Biomed. Papers 148(2), 245–247 (2004)

PRODUCTION OF H_2O_2 IN HUMAN EMBRYOS AFTER LONG-LASTING CRYOPRESERVATION AND SUBSEQUENT CULTURE

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Received: September 20, 2004

Key words: Human embryo/Cryostorage/In vitro/H_2O_2 production

The production of hydrogen peroxide (H_2O_2) was investigated by means of cytochemical reaction with cerium chloride in human embryos cryostoraged for a long time period. The sites of H_2O_2 generation were demonstrated at submicroscopic level in both freshly thawed embryos and in embryos and blastocysts formed after subsequent culture. The intact blastomeres as well as cells of well developed blastocysts did not produce any H_2O_2. Two main intracellular sites of H_2O_2 production were identified: mitochondria and plasma membranes. Some alterations and often destruction of plasma membrane integrity accompanied by massive H_2O_2 generation are believed to be caused by the freezing and thawing.

INTRODUCTION

It has been accepted that in vitro development of mammalian embryos is mostly accompanied by increased reactive oxygen species (ROS) production[1]. ROS generation may be still more enhanced during the freezing, cryopreservation and thawing procedures[2]. Since we have had a unique oportunity to investigate human embryos which were cryopreserved for a long time period and then could not be transferred, the aim of this study was

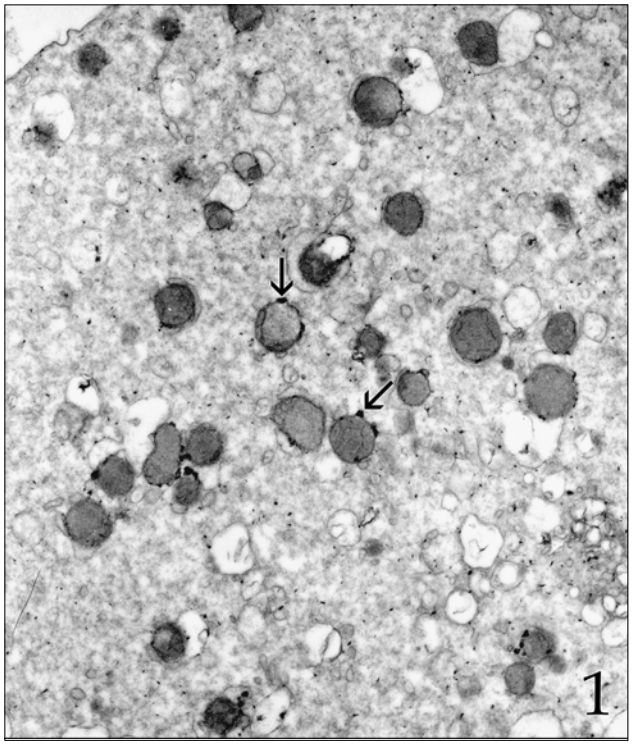

Fig. 1. Mitochondrial H_2O_2 production was demonstrated by means of cytochemical reaction in the form of dense cerium perhydroxide granules present in limited number on the outer mitochondrial surfaces in degenerated blastomeres or cell fragments (arrows). 13,000 x

