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Steroidogenic acute regulatory protein (StAR) and peripheral-type benzodiazepine receptor (PBR) are decreased in human apoptotic embryos

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Fragmentation in human pre-implantation embryos has been suggested as the process of apoptosis. We have previously demonstrated a direct relationship between the increased reactive oxygen species (ROS) and apoptosis in human pre-implantation embryos. ROS is known to suppress the function of mitochondria in which steroidogenic acute regulatory protein (StAR) and peripheral-type benzodiazepine receptor (PBR) are presented. Therefore, the purpose of this study was to examine the expression of StAR and PBR in human pre-implantation embryos and to evaluate whether reduction of these proteins is associated with apoptosis. Apoptosis was detected by annexin V-fluorescein isothiocyanate (FITC) and mitochondrial membrane potential was measured by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1). Immunofluorescence staining and Western blotting were applied to examine the expression of StAR and PBR in the embryos. Lipid droplets in the embryos were stained with Oil Red O. The fragmented pre-implantation embryos were stained with annexin V-FITC, but not the normal ones. The mitochondria with active membrane potential were present less in the fragmented embryos compared with the non-fragmented embryos. We also confirmed that both StAR and PBR were expressed in the embryos and their expression levels were lower in the fragmented ones. In addition, the number and size of lipid droplets were increased in the fragmented embryos. The present study provides evidence that reduction of StAR and PBR in human pre-implantation embryos is associated with an increase in the lipid droplets leading to apoptosis.

Keywords: apoptosis; human fragmented embryo; lipid droplets; PBR; StAR

Introduction

Many studies have been conducted over the past decade to find the reasons for apoptosis of human pre-implantation embryos (Jurisicova et al. 1998; Delimitreva et al. 2005). One of the reasons for apoptosis is known to be the lack of growth factors or survival factors needed for the development of embryos at the early stage (Tilly 2001; Haouzi and Hamamah 2009). We also have demonstrated that an increased level of reactive oxygen species (ROS) in human pre-implantation embryos induces apoptosis (Yang et al. 1998). Moreover, it was reported that the high level of ROS in the embryos reduces the active membrane potential of mitochondria, leading to apoptosis (Kwon et al. 1999; Sohn et al. 2002). The mitochondrial membrane contains not only apoptosis-related proteins such as Bax and Bcl-2 (Reed 1994; Kunson and Korsmeyer 1997) but also steroidogenesis-related proteins (Stocco and Clark 1996; West et al. 2001). Although key proteins that are responsible for controlling steroidogenesis have not been clearly identified, the mitochondrial proteins such as steroidogenic acute regulatory protein (StAR) and peripheral-type benzodiazepine receptor (PBR) are thought to be important regulating factors.

StAR plays an important role in steroid hormone biosynthesis through its action in mediating cholesterol transfer to the inner mitochondrial membrane and the cholesterol side chain cleavage enzyme system (Stocco 2000). It is primarily present in steroid-producing cells, including theca cells and luteal cells in the ovary and Leydig cells in the testis (Stocco and Clark 1996). StAR knockout male mice were reported to show the inhibition of sperm maturation and germ cell apoptosis along with decreased biosynthesis of androgen (Hasegawa et al. 2000), suggesting that StAR protein affects not only steroid production but also germ cell apoptosis. In addition, it has been reported that suppression of expression of StAR protein in Leydig cells increases apoptosis, resulting in impaired Leyding cell function (Luo et al. 2011).

PBR was originally discovered because it binds benzodiazepine with relatively high affinity (Papadopoulos 1993). It was found to be particularly high in steroid-producing tissues where PBR was primarily localized in the outer mitochondrial membrane (Anholt et al. 1986; Papadopoulos et al. 1999). PBR is well known to be a functional component of the steroidogenic machinery (Papadopoulos et al. 1990; Papadopoulos 1998) mediating cholesterol delivery from the

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outer to the inner mitochondrial membrane (Krueger and Papadopoulos 1990). Recently, PBR has been renamed the translocator protein (TSPO) due to its channel-like properties (Papadopoulos et al. 2006). Knockdown of TSPO by genetic manipulation was reported to show anti-apoptotic effects, suggesting a potential pro-apoptotic function of TSPO (Veenman et al. 2007). On the other hand, it was reported that rat mammary tumor cells express evaluated levels of PBR, a cancer-promoting gene. When treated with PK11195, a PBR antagonist, the proliferation, invasion and migration appear to decrease, causing an increase in apoptosis of breast cancer (Mukhopadhyay et al. 2010).

