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Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos

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In human in-vitro fertilization (IVF)–embryo transfer, the in-vitro culture environment differs from in-vivo conditions in that the oxygen concentration is higher, and in such conditions the mouse embryos show a higher concentration of reactive oxygen species (ROS) in simple culture media. ROS are believed to cause damage to cell membranes and DNA fragmentation in somatic cells. This study was conducted to ascertain the level of H_2O_2 concentration within embryos and the morphological features of cell damage induced by H_2O_2. A total of 62 human oocytes and embryos (31 fragmented, 15 non-fragmented embryos, 16 unfertilized oocytes) was obtained from the IVF–embryo transfer programme. The relative intensity of H_2O_2 concentrations within embryos was measured using 2′,7′-dichlorodihydrofluorescein diacetate by Quanti cell 500 fluorescence imaging and DNA fragmentation was observed with transmission electron microscopy and an in-situ apoptosis detection kit. The H_2O_2 concentrations were significantly higher in fragmented embryos (72.21 ± 9.62, mean ± SEM) compared to non-fragmented embryos (31.30 ± 3.50, $P < 0.05$) and unfertilized oocytes (30.75 ± 2.67, $P < 0.05$). Apoptosis was observed only in fragmented embryos, and was absent in non-fragmented embryos. Electron microscopic findings confirmed apoptotic bodies and cytoplasmic condensation in the fragmented blastomeres. We conclude that there is a direct relationship between increased H_2O_2 concentration and apoptosis, and that further studies should be undertaken to confirm these findings.

Key words: apoptosis/embryo fragmentation/reactive oxygen species

Introduction

In human in-vitro fertilization (IVF)–embryo transfer programmes only a few oocytes develop to be good quality embryos, depending on the incubation conditions and the quality of the ovum and spermatozoon, while the rest show abnormal morphology due to unequal cell division or fragmentation of the cell (Veeck, 1988; Goyanes *et al.*, 1990). Fragmented embryos have limited developmental potential and rarely result in implantation (Plachot and Mandelbaum, 1990; Erenus *et al.*, 1991). Such abnormal embryo development has been reported to be due to an inadequate culture environment. The in-vitro culture environment differs from in-vivo conditions in that the oxygen concentration is higher and, in such conditions, mouse embryos show a higher reactive oxygen species (ROS) concentration in simple culture media (Nasr-Esfahini *et al.*, 1990a; Goto *et al.*, 1993). ROS such as superoxide anion radicals, hydroxyl radicals, hydrogen peroxide, organic peroxide radicals and singlet molecular oxygen are thought to cause damage to the cell membrane (Aitken *et al.*, 1989; Halliwell and Chirico, 1993) and to cause DNA fragmentation (Halliwell and Aruoma, 1991) in somatic cells, and may participate in the process of apoptosis (Hockenbery *et al.*, 1993). Recently, apoptotic configurations in fragmented human embryos were observed at a stage prior to blastocyst formation and these have been suggested as the process of programmed cell death (Jurisicova *et al.*, 1996). This study was conducted to ascertain the relationship between the H_2O_2 levels and the morphological features of cell damage within embryos in in-vitro culture under a 5% CO_2 atmosphere.

Materials and methods

IVF and embryo culture

Spare human pre-implantation embryos and unfertilized oocytes were obtained from the IVF–embryo transfer programme, Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, Ajou University Hospital. Patients who did not wish to freeze their spare embryos for future transfers were asked to donate them for research and their informed consent was obtained. This research was approved by the Human Ethics Committee of the Ajou University Hospital.

Ovarian stimulation was performed under a long protocol regimen with gonadotrophin releasing hormone (GnRH) agonist (buserelin acetate; Suprefact, Hoechst AG, Frankfurt, Germany), follicle stimulating hormone (FSH; IBSA, Lugano, Switzerland), and human menopausal gonadotrophin (HMG; IBSA). Transvaginal ultrasound and serum oestradiol measurements were used to monitor the response to hormone therapy, and 10 000 IU human chorionic gonadotrophin (HCG; Profasi, Serono S.A., Aubonne, Switzerland) was administered. When two or more follicles were >18 mm in diameter and the serum oestradiol concentration was >600 pg/ml, oocyte aspiration was carried out 36 h after the HCG injection, using a standard transvaginal ultrasound-guided approach.

Commercial IVF culture media (Medi-Cult IVF media; Medi-Cult, Copenhagen, Denmark) was employed in the insemination and growth of embryos cultured at 37°C with an atmosphere of 5% CO_2 and 20% O_2. Cleavage and fragmentation of the embryos was observed

42–48 h after insemination, and the embryos were classified and transferred. A total of 53 human oocytes and spare embryos (16 unfertilized oocytes, 31 fragmented or partially fragmented, and 15 non-fragmented embryos) were employed in this study.

