

# Fragmentation of embryos is associated with both necrosis and apoptosis

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**Objective:** To explore the association between embryo fragmentation and necrosis and apoptosis.

**Design:** A prospective study.

**Setting:** Mizmedi Hospital.

**Patient(s):** None.

**Intervention(s):** None.

**Main Outcome Measure(s):** Staining with annexin V (a marker of apoptosis) and propidium iodide (PI, a marker of necrosis), DNA integrity and mitochondrial distribution, and a beneficial effect of fragment removal in human fragmented embryos.

**Result(s):** Most of the mouse and human fragmented embryos were stained with PI but not with annexin V. The comet assay revealed severe DNA fragmentation of the fragmented human embryos but not of the unfragmented embryos. Fewer mitochondria were observed in the fragmented compared with the normal blastomeres, indicating a rapid depletion of ATP in the fragmented embryos. Microsurgical fragment removal from the embryos had a beneficial effect on their subsequent development.

**Conclusion(s):** Fragments of human embryos exhibited various characteristics of necrosis, such as staining with PI, DNA fragmentation, rapid depletion of ATP, and harmful effects on neighboring blastomeres. We suggest that the fragmentation of embryos is closely associated with both necrosis and apoptosis. Whether this fragmentation is associated with primary or secondary necrosis remains to be elucidated. (Fertil Steril® 2011;96:187–92. ©2011 by American Society for Reproductive Medicine.)

**Key Words:** Embryo fragmentation, apoptosis, secondary necrosis, necrosis, fragment removal

The presence of anucleate fragment limits the subsequent development of embryos (1–4) and can lead to one of two forms of cell death (5): apoptosis and necrosis. Apoptosis is a programmed cell death that is characterized by chromatin condensation, cell shrinkage, membrane blebbing, prelytic DNA fragmentation, requirement of ATP, phagocytosis by adjacent cells, and beneficial effects on neighboring cells (6–9). On the other hand, necrosis is cell death by injury and is characterized by cell swelling, increased permeability of the damaged membrane, rapid depletion of ATP, postlytic DNA fragmentation, cell lysis, and harmful effects on neighboring cells (7–9). Possible causes of apoptosis in preimplantation embryos are chromosomal abnormalities (10), imbalance of growth factors (11), reactive oxygen species (12, 13), and suboptimal culture conditions (10, 14, 15). Potential causes of necrosis are oxidative stress, endotoxin, and ATP depletion (8).

Unlike necrosis, apoptosis plays a protective role in the developing embryo by eliminating defective blastomeres (14, 16), but if the ratio of apoptotic blastomeres is elevated, the development of the entire embryo could also be arrested, or it may die, as a necrotic cell does. Secondary necrosis occurs in the terminal stages of apoptotic cell death in cells that fail to be engulfed by phagocytes

(9, 17). It is characterized by condensation of the chromatin, nuclear fragmentation, and necrotic features such as an increase in membrane permeability and distension of the cytosolic membrane structure (9). Owing to the similarity between primary and secondary necrosis, it is possible to misinterpret which is the cause of cell death. Although apoptosis has been regarded as the major cause of embryo fragmentation, some investigators have suggested a relationship between embryo fragmentation and necrosis. Fragments of embryo release toxic substances that are harmful to neighboring blastomeres (1, 5, 18), which is one of the typical characteristics of necrosis. This assertion is supported by the finding that fragment removal enhances the viability and development of neighboring blastomeres (19, 20). Therefore, the present study explored the association of embryo fragmentation with necrosis and apoptosis.

## MATERIALS AND METHODS

### Institutional Review Board

This study was approved by Institutional Review Board (IRB) of Mizmedi Hospital and the IRB of the Ministry of Health and Welfare in Korea.