However, little is known about the expression of StAR and PBR in human pre-implantation embryos and the relationship between these proteins and apoptosis of the embroys. Therefore, we investigated whether StAR and PBR are expressed in human pre-implantation embryos using confocal image and Western blot analysis and whether the reduction of these proteins induces apoptosis in the pre-implantation embryos.

Materials and methods

Human pre-implantation embryos and culture

Human pre-implantation embryos and unfertilized oocytes were collected from the patients who underwent IVF–ET from 2002 to 2003. 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 Institutional Review Board of the Eulji University Medical Center. A total of 72 human pre-implantation embryos and unfertilized oocytes (24 unfertilized oocytes, 31 fragmented or partially fragmented embryos, and 17 non-fragmented embryos) were employed in this study.

Detection of apoptosis by annexin V-FITC

The unfertilized oocytes and embryos were divided into two groups: those showing fragmentation and normal embryos. Apoptosis was detected using the annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BD Biosciences, USA). After washing the oocytes and embryos in PBS, they were incubated with annexin V-FITC for 20 min and counterstained with DAPI. The oocytes and embryos after reaction were washed with PBS, mounted with fluorescent mounting medium (Dako, USA), and observed using a confocal laser scanning microscope (Olympus, Japan).

Measurement of mitochondrial membrane potential by JC-1

To assess the mitochondrial function, the oocytes and embryos were stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl-carbocyanine iodide (JC-l, Molecular Probes, USA), which can specifically detect the mitochondria with active membrane potential. The obtained oocytes and embryos were stained with 50 μl of JC-1 (10 μg/ml) in the medium for 10 min in an incubator. They were rinsed in dye-free culture medium and observed under a fluorescent microscope (Carl Zeiss, Germany).

Immunofluorescent staining of StAR and PBR proteins

The oocytes and embryos were fixed in 10% neutral buffered formalin (NBF) for 10 min, washed with PBS, and reacted in 2% BSA solution for 10 min to prevent non-specific binding. Each oocyte and embryo was incubated with rabbit anti-rat StAR antibody protein (BD Biosciences, USA) and rabbit anti-rat PBR antibody (BD Biosciences, USA) overnight at 4°C. After washing with PBS, they were reacted with FITC-conjugated goat anti-rabbit IgG (BD Biosciences, USA) or rhodamine-conjugated goat anti-rabbit IgG for 1 h at room temperature. The oocyte and embryo after reaction were washed with PBS, mounted with fluorescent mounting medium (Dako, USA), and observed using a confocal laser scanning microscope (Olympus, Japan).

Western blot analysis of StAR and PBR proteins

For each group, five oocytes or embryos were placed into 500 μl of lysis buffer (10 mM Tris, 250 mM sucrose, 100 mM EDTA, pH 7.4) containing protease inhibitors (1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 mg/l leupeptin, 2 mg/l aprotinin). The mixture was dissolved by homogenating at 4°C and centrifuged at 100,000 × *g* for 30 min to obtain a mitochondria fraction. The pellets were dissolved in sample buffer (25 mM Tris-HCl, 1% SDS, 5% mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) and electrophoresed on SDS-polyacrylamide gel (Bio-Rad, USA). After electrophoresis, the gel was separated and transferred to nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech) in transfer buffer (48 mM Tris-HCl, 39 mM glycine, 0.037% SDS, 20% methanol). This membrane was stained in ponceau S solution for 5 min, washed 2–3 times with distilled water to remove excess stain solution, reacted with TBS (10 mM Tris, 0.2 M NaCl, 0.1% Tween-20) containing 5% defatted milk for 1 h, and washed several times with TBS. The washed membrane was incubated with rabbit anti-tubulin antibody (BD Biosciences, USA) as the internal

control, rabbit anti-rat StAR antibody protein (BD Biosciences, USA), and rabbit anti-rat PBR antibody (BD Biosciences, USA) overnight at 4°C. It was washed with TBS and incubated with HRP-conjugated goat anti-rabbit IgG (BD Biosciences, USA) for 1 h. After washing the membrane with TBS several times, it was reacted in ECL (Enhanced Chemi-luminescence, GE Healthcare, USA) solution for 1 h. After the completion of reaction, it was exposed to X-ray film for 10 s in a darkroom to confirm the band. The relative expression amount of StAR and PBR proteins was quantified by comparing it with the density of the internal control tubulin.

