REPRODUCTIVE
BIOLOGY

Evaluation of mouse preimplantation embryos exposed to oxidative stress cultured with insulin-like growth factor I and II, epidermal growth factor, insulin, transferrin and selenium

Rafał Kurzawa,[1,2], Wojciech Głąbowski[3], Tomasz Bączkowski[2], Paweł Brelik[2]
[2]Clinic for Reproduction and Gynecology, Pomeranian Academy of Medicine;
[3]Department of Histology and Embryology,
Pomeranian Academy of Medicine, Szczecin, Poland

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SUMMARY

Blastocyst culture requires strictly defined culture media to sustain its viability and quality. Although blastocyst media are commercially available, they do not meet all the needs and research focused on blastocyst-promoting agents is on the way. The aims of the study were to evaluate the significance of insulin-like growth factors I (IGF-I) and II (IGF-II); epidermal growth factor (EGF) and a mixture of insulin, transferrin and selenium (ITS) on the development of embryos exposed to oxidative stress. C3B6F1 mice were stimulated with 5 IU of pregnant mare serum gonadotropin following by administration of 5 IU of equine chorionic gonadotropin and mating with DBA males. The mice were killed 40 h after eCG injection by cervical dislocation and then the 2 cell embryos were flushed out from the fallopian tubes. To evaluate whether the growth factors may compensate the unfavorable – oxidative milieu created by hydrogen peroxide (H_2O_2), the embryos were transferred to 1/ control medium, 2/ control medium + 0.1 mM (H_2O_2) or 3/ control medium + H_2O_2 enriched with 10^{-7} g/ml of IGF-I, IGF-II,

[1] Corresponding author: Clinic for Reproduction and Gynecology, Pomeranian Academy of Medicine, 1 Unii Lubelskiej St., 71-252 Szczecin, Poland; e-mail: kurzawa@sci.pam.szczecin.pl

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EGF or a mixture of insulin (5×10^{-6} g/ml), transferrin (5×10^{-6} g/ml) and selenium (5×10^{-9} g/ml; ITS). Embryos were evaluated 96 – 144 hours following eCG injection. In the study the dynamics of embryo development and blastocyst cell numbers (including inner cell mass) were assessed. The morphological evaluation comprised viability and apoptosis (TUNEL). In oxidative stress setting, IGF-I, IGF-II, EGF and ITS minimized the negative influence of H_2O_2, and embryos developed faster than in control conditions. Blastocysts cultured with hydrogen peroxide and growth factors or ITS displayed normal morphology and had more cells – also within the inner cell mass – than those treated only with H_2O_2. The positive TUNEL reactions were sporadically observed in embryos cultured with hydrogen peroxide supplemented with growth factors. IGF-I, IGF-II, EGF and ITS have a positive effect on pre-implantation embryo development in detrimental culture conditions of oxidative stress. *Reproductive Biology 2002 (2): 143 – 162*
Key words: embryo; growth factors; *in vitro* culture

INTRODUCTION

In vitro fertilization (IVF) is an accepted method of infertility treatment. Regular *in vitro* fertilization and embryo transfer (IVF-ET) enable infertile women with an anatomical background of disease to become pregnant. Intracytoplasmic sperm injection (ICSI) helps men with severe semen, testicular or epididymal abnormalities to conceive healthy children. Traditional IVF comprises the following steps: ovarian stimulation, oocyte retrieval, *in vitro* fertilization of gametes (spontaneous or by ICSI), culture of embryos, and embryo transfer with implantation to be the crucial point of this procedure. The current clinical and laboratory strategies in IVF should focus on the best preparation of blastocysts for implantation [3, 17, 73]. Blastocyst transfer is associated with 40-60% of implantation rates compared to 10-20% for the transfer of the 4-8 cell embryos [18, 31]. However, the culture of pre-implantation embryos requires a strict definition of culture media.