### Human and Mouse Embryo Preparation

IVF patients (n = 3) who succeeded in giving birth to a healthy baby donated their surplus frozen embryos (16 embryos) for the present study after giving informed consent. The patients were 24, 28, and 29 years old and donated three, seven, and six frozen embryos, respectively. The embryos were frozen at 2 or 3 days by the slow-freezing protocol. Of 16 frozen-thawed embryos, 14 embryos survived and were used for comet assay (four embryos) and annexin V staining (10 embryos), respectively. One hundred eighty-five two-cell-stage mouse embryos were isolated, and then the embryos that had developed to the morulae stage in vitro were used for annexin V staining

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before the use of human embryos in this study. Since most of the morulae-stage mouse embryos were unfragmented, 43 fragmented embryos and 10 unfragmented embryos were used for annexin V staining.

### Microsurgical Fragment Removal from Human Fragmented Embryos

Assisted hatching and fragment removal were performed on day 2 human embryos at the four-cell stage. Before fragment removal, the fragmented embryos were incubated in a prewarmed  $\text{Ca}^{++}$ - and  $\text{Mg}^{++}$ -free phosphate buffered saline (PBS) supplemented with 10% synthetic serum substitute (Irvine Scientific) under mineral oil for 30 minutes. Microsurgical fragment removal was performed in the same PBS droplet. A pipette with an inner diameter of 10–15  $\mu\text{m}$  was prepared to suck the fragments, and it was attached to a mouth suction unit.

### Ultrastructure of Human Fragmented Embryos

The ultrastructure of the human fragmented embryos was investigated with the aid of transmission electron microscopy (TEM). The fragmented embryos were fixed in 2.5% glutaraldehyde (Sigma) for 24 hours at room temperature. Postfixation in 1% osmium tetroxide was followed by dehydration using ethyl alcohol and substitution using ethyl alcohol and xylene (Sigma). Embedding was performed in Epon at 60°C for 48 hours, and thin sections (80 nm thickness) were cut using a diamond knife on a microtome stained with uranyl acetate (Sigma) and lead citrate (Sigma) and examined using TEM (magnification  $\times 200$ ).

### DNA Fragmentation of Human Fragmented Embryos

A modification of the sperm comet assay described in a previous study (21) was performed to investigate the DNA fragmentation of the human frag-

mented embryos. Images were captured at a magnification of  $\times 200$  using a digital camera imaging system (DS-U1, Digital Sight series, Nikon) attached to a fluorescence inverted microscope.

### Annexin V and PI Staining

The hallmark of apoptosis is the translocation of phosphatidylserine (PS) on the outer leaflet of the plasma membrane, and this can be detected using annexin V staining. Necrotic cells are easily stained with the cell-impermeant dye PI owing to extensive damage to the membrane permeability (7, 13). Thus, apoptotic cells stain positive for annexin V (green) but not PI, and necrotic cells stain positive for PI (red) but not annexin V, while viable cells with an intact plasma membrane and no translocated PS stain negative for both annexin V and PI (colorless). An ApoAlert annexin V apoptosis detection kit (Clontech) was used to stain the fragmented embryos according to the manufacturer's recommendations. The embryos were photographed under a fluorescence microscope (Nikon) under bright light and with a FITC/Texas Red dual filter.