Oil Red O staining

After the oocytes and embryos were fixed in 10% NBF for 10 min, they were washed thoroughly with PBS and stained with 0.5% Oil Red O (Sigma, USA) in propylene glycol (Sigma, USA) for 15 min. After the reaction, they were washed with PBS to remove excess staining solution, mounted with aqueous mounting medium (Sigma, USA), and observed under a light microscope.

Statistical analysis

Significance of the results was confirmed using the Student *t*-test. *P* values less than 0.05 were considered to be significant. All data are represented as means $\pm$ SEM.

Results

The fragmented embryos were stained with annexin V

When annexin V staining was done in five embryos in each group, no fluorescent staining was observed in those embryos showing no fragmentation (Figure 1a, b), whereas green fluorescent staining was observed in those embryos showing fragmentation (Figure 1c, d).

Mitochondrial membrane potential was reduced in the fragmented embryos

When the embryos were stained with JC-1 dye and examined under a fluorescent microscope, the non-fragmented embryos showed a great number of active mitochondria, which were evenly distributed throughout the blastomere (Figure 2a, b). On the other hand, the fragmented embryos displayed fewer active mitochondria, which were distributed to the outer edge of the blastomere (Figure 2c, d).

The levels of StAR and PBR proteins were decreased in the fragmented embryos

In immunofluorescence staining of StAR and PBR, they were expressed throughout the cytoplasm except in the nucleus of the embryo. The degree of expression of both proteins was lower in those embryos showing fragmentation compared with normal embryos showing no fragmentation (Figure 3). Western blotting was performed

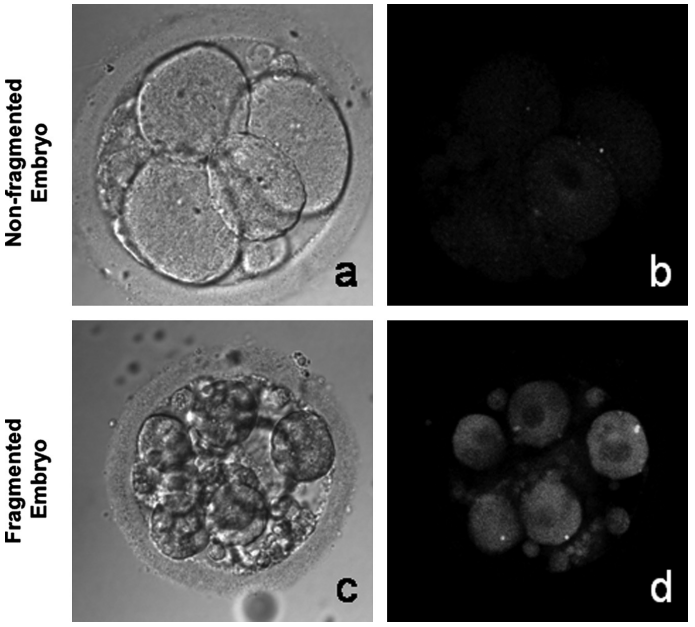

Figure 1. Detection of apoptosis in human pre-implantation embryos. The embryos were stained with annexin V-FITC and observed under a confocal microscope. The fragmented embryos were intensively stained with annexin V-FITC as green fluorescence (d), whereas no fluorescent staining was observed in the embryos showing no fragmentation (b). Bright field (a, c) and fluorescence (b, d) microscopic images. Original magnification 400 $\times$.