Measurement of reactive oxygen species

The development of embryos was assessed in each group 2 days after insemination. In order to determine the quantity of ROS produced by the embryos, the H_2O_2 concentration within the embryos was measured by 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA; Molecular Probes Inc., Eugene, OR, USA). The principle underlying this procedure may be described briefly as follows: non-ionized DCHFDA is membrane permeant and therefore is able to diffuse readily into cells. Within the cell, the acetate groups are hydrolysed by intracellular esterase activity forming 2′,7′-dichlorodihydrofluorescein (DCHF) which is polar and thus trapped within the cell. DCHF fluoresces when it is oxidized by H_2O_2 or lipid peroxides to yield 2′,7′-dichlorofluorescein (DCF). The level of DCF produced within the cells is related linearly to that of peroxides present and thus its fluorescent emission provides a measure of the peroxide levels (Nasr-Esfahani *et al.*, 1990).

The DCHFDA was prepared in dimethyl sulphoxide (DMSO) at 1×10^{-3} M just before the start of each experiment and kept in the dark and used over a maximum period of 48 h. In each experiment, the concentration was adjusted to 1×10^{-5} M by diluting with human tubal fluid (HTF) containing 0.4% bovine serum albumin (BSA) and then exposing the embryos for 15 min. The embryos were then completely washed to remove surface fluorescence with HTF, and the relative concentrations of H_2O_2 produced by the embryos and unit blastomeres were measured by the Quanti-cell 500 technique (Applied Imaging Co., Sunderland, UK) after excitation at 480 nm and emission at 510 nm with an inverted fluorescent microscope (Nikon Diaphot 300, Nikon Co., Tokyo, Japan) in which the background was calibrated to zero using a xenon lamp (100 W) and fluorescein isothiocyanate filter set.

Confirmation of DNA fragmentation

To determine the DNA fragmentation of the unfertilized oocytes and embryos, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP–digoxigenin nick end-labelling (TUNEL; ApopTag kit, Oncor Inc., Gaithersburg, MD, USA) was performed. For TUNEL, the embryos were fixed with 4% neutral buffered formalin and washed with Tris buffer. The embryos were then processed with an equilibrium buffer for 5 min (from the ApopTag kit) and then reacted in reaction buffer with TdT and dUTP–digoxigenin at 37°C for 24 h. The reaction was terminated by adding stop/wash buffer and standing at room temperature for 10 min and then washed three times with Tris buffer. Anti-digoxigenin–fluorescein was added and reacted at 37°C for 30 min. Washing was again performed with this buffer, after which apoptosis within the embryos was observed under a fluorescent microscope.

Transmission electron microscopy

The unfertilized oocytes and embryos were fixed for 2 h with Karnovsky's fixative solution (1% paraformaldehyde, 2% glutaraldehyde, 0.002 M calcium chloride, 0.1 M cacodylate buffer, pH 7.4), washed with cacodylate buffer and then further fixed with 1% osmium tetroxide and 1.5% potassium ferrocyanide. After dehydration with 50–100% alcohol and embedding in a Poly/Bed 812 resin (Polysciences Inc., Warrington, PA, USA), they were polymerized for 6 h at 36°C, 12 h at 48°C and 24 h at 60°C. The tissue blocks were cut into 1 μm slices and stained with 1% toluidine blue and observed under a fluorescent microscope. For observation under the electron microscope, the tissue was cut into 70 nm slices and double stained with uranium and lead salts (Ultrostain 1H, 2; Leica, Cambridge, UK) and then observed under the electron microscope (Zeiss EM 902A; Transmission Electron Microscopy, Oberkochen, Germany).

Statistical analysis

Statistical analysis was performed by the Scheffé test and values obtained as mean ± SEM. P values < 0.05 were considered to be significant.

Results

A total of 16 unfertilized oocytes and 46 human embryos, at different stages of development ranging from the two cell to eight cell stage with varying degrees of fragmentation, was studied for the detection of H_2O_2 and apoptosis. Using Quanticell 500, the relative fluorescence intensity of DCF is shown as a pseudocolour image where the lowest and highest intensities are coded blue and red respectively. Non-fragmented embryos were stained blue, indicating the low level, but fragmented embryos stained yellow to red, indicating the higher level (Figure 1). The relative concentration of H_2O_2 was significantly higher in fragmented embryos ($n = 31$, 72.21 ± 9.62) compared to non-fragmented embryos ($n = 10$, 31.30 ± 3.50, $P < 0.05$) and unfertilized oocytes ($n = 12$, 30.75 ± 2.67, $P < 0.05$) (Table I).