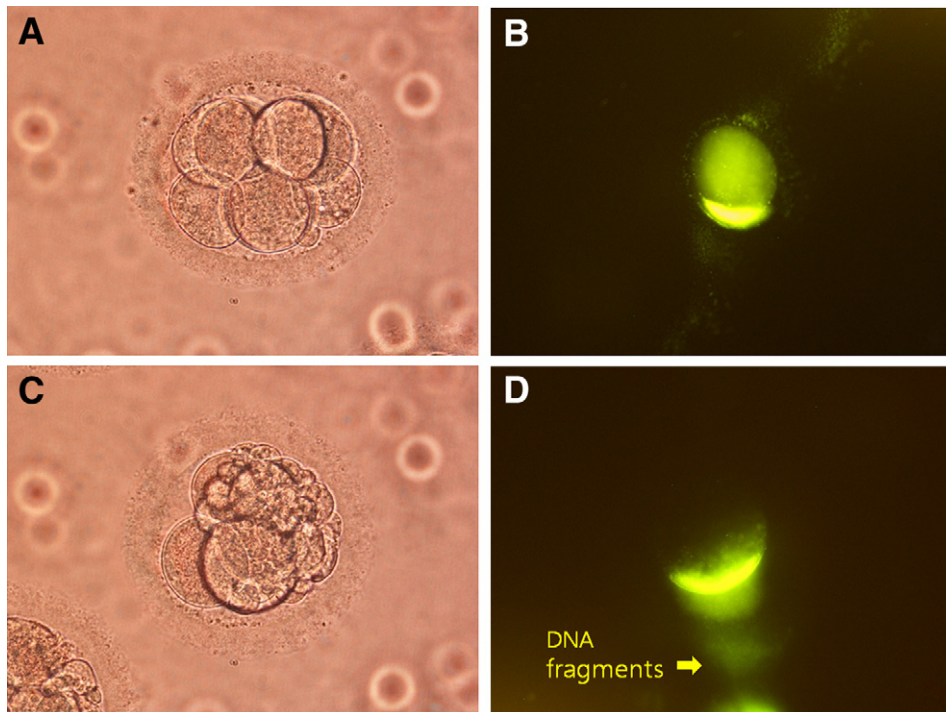
## RESULTS

### DNA Fragmentation of Human Fragmented Embryos

DNA fragmentation is a characteristic that is common to both apoptotic and necrotic cells. The DNA integrity of the human fragmented (two embryos) and unfragmented embryos (two embryos) was investigated using a modified comet assay (Fig. 1). Unlike many other studies, we did not remove the embryos' zona pellucida for the comet assay, because this membrane does not disturb the migration of DNA particles during gel electrophoresis. The unfragmented embryos showed no sign of a comet tail, but the

## FIGURE 1

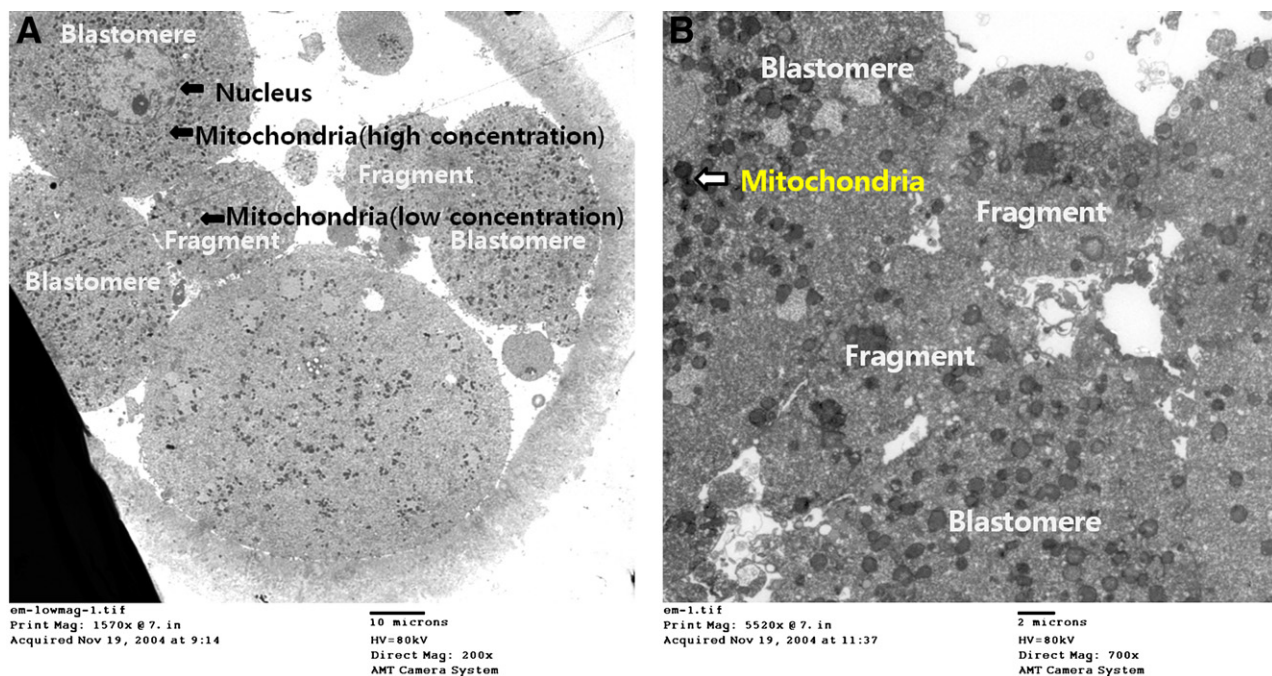
DNA integrity of unfragmented (A, B) and fragmented (C, D) human embryos, as assessed by the comet assay. Magnification, A, C:  $\times 400$ ; B, D:  $\times 200$ .