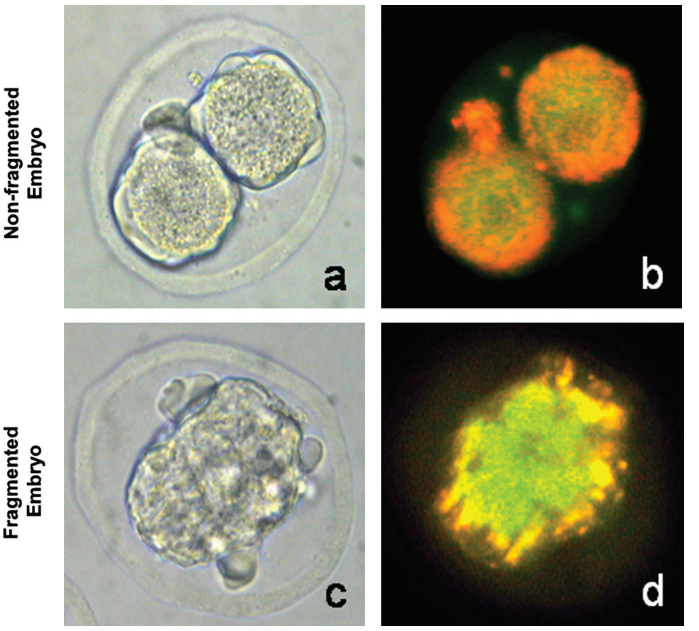

Figure 2. Measurement of mitochondrial membrane potential in human pre-implantation embryos. The embryos were stained with JC-1 dye and observed under a fluorescent microscope. The fragmented embryos displayed fewer active mitochondria, which were distributed to the outer edge of blastomere (d). On the other hand, the non-fragmented embryos showed a great number of active mitochondria, which were evenly distributed throughout the blastomere (b). Bright field (a, c) and fluorescence (b, d) microscopic images. Original magnification 400 ×.

for StAR and PBR proteins to confirm the results of staining in the normal and fragmented embryos. The StAR protein was detected in the molecular weight region of 30 kDa as reported previously and PBR protein, 18 kDa. When the relative expression levels of StAR and PBR were analyzed using an image analysis program, using tubulin as an internal control, the degrees of expression of StAR and PBR protein were significantly higher in non-fragmented embryos (0.71 ± 0.04 and 0.55 ± 0.05, respectively) than in the embryos showing fragmentation (0.68 ± 0.05 and 0.49 ± 0.02, respectively) (Figure 4).

The amount of lipid droplets was increased in the fragmented embryos

When the embryos were stained with Oil Red O, which selectively stains neutral triglycerides and lipids, the number and size of lipid droplets stained in red were increased in the fragmented embryos (Figure 5c, d), but a few lipid droplets were not observed in non-fragmented embryos (Figure 5a, b).

Discussion

Fragmentation of human pre-implantation embryos during in vitro fertilization and embryo transfer (IVF-ET) is known to affect the development and implantation of embryos (Wu et al. 2000; Check et al. 2007). The study of the fragmentation of the pre-implantation is clinically very important. We reported previously that fragmentation of pre-implantation embryos is very similar to apoptosis morphologically and induced by increased ROS in the embryos during in vitro culture (Yang et al. 1998).

Many studies demonstrated that an increased level of ROS within cells decreases active mitochondrial membrane potential, which, in turn, leads to apoptosis (Wilding et al. 2001; van Blerkom et al. 2006). It was reported that the level of ROS increases drastically while the active mitochondrial membrane potential decreases within embryos that are in vitro cultured and abnormality is induced in the mitochondria (Chen et al. 2003). These changes would stop the embryonic development and induce apoptosis (Sohn et al. 2002; Chen et al. 2010). In this study we confirmed that mitochondrial membrane potential was reduced in the human pre-implantation embryos showing fragmentation. These results suggest that the increased ROS level within embryos could play an important role in regulating the mitochondrial function, eventually leading to apoptosis of in vitro cultured pre-implantation oocytes.