Positive TUNEL labelling in apoptotic cells was defined as a bright and punctuate fluorescence staining pattern. Positive labelling was observed in 16 out of 21 fragmented embryos, but in non-fragmented embryos ($n = 10$) and unfertilized oocytes ($n = 12$), a positive labelling pattern could not be identified (Figure 2).

Evidence of apoptosis was confirmed by transmission electron microscopy; 10 fragmented embryos which had arrested with variable numbers of cells were serially sectioned. In six embryos, we observed several apoptotic bodies or dense cytoplasm which contained normal appearing cytoplasmic organelles but in non-fragmented embryos ($n = 5$) and unfertilized oocytes ($n = 4$), such characteristic patterns could not be observed (Figure 3).

Discussion

The arrest of embryo development in in-vitro culture has been reported to be a consequence of an abundance of ROS induced by an ambient oxygen concentration environment and a relative absence of free radical scavengers compared to in-vivo conditions (Pabon *et al.*, 1989; Nasr-Esfahini *et al.*, 1990a; Goto *et al.*, 1993). Substances such as hypoxanthine, catecholamines, thiols and flavin in developing cells or culture media react with oxygen to produce oxygen free radicals (Ballou *et al.* 1969; Misra and Fridovich, 1972; Baccanari, 1978; Loutradis *et al.*, 1987). Consequently, the addition of free radical scavengers and metal chelators such as superoxide dismutase, transferrin, EDTA and thioredoxin to the culture media under an ambient oxygen concentration led to enhanced embryo development (Abramczuk *et al.*, 1977; Nasr-Esfahani *et al.*, 1990b; Noda *et al.*, 1991; Nonogaki *et al.*, 1991; Goto *et al.*, 1992; Nasr-Esfahani and Johnson, 1992), and low oxygen

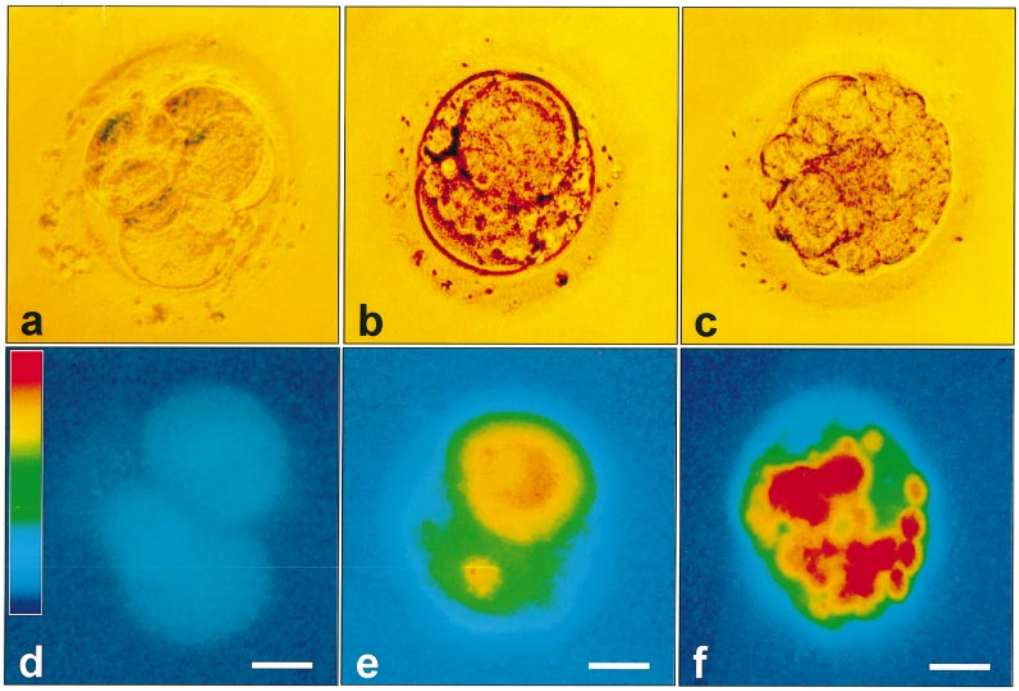

Figure 1. Bright field and pseudocolour images of fragmented and non-fragmented embryos. Fluorescence intensity due to H_2O_2 is expressed in pseudocolour using Quanti-cell 500 according to the colour bar in (**d**). (**a**, **d**) Non-fragmented embryo; the blue colour represents the low H_2O_2 concentration. (**b**, **e**) Partially fragmented embryo stains a yellow colour. (**c**, **f**) Fragmented embryo; red colour indicates the highest H_2O_2 level.

