonic morphogenesis especially in the head regions. From these recent studies, newly designed C60

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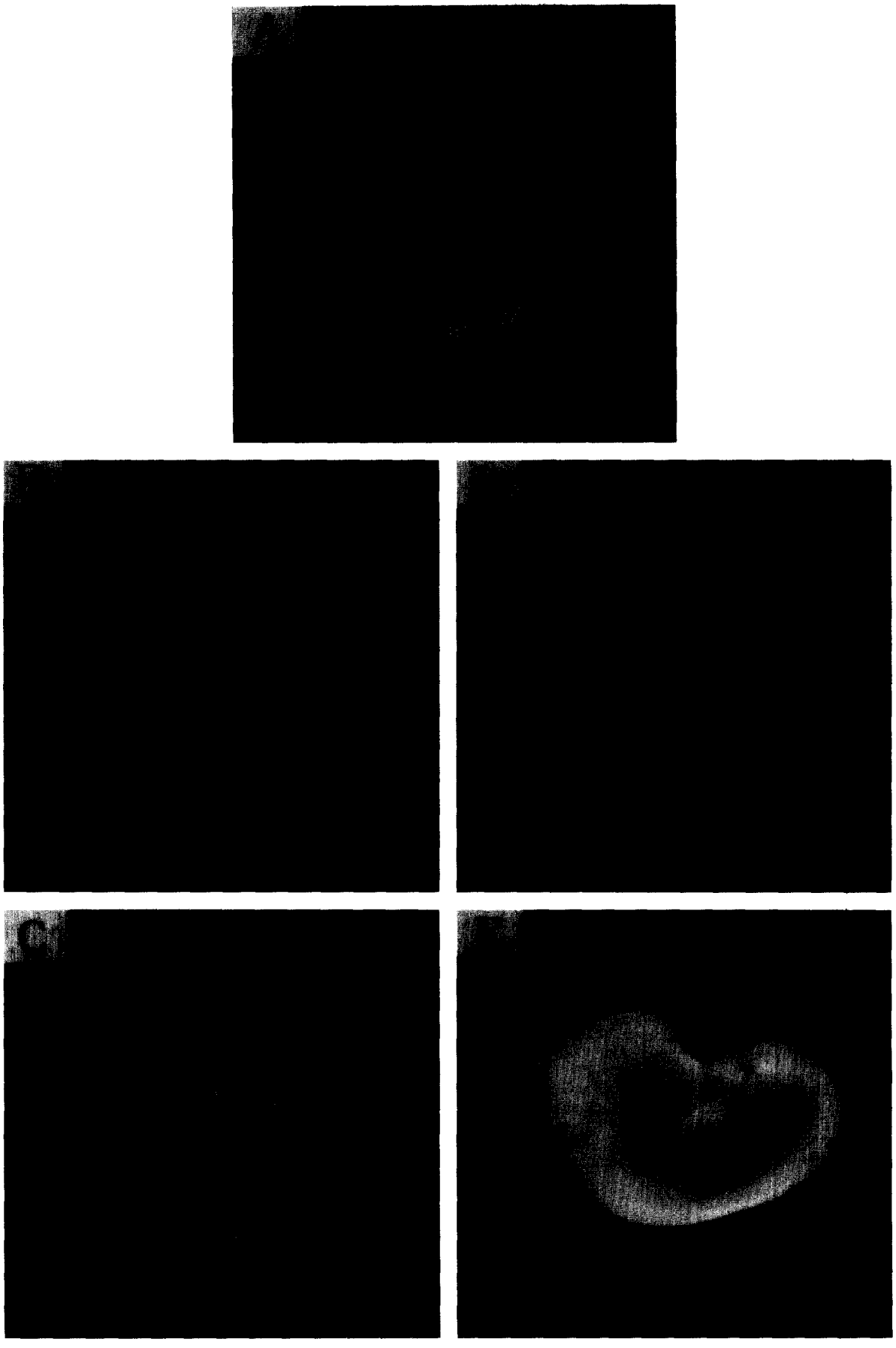
Fig. 5. Appearance of the mouse embryos after in vivo exposure of C60 solubilized PVP or PVP. C60 (25 or 50 mg/kg) in PVP, PVP (3.3 or 6.6 g/kg) in isotonic sodium chloride solution or control vehicle (isotonic sodium chloride solution, 25 ml/kg) was intraperitoneally administered to pregnant female mice on day 10 of gestation. After 18 h, the embryos were removed from the uteri, and pooled from two test or control animals. (A) Control (isotonic sodium chloride solution, 25 ml/kg); (B) C60 solubilized with PVP (C60, 25 mg/kg+PVP, 3.3 g/kg+isotonic sodium chloride solution, 25 ml/kg). Arrow shows enlargement of head; (C) C60 solubilized with PVP (C60, 50 mg/kg+PVP, 6.6 g/kg+isotonic sodium chloride solution, 25 ml/kg). Arrow shows abnormal head; (D) PVP (3.3 g/kg) in isotonic sodium chloride solution (25 ml/kg); (E) PVP (6.6 g/kg) in isotonic sodium chloride solution (25 ml/kg) (magnification ×10).