With the revelation of the fact that the mitochondria are involved in the apoptotic process, studies have been conducted in proteins present within the mitochondria. The mitochondria contain not only Bcl-2 family proteins involved in apoptosis, but also proteins involved in steroidogenesis (Reed 1994; Ginger et al. 1999; Kim 2000). Therefore, the present study was

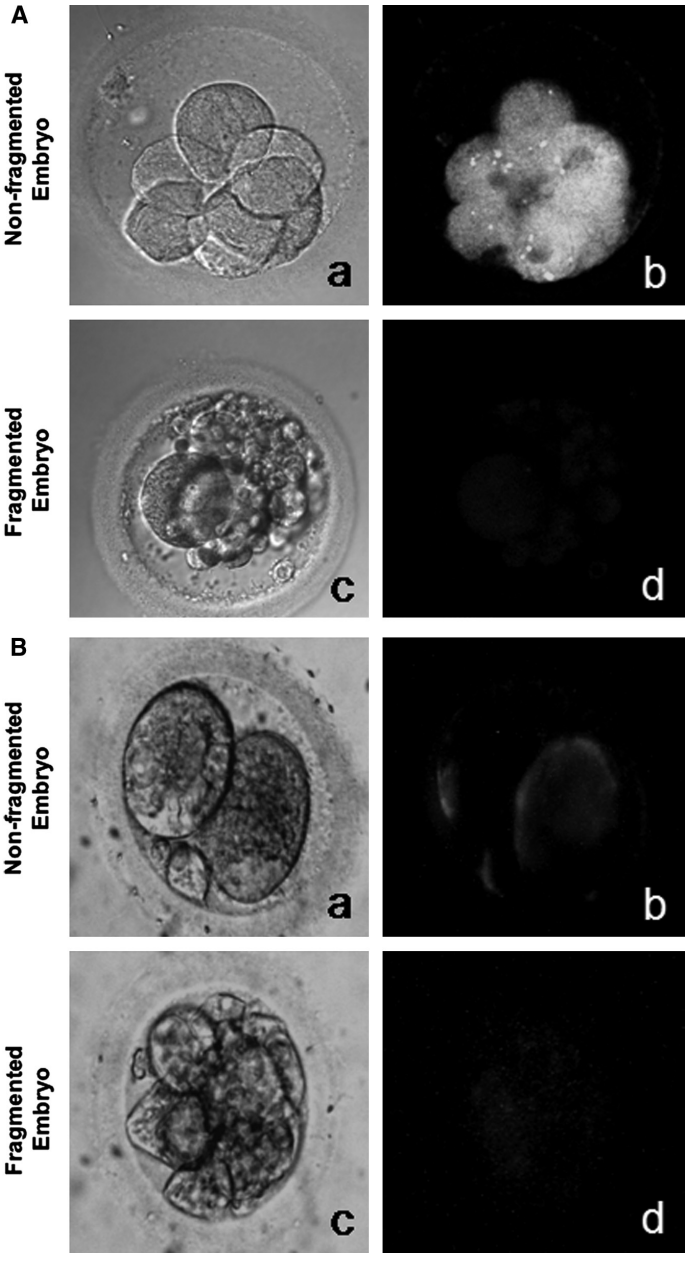

Figure 3. Localization of StAR and PBR in human pre-implantation embryos. The embryos were stained with antibodies against StAR or PBR and observed under a fluorescence microscope. StAR was detected in the human pre-implantation embryos and its degree of expression was lower in the embryos showing fragmentation compared with normal embryos showing no fragmentation (A). Similar to StAR, PBR was expressed in the embryos showing the decreased degree of expression in the fragmented embryos (B). Bright field (a, c) and fluorescence (b, d) microscopic images. Original magnification 400 ×.

conducted to determine whether StAR and PBR, known to regulate steroidogenesis, are also present in human pre-implantation embryos and the relationship of these proteins with apoptosis of the fragmented embryos observed frequently during IVF-ET. We confirmed the expression of StAR and PBR in human pre-implantation embryos and observed the decreased expression levels of these proteins in fragmented embryos. Although StAR is well known to play a crucial role during steroidogenesis by transporting cholesterol to P450scc enzyme in the inner membrane of mitochondria (Sugawara et al. 1995; Stocco and Clark 1996), many recent studies show that StAR is also involved in apoptosis. StAR knockout male mice showed the inhibition of germ cell apoptosis along with decreased biosynthesis of androgen (Hasegawa et al.