Table I. H_2O_2 concentrations and apoptosis in fragmented and non-fragmented embryos

Human embryos and oocytes	Concentration[a] of H_2O_2	TUNEL[b] positive staining	TEM[c] apoptosis
Fragmented embryos	72.21 ± 9.62* (n = 31)	16/21	6/10
Non-fragmented embryos	31.30 ± 3.50 (n = 10)	0/10	0/5
Unfertilized oocytes	30.75 ± 2.67 (n = 10)	0/12	0/4

[a]The relative intensity of H_2O_2 concentration within embryos was measured using 2′,7′-dichlorodihydrofluorescein diacetate by the Quanti cell 500 technique.
[b]Number of positive stained embryos/total observed embryos by TUNEL.
[c]Number of embryos with chromatin condensation and apoptotic body/total observed embryos by TEM.
TUNEL = transferase-mediated DNA end labelling; TEM = transmission electron microscopy.
*$P < 0.05$ significant difference between fragmented embryos and the other groups.

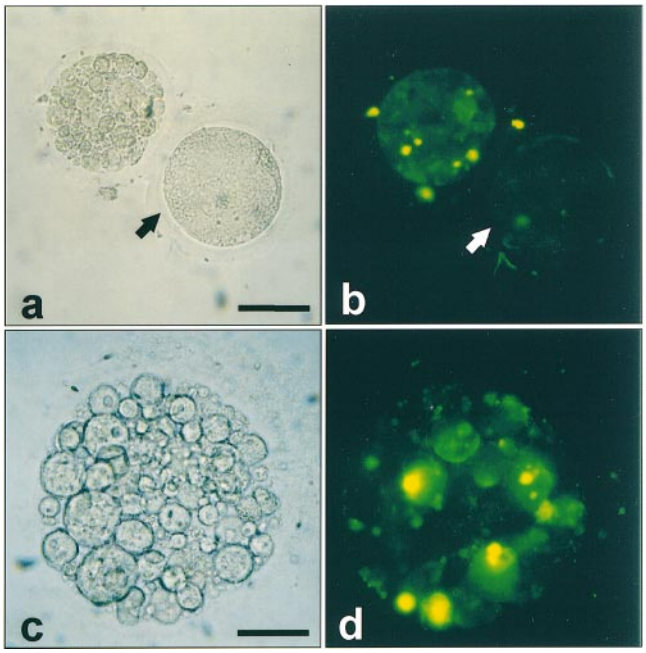

Figure 2. Detection of apoptosis in unfertilized oocytes and fragmented embryos by TUNEL method. (**a**, **b**) Unfertilized oocyte (arrow) showing absence of DNA fragmentation. Bar, 70 µm. (**c**, **d**) Fragmented embryo positively stained showing many fragments. Bar, 35 µm.

concentration culture conditions of 5% O_2 have been shown to enhance development in mice embryos (Quinn and Harlow, 1978; Pabon *et al.*, 1989), and this has also been demonstrated in human in-vitro studies (Noda *et al.*, 1994; Dumoulin *et al.*, 1995).

In this study, we were able to observe that increased fragmentation rate of human embryos was directly correlated with increased H_2O_2 concentration especially at the site of fragmentation, and therefore it is thought that H_2O_2 plays an important role in the fragmentation of embryos. The fragmentation phenomenon is similar to one of the morphological characteristics of apoptosis. In cell necrosis, the cell membrane loss of permeability leads to swelling and rupture, whereas in fragmentation, the cell condenses and is divided into several fragments leading to cytoplasmic condensation and condensed nuclei which are called apoptotic bodies (Kerr *et al.*, 1972; Wyllie, 1980). One of the distinct features of