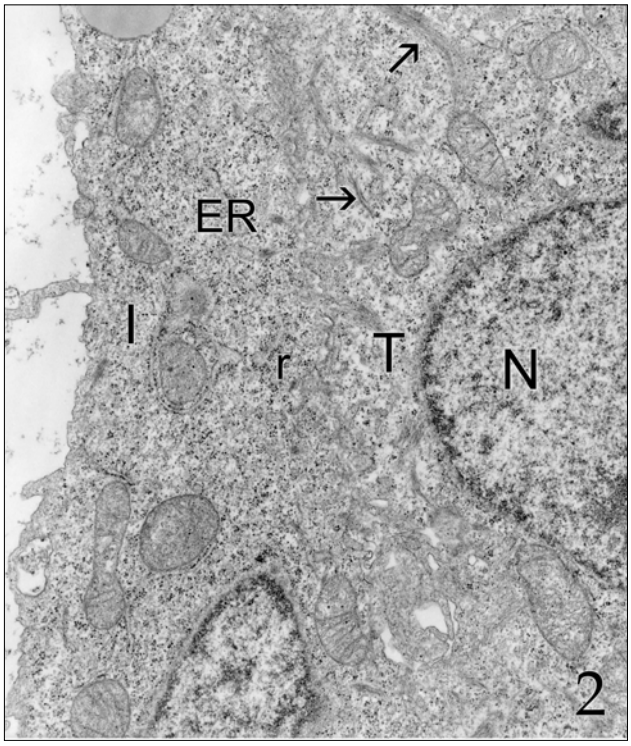

Fig. 2. A part of well developed blastocyst. An inner cell (I) with numerous ribosomes (r), and granular endoplasmic reticulum (ER); a trophectoderm cell (T) with nucleus (N) and cytokeratin filaments (arrows). No granules of cerium perhydroxide are visible. 12,000 x

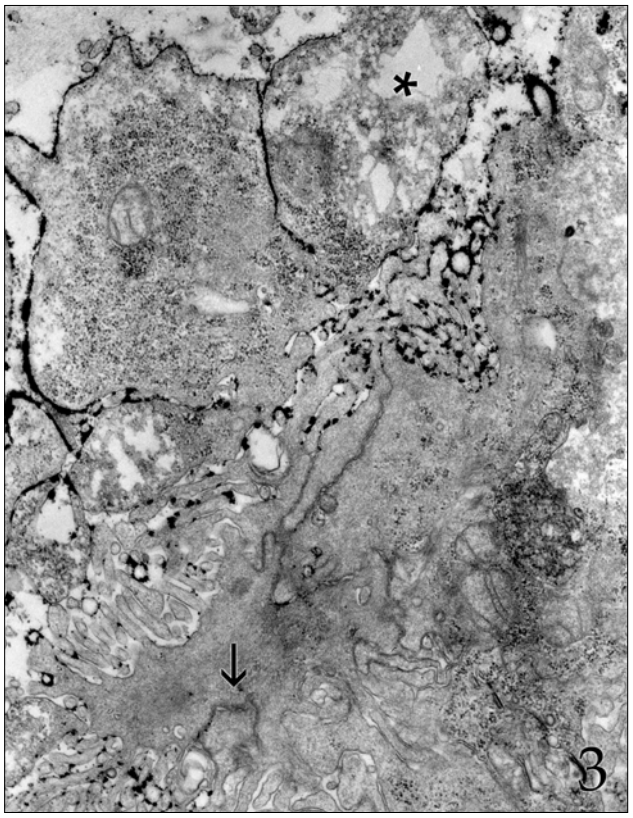

Fig. 3. A group of the trophectoderm cells at the embryonic pole of a blastocyst. The H_2O_2 production is dected on the surface of some cells showing initial necrotic changes (swollen and vacuolised cytoplasm) (*). Note well developed junction complexes among normal cells (arrow). 15,000 x