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## FIGURE 2

TEMs of human fragmented embryos. The normal blastomere contains a nucleus and a high concentration of mitochondria, but the anucleate fragments contain a low concentration of mitochondria (ATP depletion). Magnification, A:  $\times 1,570$ , B:  $\times 5,520$ .



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fragmented embryos (2/2, 100%) exhibited a long comet tail resulting from the migration of fragmented DNA.

### Ultrastructure of Human Fragmented Embryos

The ultrastructure of human fragmented embryo was investigated by TEM (Fig. 2). The observed internal location of the fragments may be the result of breakage of cytoplasmic bridges and blastomere movement and rotation during embryo cleavage. The anucleate fragments contained fewer mitochondria in their cytoplasm compared with normal blastomeres. The apparent deficiency of mitochondria in the fragments may lead to a depletion of ATP, resulting in cell lysis.

### Harmful Effect of Fragments on the Development of Neighboring Blastomeres

To confirm the harmful effects of fragments, complete fragment removal of human fragmented embryos was performed microsurgically on day 2, and the embryos were subsequently cultured for 24 hours in vitro (Fig. 3). After fragment removal, the morphological grade of the embryos was significantly improved. Most of the fragment-removed embryos did not exhibit a reoccurrence of fragmentation during the subsequent 24 hours of their development. The beneficial effect of fragment removal indicates that fragments have a harmful effect on the development of neighboring blastomeres. After fragment removal, the embryos were cultured for 24 hours, and then three embryos were transferred into the uterus of the patient and the surplus embryos were cryopreserved.

### Annexin V and PI Staining

Mouse (53 embryos) and human unfragmented and fragmented embryos (10 embryos) were stained with annexin V and PI (Fig. 4). Unfragmented mouse and human embryos were not stained by either annexin V (green) or PI (red). However, most of the mouse (39/43, 90.6%) and human fragmented embryos (6/7, 85.1%) stained positively for PI (red color). The intensity of the red staining increased with the degree of embryo fragmentation. One of the mouse fragmented embryos (1/39, 2.5%) stained positively for both annexin V and PI. Furthermore, a blastomere with a blebbing membrane was, if only very weakly, stained by annexin V. One of the human fragmented embryos (1/6, 16.6%) was also positive for both annexin V and PI, but the green stain was more prominent than the red. The embryo (six-cell-stage embryo) was thawed and then cultured for 24 hours in vitro; the embryo was not subsequently cleaved but developmentally arrested.