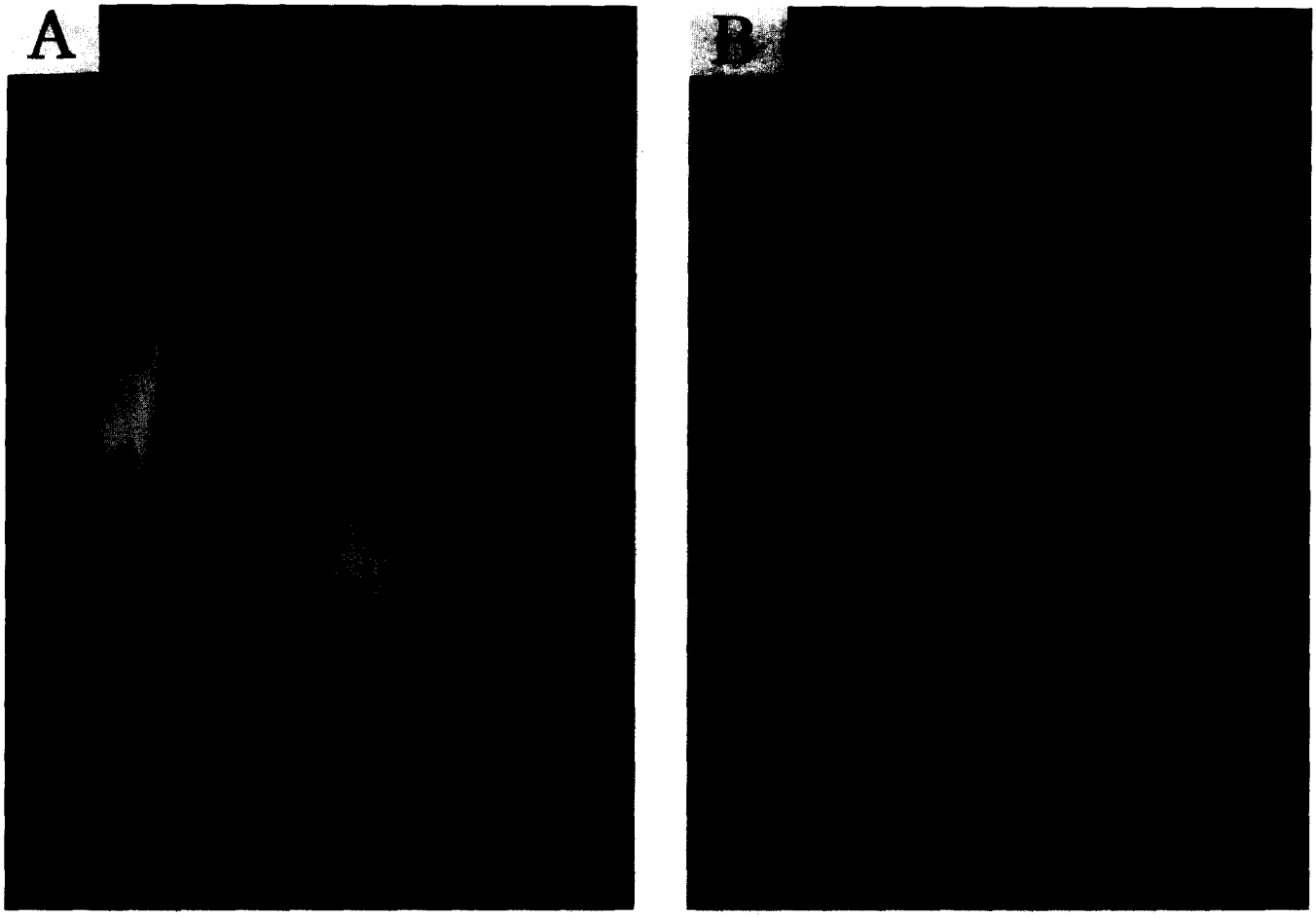


Fig. 6. Appearance of dorsal regions of the embryos after in vivo exposure of C60 solubilized with PVP or control vehicle. C60 in PVP or control vehicle was administered to the pregnant female mice and the embryos werre removed from the uteri following the procedure described in the legend to Fig. 5. (A) Control vehicle (isotonic sodium chloride solution, 25 ml/kg); (B) C60 (50 mg/kg) with PVP (6.6 g/kg) in isotonic sodium chloride solution (25 ml/kg). Arrow shows imperfect fusion and irregular shape of neural tube (magnification ×20).

derivatives which produce lesser amounts of active oxygen may be useful drugs for curing cartilage tissue diseases and also diminish the harmful effects on embryos.