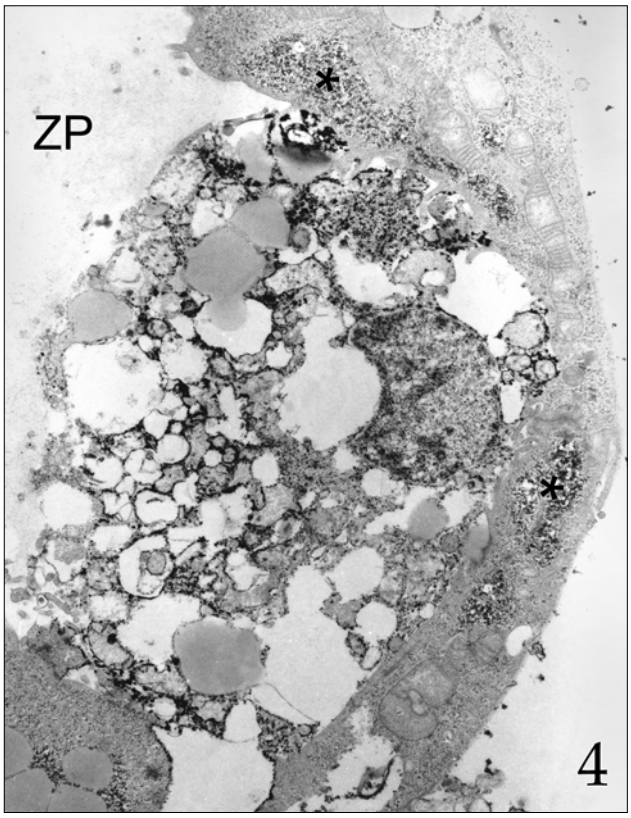

Fig. 4. A disintegrated necrotic cell with granules of reaction product in rests of the ground cytoplasm. Trophectoderm cells with glycogen (*). Zona pellucida (ZP). 12,000 x

to detect the sites of ROS production partly immediately after their thawing and partly after subsequent culture. For identification of ROS generation sites, cytochemical demonstration of hydrogen peroxide (one of the three main ROS) was used.

MATERIALS AND METHODS

A total number of 52 two- and four-cell embryos cryoprotected by 1,2-propanediol, frozen in Planer Kryo F10, and stored for five or more years in liquid nitrogen were thawed and a part were cultured under standard conditions for a time period of 48 or 72 h. Embryos were prepared by standard protocol for transmission electron microscopy either immediately after thawing (12) or after cultivation (40). In the course of this procedure, H_2O_2 was detected using cerium chloride medium[3]. Generation of H_2O_2 was evidenced by the formation of electron dense granules of cerium perhydroxides.

RESULTS

In the group of embryos processed immediately after thawing, the embryos showing normal submicroscopic structure did not produce any H_2O_2. Its production was regularly demonstrated in all embryos with signs of degeneration or fragmentation. Major and mostly sole intracellular sources of H_2O_2 were mitochondria (Fig. 1). Sometimes, massive amounts of H_2O_2 and their release were discovered on damaged cell membranes of some blastomeres or cell fragments.

After thawing and culture, well developed blastocysts (about 10 % of embryos) occurred only in the group of four-cell cryostoraged embryos. Both the trophectoderm and inner cells did not produce H_2O_2 in such blastocysts (Fig. 2). In developmentally retarded embryos, the injured (Fig. 3) and dying (Fig. 4) cells were identified as sources of a massive H_2O_2 production. Major intracellular sites of H_2O_2 production were cell membranes (Fig. 3) in damaged (mostly necrotic) cells. In late phases of necrosis, the H_2O_2 was detected often elsewhere in the nucleus and cytoplasm (Fig. 4). Cell fragments and apoptotic bodies present typically in arrested or fragmented embryos

and occasionally in well developed blastocysts may also produce H_2O_2. If the H_2O_2 sources were present in high numbers, massive amounts of H_2O_2 were demonstrated especially in the intercellular spaces or blastocyst cavity.

DISCUSSION

The results of this study indicate that long-lasting cryostorage of human embryos need not compromise their development after thawing and subsequent culture, but the developmental potential of the embryos declines. If well differentiated two- and four-cell embryos were used, only about 10% developed to the blastocyst stage. This finding is in agreement with results obtained under similar conditions in mouse embryos[2]. The results of ultracytochemical detection of H_2O_2 confirmed our previous findigs on unfertilised oocytes and arrested or blocked human embryos[4]. Injured and dying cells or their fragments were recognised as a main source of H_2O_2 in arrested embryos. Two intracellular sites of ROS generation – mitochondria and cell membrane – were detected in both freshly thawed embryos and embryos after subsequent culture. Increased ROS generation and their release into the environment was especially typical of cells with damaged, often discontinuous, cell membranes. Such alterations of cell membranes may be caused by the freezing and thawing of the embryos[2].

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