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Lower total cell numbers in mouse preimplantation embryos cultured in human assisted reproductive technique (ART) media are not induced by apoptosis

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ABSTRACT

A common feature of assisted reproductive techniques such as IVF or intracytoplasmic sperm injection is the IVC of oocytes or preimplantation embryos in artificial culture media. The IVC conditions are selected to mimic the environment of the female genital tract. We have shown that murine preimplantation embryos respond to different culture media with changes in developmental rates, cellular lineage composition, and gene expression patterns. In this study, we explored whether apoptosis is responsible for cell loss in mouse preimplantation embryos after exposure to different human culture media. We examined total embryonic cell count as well as the pattern of protein expression for caspase-9 (intrinsic pathway), caspase-8 (extrinsic pathway), and the executioner caspase-3 via immunohistochemical staining. Total cell counts decline in embryos cultured either in innovative sequential medium 1 and Blast Assist (Origio) or human tubal fluid and MultiBlast (Irvine Scientific) when compared to KSOM(aa). Few cells were caspase-9 and -3 positive in all experimental groups. Staining for caspase-8 was almost undetectable. We conclude that embryonic cell loss is not associated with higher rates of intrinsic apoptotic cell loss. Our results suggest that the culture medium–dependent decline in total cell count and the developmental restriction in embryos cultured in innovative sequential medium 1/Blast Assist and human tubal fluid/MultiBlast are related to processes affecting cell proliferation.

1. Introduction

Assisted reproductive techniques (ARTs) are used worldwide. In Europe, the prevalence of children born from ARTs ranges from 1.4% to 5.9% depending on the country, and it increases by 0.1% to 0.2% each year [1–4].

The most frequently used methods in ARTs are IVF, intracytoplasmic sperm injection (ICSI), and the cryopreservation and transfer of embryos [5]. A common feature of all techniques is the IVC of oocytes or preimplantation embryos in a synthetic medium, on the assumption that the artificial environment of the medium mimics the natural environment of the female genital tract [6]. Notably, the number of ART media has grown from a couple, which were available in the 1980s to a palette of about 20 which are available now. The driving force of this increase is the goal to enhance ART success as measured by the baby-take-home rate, which is still very low with only 20.4% (IVF/ICSI, fresh cycles) [5].

On the assumption that the oviduct is probably the optimal microenvironment for a preimplantation embryo,

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many studies analyzed the composition of the oviductal fluid or the gene expression of IVC embryos compared to *in vivo* embryos, to base the development of embryo culture media on objective criteria [7]. Although early-cleavage embryos are able to grow in a huge variety of culture conditions, these conditions do matter and can effect gene expression [8]. However, the high plasticity of embryos throughout the preimplantation and peri-implantation developmental phase may obscure long-term effects on behavior, growth, and development of the offspring in infancy or also at adult age [6,7,9].

The potential impact of different culture conditions or different components of embryo culture media on preimplantation embryos has been the subject of intense discussions [10]. On the basis of studies in the mouse, we showed that blastocyst rates, cell number, lineage composition (primitive ectoderm and endoderm, trophectoderm) and gene expression varied across multiple commercially available embryo culture media [11]. Interestingly, once these blastocysts were transferred to the genital tract, no significant differences in fetal growth or weight were detected, suggesting either compensatory growth [12] or selection [13]. Whether IVC poses a risk for the embryo is controversial with studies either corroborating [14] or confuting this possibility [15–17]. However, detailed mechanisms by which ARTs such as IVF or ICSI and the culture of preimplantation embryos may have an effect on the developing organism; its biological and molecular processes are at this point not fully elucidated.

During development of preimplantation embryos, programmed cell death is a crucial but physiological normal process. During early mouse embryo development, spontaneous apoptosis can be observed from the four-cell stage onwards, just shortly after embryonic gene activation at the two-cell stage [18]. Apoptosis is regulated via two main pathways, the intrinsic and extrinsic pathways [19]. The extrinsic pathway is activated via binding of specific ligands to death receptors of the tumor necrosis factor superfamily [20]. Current models propose that after death receptor activation, the activation of procaspase-8 is enabled [21]. Once caspase-8 is activated, the caspase cascade is initiated and the cleavage of the effector procaspase-3 directly leads to apoptosis [22]. The intrinsic pathway is initiated via nonreceptor-mediated stimuli, which lead to production of intracellular signals. Hence, cytochrome c is released from the mitochondria, and the initiator caspase-9 is recruited, dimerized, and activated, which then leads to cleavage and activation of the effector caspase-3 [23]. Caspase-activated DNase as one of the most important apoptotic nucleases then causes DNA fragmentation and chromatin condensation in the dying cell [24].

In the present study, we tested the hypothesis that different human ART culture media have an effect on apoptosis in murine preimplantation embryos, spanning from zygote to blastocyst during IVC. Specifically, we examined whether the total cell count of individually *in vitro*–cultured blastocysts is altered because of higher apoptotic rates, which emerge by using different human embryo culture media. Furthermore, we aimed to elucidate whether apoptosis in preimplantation embryos is regulated either via the intrinsic mitochondrial pathway or the extrinsic death receptor pathway. Some methods for detection of apoptosis (terminal deoxynucleotidyl transferase dUTP nick end labeling [TUNEL], annexin V) lack specificity because it is not possible to distinguish accurately between necrosis and apoptosis. A combination of a staining against active caspases, playing a central role in apoptosis, and a DNA staining to observe typical apoptotic morphology of the nucleus is considered as one of the best methods to evaluate apoptosis in murine preimplantation embryos [25].

2. Materials and methods

2.1. Mice

B6C3F1 females were used as source of *in vivo*–fertilized oocytes, C57Bl/6 males were used for mating, and CD1 females were used as recipients for embryo transfer (ET).

These mice were produced, reared, and housed at the central animal facility of the medical faculty of the University of Münster according to Federation of European Laboratory Animal Science Associations recommendations and to the ethical permit issued by the Landesamt für Natur-, Umwelt-und Verbraucherschutz (LANUV) of the state North Rhine-Westphalia, Germany (LANUV permit number: 84–02.04.2011.A140). The mice were kept under 12:12 photoperiod and were fed ad libitum with the Altromin 1324 standard diet (Altromin International, Lage, Germany).

2.2. Embryo production

The B6C3F1 females (aged 6–8 weeks) were hormonally stimulated with 10 IU pregnant mare serum gonadotropin (Intervet, Unterschleißheim, Germany) and 10 IU hCG (Intervet) injected intraperitoneally 50 hours apart. The females were then mated overnight with the C57Bl/6 males of proven fertility and checked for presence of a copulation plug on the next morning. At 22 hours after hCG treatment, fertilized oocytes with two pronuclei and a second polar body were retrieved from the oviductal ampullae and collected in HEPES-buffered M2 medium with albumins (Sigma, Taufkirchen, Germany; [26]). These zygotes were randomly allocated to the different experimental groups within 1 hour after retrieval.

2.3. Embryo culture

2.3.1. Embryo culture media

On the basis of our comparative studies of up to 13 ART culture protocols [11,12], two protocols for embryo culture were selected to culture zygotes in the present study. These protocols were a sequential media protocol based on innovative sequential medium 1 (ISM1) and Blast Assist (BA; Origio, Berlin, Germany), which previously revealed low blastocyst and fetal rates, and a sequential protocol based on human tubal fluid (HTF) and MultiBlast (MB; Irvine Scientific, MTG, Bruckberg, Germany), which previously scored high blastocyst and fetal rates [11]. ISM1 contains a high level of pyruvate as well as essential and nonessential amino acids, notably methionine which is involved in methylation processes and imprinting. BA has a