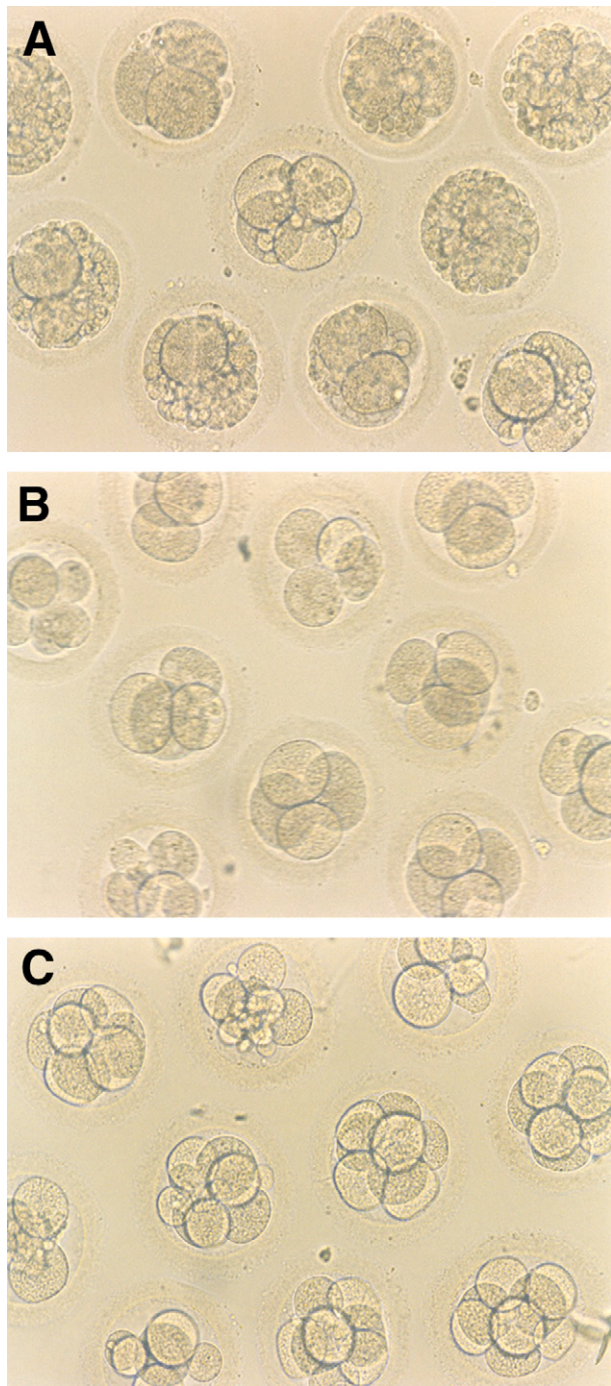
### DISCUSSION

Some authors have removed the zona pellucida before the comet assay of embryos (22, 23), whereas others performed zona thinning (24) or did not remove this membrane (25). We also performed the comet assay without removal of this membrane because it would otherwise have been impossible to perform a successful comet assay in this case owing to scattering of the blastomeres and fragments. We observed the comet tail of DNA fragmentation in the fragmented human embryo but not in the unfragmented normal embryo.

Van Blerkom et al. (3) have demonstrated that fragmentation in some early embryos disappeared at later stages after resorption by

### FIGURE 3

Beneficial effect of fragment removal on the subsequent development of neighboring blastomeres. (A) Human embryos before fragment removal (day 2), (B) after fragment removal (day 2), and (C) after culture for 24 hours (day 3). Magnification,  $\times 200$ .



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sis of the apoptosing cell is performed by scavenger cells such as macrophages (9). The normal phagocytic system can not be present in embryos even in the *in vivo* environment because embryo blastomeres are entirely cut off from outsider cells by the zona pellucida. Moreover, we observed that the fragments were not re-sorbed but rather hidden in the inner spaces, enclosed by the blastomeres. Individually separated fragments at the apical surface of the early-stage embryo appear to move inward with ease with the division and rotation of blastomeres during embryonic cleavage.

The number of mitochondria in the cytoplasm was lower in anucleate fragments in the present study than in normal blastomeres. Van Blerkom et al. (3) reported that mitochondria were absent from most of the fragments. Mitochondrial deficiency of the fragments indicates depletion of ATP in the cells; depletion of ATP is one of the characteristics of necrotic cells and could lead to cell lysis. Previous ultrastructural observations indicate that blastomeres adjacent to the fragments exhibit early signs of vascular degeneration (26). This has raised the possibility that lysing fragments release toxic substances that damage neighboring blastomeres (1).