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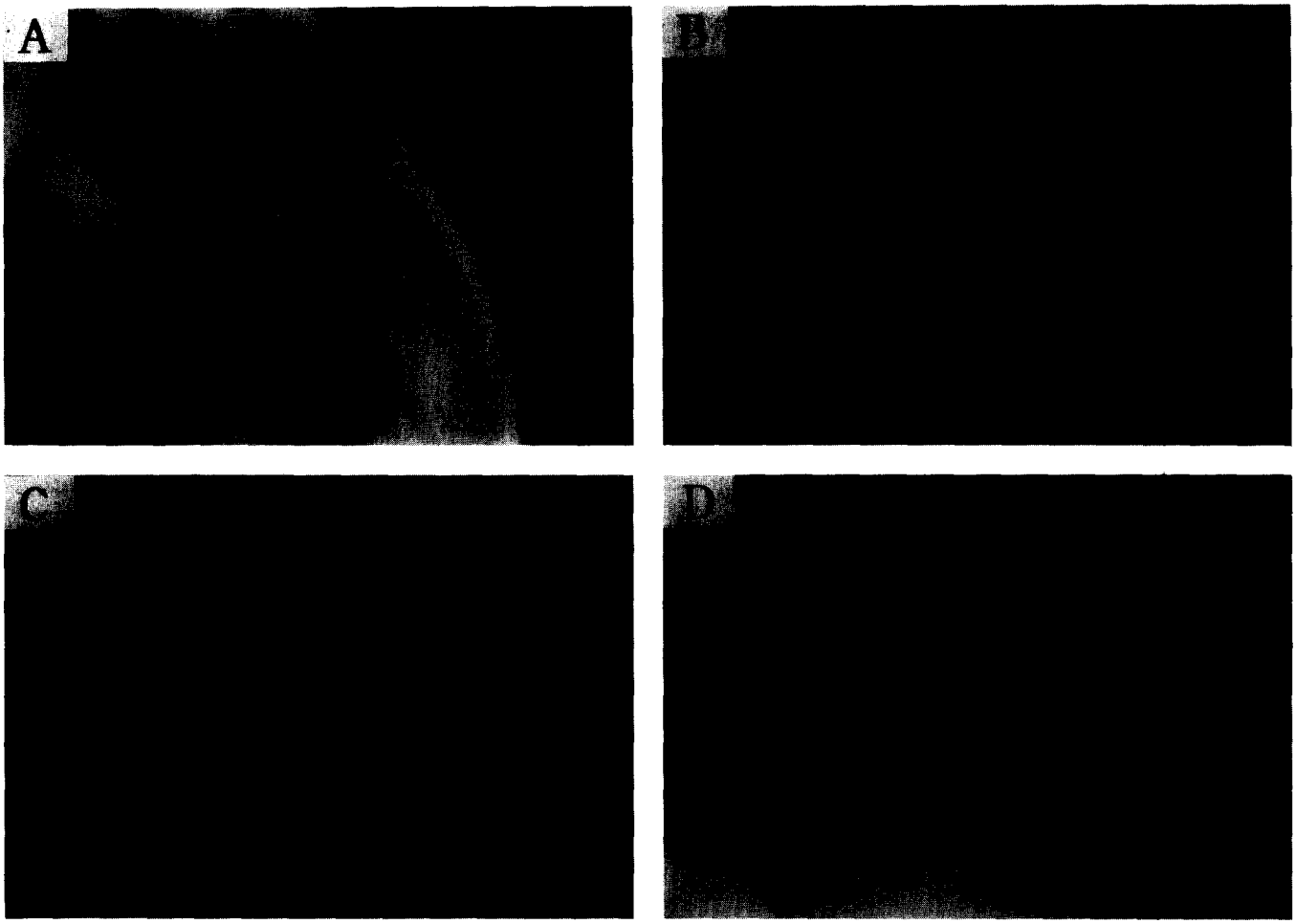

Fig. 7. Appearance of the yolk sacs removed from the uteri after in vivo exposure to C60 solubilized with PVP, PVP or control vehicle. C60 in PVP, PVP or control vehicle was administered to the pregnant female mice (day 10 of gestation) and the yolk sacs were removed from the uteri (day 11 of gestation) following the procedure described in the legend of Fig. 5. (A) Control vehicle; (B) PVP 6.6 g/kg; (C) C60 (25 mg/kg) in PVP (3.3 g/kg); (D) C60 (50 mg/kg) in PVP (6.6 g/kg). Arrow shows shrunken membrane and narrow blood vessels of the yolk sac (magnification ×45).