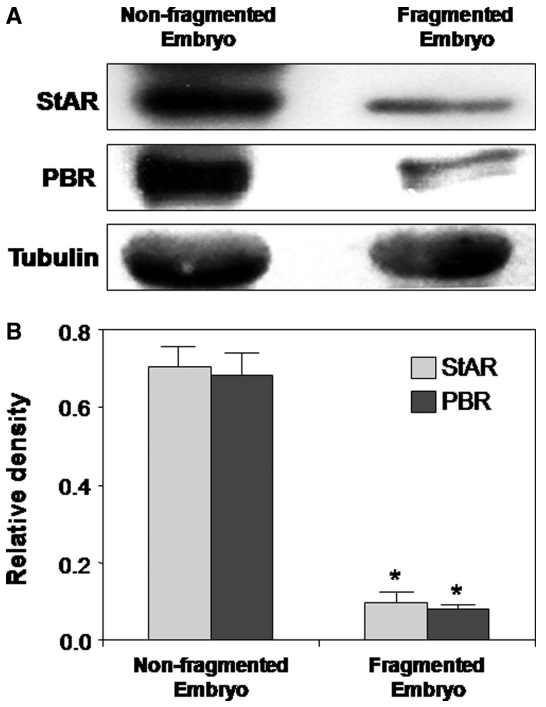

Figure 4. Western blot analysis of StAR and PBR proteins in human pre-implantation embryos. (A) Representatives of Western blots of StAR and PBR and tubulin as an internal control. (B) Relative density of StAR and PBR expressed in human pre-implantation embryos. The relative expression amount of StAR and PBR was calculated by using a densitometer and image analysis program and quantified by comparing it with the density of the internal control tubulin. Values are the mean $\pm$ SEM. *, significant difference at $P < 0.001$ compared with non-fragmented embryos.

2000). This result suggests that decreased expression of StAR may stimulate the apoptosis process of human pre-implantation embryos.

PBR is an 18 kDa protein present in various tissues including the kidney, lung, heart, adrenal cortex, testis, endocrine tissues including the pituitary gland, uterus, fallopian tube, and term placenta (Fares et al. 1987; Tong et al. 1991). However, the presence of this protein has not been reported in human pre-implantation embryos. Thus, we found the expression of PBR as well as StAR in early embryos for the first time in the present study. Furthermore, considering the finding that PBR decreases in fragmented embryos, PBR not only participates in the process of cholesterol transport needed for the production of steroid (Sugawara et al. 1995; Stocco and Clark 1996) but would also selectively participate in the progression of the cell cycle and apoptosis. The hypothesis that PBR would affect the regulation of the cell cycle and apoptosis can be supported by the study reporting that the cell cycle would stop and apoptosis would occur when tumor tissues were treated with specific ligands binding with PBR (Maaser et al. 2001).

On the other hand, PBR is an essential protein needed for steroidogenesis in the reproductive organ by transporting lipid into the inner membrane of mitochodria where the P450scc enzyme is present (Manna et al. 2002). Therefore, dysfunction of these proteins causes problems in lipid transport, preventing steroidogenesis within the mitochondria and increasing lipid droplets in the form of cholesterol ester within cytoplasm (Sridaran et al. 1999). This increased level of lipid droplets within cytoplasm would undergo lipid oxidation affected by ROS, resulting in apoptosis. In this study, we also confirmed the distribution of lipid droplets in the embryos showing the accumulation of a large quantity of lipid droplets within the cytoplasm of severely fragmented embryos. This result was similar to the finding that the accumulation of lipid within cytoplasm is a result of cholesterol metabolism abnormality (Devine et al. 2000; Adrienne et al. 2001). Thus, the accumulation of lipid in fragmented embryos was probably a result of the interference in lipid metabolism due to abnormal mitochondrial function in the process of apoptosis.

Interestingly, the activity of 5-3b-hydroxysteroid dehydrogenase and 17b-hydroxysteroid dehydrogenase and the presence of endogenous progesterone and estradiol were found in immature oocytes in the human ovary (Suzuki et al. 1983). Therefore, the fact that we found StAR and PBR proteins participating in steroid production in embryos suggests that they would also possess steroidogenesis capacity. At the same time, the finding that the expression of StAR and PBR proteins was less in embryos showing fragmentation suggests that StAR and PBR proteins are essential in the normal development of embryos and may play a role in regulating apoptosis of fragmented embryos.