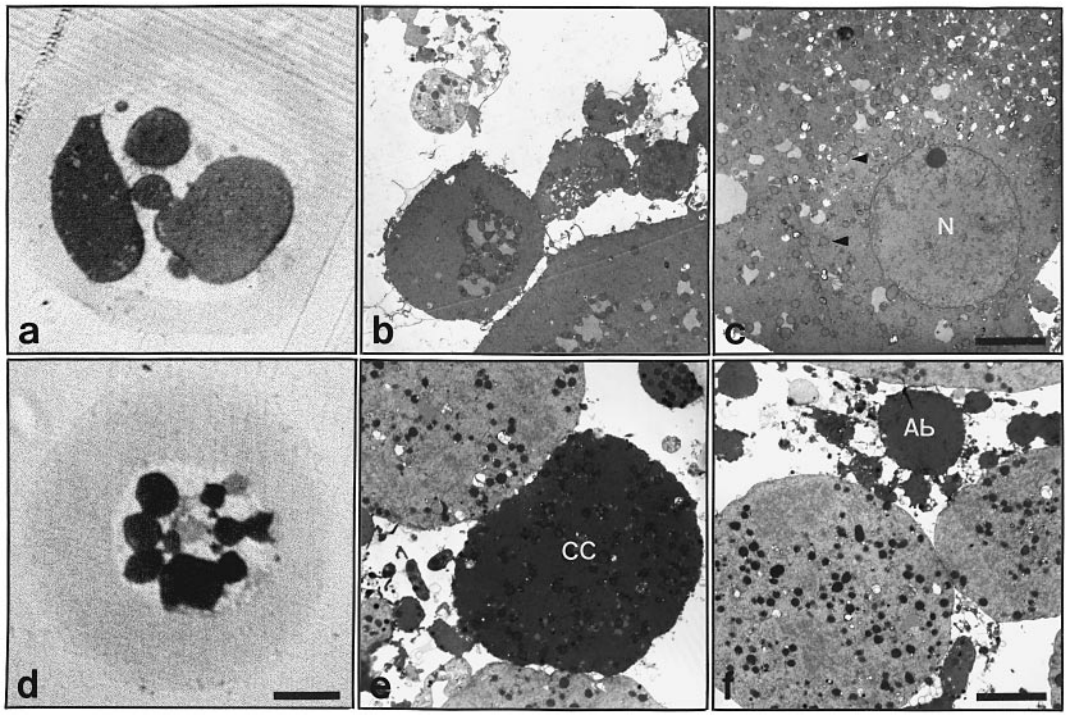

Figure 3. Transmission electron micrographs of fragmented and non-fragmented embryos. (**a**, **d**) Thin sections of embryos (**a** non-fragmented embryo, **d** fragmented embryo) stained with Toluidine Blue. Condensed cytoplasm in fragmented embryo is darkly stained. Bar, 35 μm. (**b**, **c**) A non-fragmented embryo has intact mitochondria and normal nucleus. Bar, 5 μm. (**e**, **f**) Some parts of blastomeres in fragmented embryo are very electron dense, representing condensed cytoplasm and an apoptotic body (Ab). Bar, 5 μm. Arrowheads = mitochondria; N = nucleus; CC = condensed cytoplasm.

cells undergoing apoptosis is the breakdown of the DNA into 185–200 bp fragments (Wyllie *et al.*, 1984; Arends *et al.*, 1990; Gaido and Cidlowski, 1991), and it is known that such fragmentation is a morphological forerunner of apoptotic bodies and the nuclear condensation that leads to apoptosis. In order to determine whether such phenomena occur in fragmented embryos, each unfertilized ovum and embryo was observed for DNA fragmentation using the in-situ TUNEL method. The unfertilized oocytes and embryos without fragmentation were not stained, whereas staining was observed at the sites of fragmentation in fragmented embryos. Overall, in embryos with severe fragmentation, we were able to observe that there were multiple sites of strongly stained areas. To confirm that apoptosis had taken place, embryos were sectioned and observed under the electron microscope; this showed that in unfertilized oocytes and non-fragmented embryos, normal nuclei and mitochondria, and preservation of the cell membrane, was observed. In embryos with severe cytoplasmic fragmentation, apoptotic bodies characteristic of apoptosis were observed. These results suggest that early human embryos which develop in high oxygen culture conditions undergo cytoplasmic fragmentation due to the elevation of H_2O_2 and eventually undergo apoptosis. These findings are in agreement with several studies in which the incubation of human pre-implantation embryos of poor quality was associated with a significant decline in the total antioxidative capacity of culture medium determined by enhanced chemiluminescence (Paszkowski and Clarke, 1996). In another study, it was demonstrated that the morphological appearance of apoptosis in fragmented human embryos, with TUNEL and transmission electron microscopy, and suggestive of programmed cell death, was triggered in human embryos at a stage prior to blastocyst formation (Jurisicova *et al.*, 1996).

The above results suggest that ROS are important factors to the detriment of embryo development, causing morphological changes such as cytoplasmic condensation and apoptotic bodies. Therefore, the increase in ROS concentration by embryos cultured in an ambient oxygen environment may lead to embryo cytoplasmic fragmentation and apoptosis. Our study also suggests that in IVF programmes, suppression of the ROS concentration in embryos resulting from a low oxygen concentration environment, or addition of free radical scavengers to culture media, may enhance normal embryo development and therefore increase the number of successful pregnancies.

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