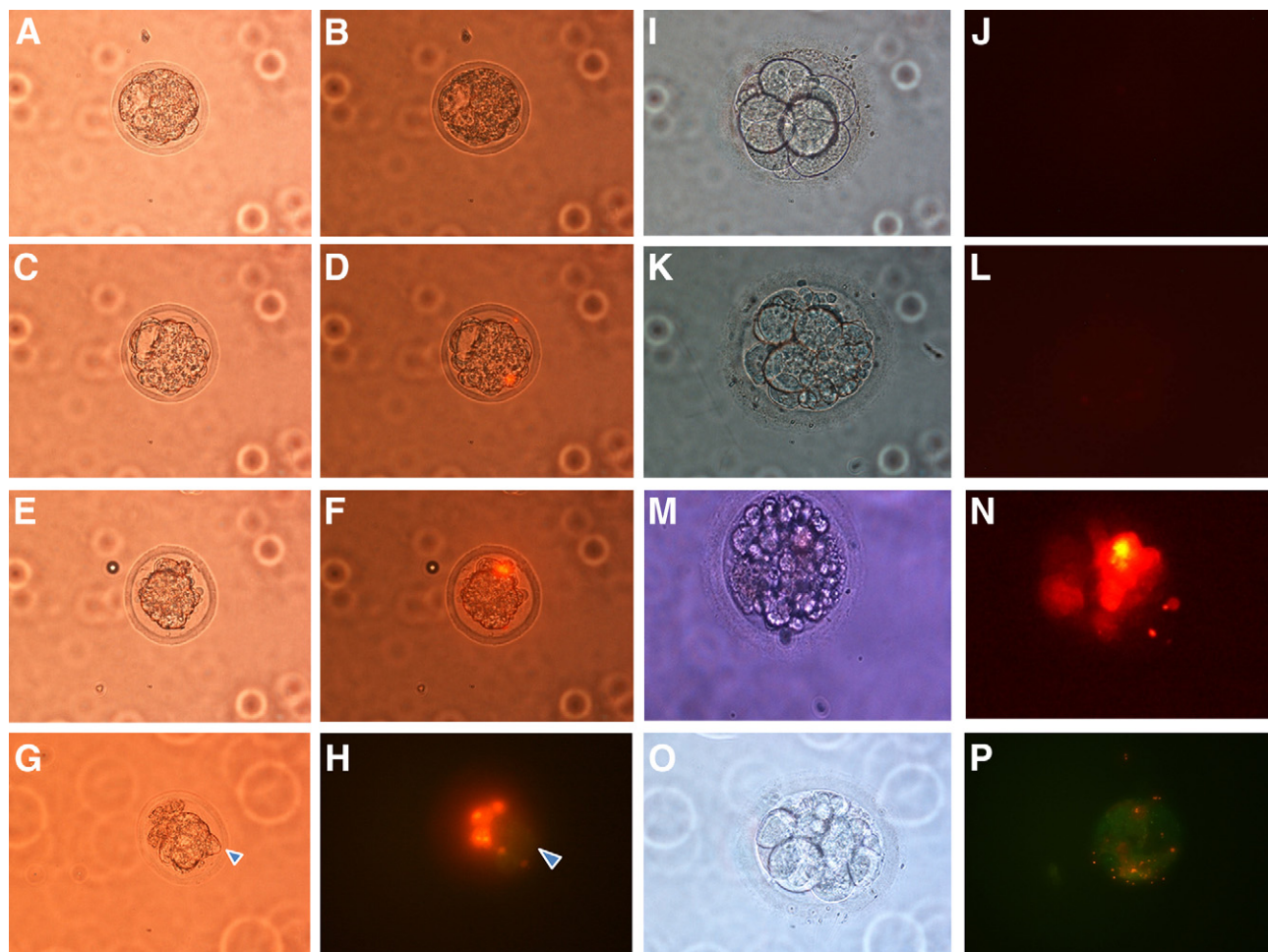
Many investigators have reported not only the detrimental effects of fragments on neighboring blastomeres including loss of essential organelles (27), reduced blastocyst formation (2), decreased chances of implantation (1, 4), and increase in abortion rate (4, 26) but also the beneficial effects of fragment removal on the cells such as improvement of embryo quality (18), blastocyst development (28), and clinical outcome (29). The beneficial effects of fragment removal were also observed in the present study, indicating that the fragments do indeed have a detrimental effect on nearby blastomeres. However, we have shown previously that fragment removal does not exert any beneficial effect when over 25% of the embryo is fragmented. Thus, fragmented embryos may not be rescued by fragment removal when they have undergone excessive loss of cytoplasm, been lethally damaged by the release of toxic substances from a large population of fragments, or have a genetic problem. Keltz et al. (18) reported that fragment removal exerts no beneficial effect on clinical outcome. This finding may be attributable to incomplete fragment removal, unlike in the present study. If the extracellular fragments are removed while simultaneously making efforts not to damage the proximal blastomeres, the intracellular fragments therein may not have been removed completely. If so, the remnant intracellular fragments may subsequently release toxic substances that will counteract the beneficial effects of fragment removal.