Taking these findings together, we hypothesize the apoptosis mechanism of in vitro cultured human pre-implantation embryos based on the above results (Figure 6). The decreased active mitochondrial membrane potential in the embryos may be due to the effect of ROS produced abundantly during in vitro culture. The loss of mitochondrial membrane potential may lead to abnormality in the function of StAR and PBR present on the mitochondrial membrane. This dysfunction of these proteins would inhibit cholesterol transport and accumulate lipid droplets within cytoplasm. We believe that lipid droplets formed during this process in embryos would cause lipid oxidation, leading to apoptosis. In conclusion, the present results suggest that StAR and PBR expressed in human pre-implantation embryos would play crucial roles in the

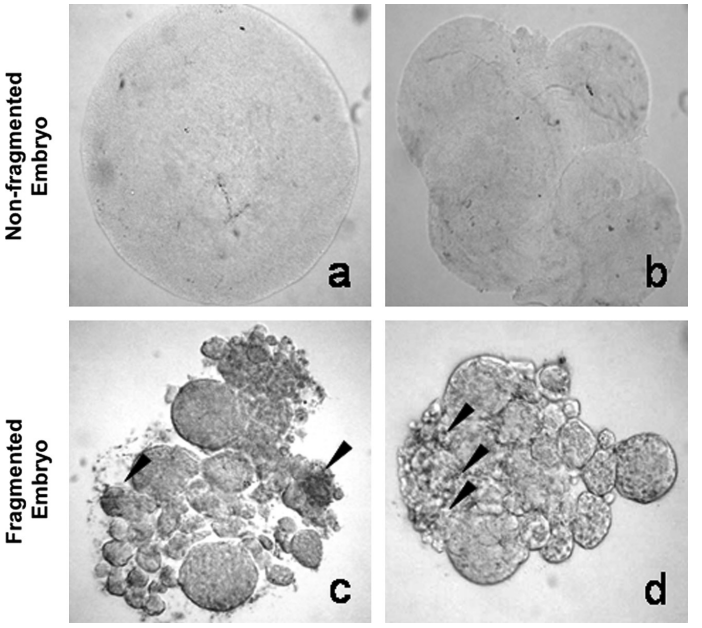

Figure 5. Detection of lipid droplets in human pre-implantation embryos. The embryos were stained with Oil Red O and observed under a light microscope. The number and size of lipid droplets stained in red were increased in fragmented embryos (c, d), but a few lipid droplets were not observed in non-fragmented embryos (a, b). Arrowheads indicate the lipid droplets stained with Oil Red O.

early development of embryos. However, further studies are needed in the future on the biochemical mechanism inducing apoptosis.

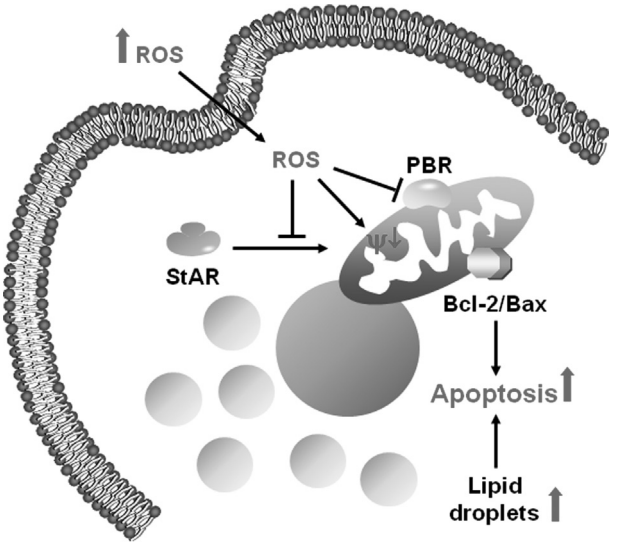

Figure 6. Schematic diagram illustrating that reactive oxygen species (ROS) acts directly on the decrease in mitochondria membrane potential, leading to dysfunction of apoptosis-related proteins presented on the mitochondrial membrane. Similar to apoptosis-related proteins, dysfunction of StAR and PBR by ROS causes an increase in lipid droplets in human pre-implantation embryos, leading to apoptosis.

Acknowledgements

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