In the present study, unfragmented viable mouse and human embryos were stained with neither annexin V nor PI. In contrast, most of the mouse and human fragmented embryos stained positively for PI. This suggests that in most fragmented embryos there is a close association with necrotic rather than apoptotic cell death. Anrczak and Van Blerkom (30) reported a similar finding of no direct correlation between fragmentation and apoptosis after the analyses of fragmented embryos with PI and annexin V markers. However, other investigators have observed both annexin V and PI staining in all arrested or fragmented human embryos (6, 31). The difference between these results and ours may be attributable to differences in the process underlying the cell death. Apoptosis may occur independently among sibling blastomeres, thus some blastomeres may proceed further to secondary necrosis after apoptosis, while others may or may not initiate apoptosis (6). Therefore, it is possible to observe both apoptotic, necrotic blastomeres and viable blastomeres within a fragmented embryo, and the

neighboring blastomeres. However, Jurisicova et al. (5) reported that the blastomeres of embryos have no phagocytic capability, since fragments were found in the intercellular spaces. Phagocyto-

**FIGURE 4**

Annexin V and PI staining of mouse and human fragmented embryos. (A) Unfragmented mouse embryo, (B) negative for annexin and PI. (C, E, G) Fragmented mouse embryos, (D, F) positive for PI (necrosis), (H) positive for annexin and PI, membrane bleb (weak green staining, apoptosis). (I) Good-quality human embryo, (J) negative for annexin V and PI. (K) Fragmented human embryo, (L) weakly positive for PI. (M) Severely fragmented and degenerating human embryo, (N) strongly positive for PI. (O) Fragmented and arrested human embryo, (P) positive for both annexin V and PI. Magnification,  $\times 400$ .



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coexistence of these different types of blastomeres may be dependent on the process underlying the cell death.

In the present study, we observed both the apoptosis and necrosis markers in the fragmented embryos, although staining for the necrosis marker was overwhelming. Apoptosis is a relatively slow cellular process, and so early stages of the apoptotic process can be detected by annexin V staining (14), but the terminal stages (secondary necrosis) cannot. Since secondary necrotic cells exhibit not only a specific morphotype associated with apoptotic features but also necrotic features such as rupture of the cytoplasmic membrane, they can be stained with PI rather than with annexin V. This similarity makes it possible to misinterpret the state of the necrotic cells between those affected by apoptotic secondary necrosis and

those affected by primary necrosis. A new method of discriminating between secondary necrosis and necrosis has recently been introduced involving the detection of caspase-3, which is released by cells undergoing secondary necrosis but not by those undergoing primary necrosis (32). We plan to use this new method in future work.

In conclusion, the fragments of embryos exhibited characteristics of necrosis, such as staining with PI, DNA fragmentation, rapid depletion of ATP, and harmful effects on neighboring blastomeres. We therefore suggest that the fragmentation of embryos is closely associated with both necrosis and apoptosis. However, whether that fragmentation is related to primary or secondary necrosis remains to be elucidated.

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