Fallopian tube mucosa and endometrium are a source of cytokines and growth factors which are necessary for the proper development of embryos [2, 9, 34, 35, 38, 40, 68, 77]. Epidermal growth factor (EGF), insulin-like growth factor I (IGF-I) and II (IGF-II), and insulin are the mitogens inducing a positive effect on pre-implantation development, and stimulating metabolism and growth of embryos. Moreover, they increase the prolifera-

tion of embryo cells, including both cells forming the inner cell mass (ICM) and the trophoectoderm. They also cooperate in the compaction and formation of blastocyst. The growth factors activate transporting systems, which are responsible for the uptake of glucose, enhance endocytosis and probably proteins turn-over. They also influence the processes of replication, translation and degradation of proteins [20, 21, 22, 25, 29, 30, 33, 36, 39, 43, 45, 54, 56, 61, 69].

Conditions of *in vitro* culture should be comparable to *in vivo* conditions. In *in vitro* culture embryos are exposed to the negative influence of the environmental factors (temperature, components of culture media and contamination) capable of inducing apoptosis and leading to the death of the embryo [1, 16, 23, 24, 47, 52, 64]. Presumably some growth factors may suppress apoptosis by enhancing embryo viability [6, 7, 27, 30, 48, 55, 66, 74]. Some positive effects of EGF, IGF I and IGF II on *in vitro* embryo development have already been reported [61], however the conclusions of these studies were based on very basic embryological data.

MATERIALS AND METHODS

Experimental design

The aim of the study was to evaluate the effects of EGF, IGF-I, IGF-II and a mixture of insulin, transferrin and selenium (ITS) on mice embryos exposed to oxidative stress. The following outcome measures were used to address this goal:

1/ numbers of embryos counted at different developmental stages: 96, 120 and 144 hours following the administration of equine chorionic gonadotropin (eCG); the embryos were evaluated by contrast-phase microscopy;

2/ inner cell mass (ICM) and total blastocyst (TBC) cell number counted 120 hours after eCG injection; structure of blastocysts was evaluated by fluorescent microscopy (differential staining);

3/ cell viability and screening for apoptosis in blastomeres, the quality of embryos was evaluated by both fluorescent microscopy and confocal microscopy.

The experiments were approved by the Ethical Commission of the Pomeranian Medical Academy.

Collection and culture of embryos

Female B6C3F1 mice (6–8 weeks old) were treated intraperitoneally with 5 IU of pregnant mare serum gonadotropin (Folligon, Intervet, Belgium), followed 48 h later by an injection of 10 IU of eCG (Chorulon, Intervet, Belgium) and mating with DBA males. The copulation plug was checked after 24 h. The mice were killed 40 h after eCG injection by cervical dislocation and then the 2 cell embryos were flushed out from the fallopian tubes. The embryos were incubated in control medium i.e. Earl's balanced salt solution (EBSS, Sigma, USA) supplemented with sodium puryvate (0.33 mM), sodium lactate (21.4 mM) and human serum albumin (4g/l; HSA, Sigma, USA). After an initial 6 h of incubation the embryos which passed the 2 cell block were transferred to 1/ control medium, 2/ control medium + 0.1 mM hydrogen peroxide (H_2O_2) or 3/ control medium + H_2O_2 enriched with 10^{-7} g/ml of IGF-I, IGF-II, EGF or a mixture of insulin (5×10^{-6} g/ml), transferrin (5×10^{-6} g/ml) and selenium (5×10^{-9} g/ml; ITS, Sigma, USA). Embryos from all groups were then incubated (37°C, 95%O_2/5%CO_2) for an additional 50, 74 or 98 hours (i.e. 96h, 120h or 144h following eCG injection, respectively). The embryos were incubated in groups of ten in microdroplets of 20 μl covered by mineral oil. The concentration of hydrogen peroxide which impaired proper embryo development was established in separate preliminary experiments (data not shown).

Contrast-phase microscopy

The following stages of the embryos were observed 96, 120 and 144 hours after eCG injection (56, 80 and 104 hours of culture): 8 cell embryos, morulas, early-, expanded-, hatched- and outgrown- blastocysts.

Fluorescent microscopy

Differential staining

Differential staining was used to determine the number of blastocyst cells and the ICM. The quality of blastocysts was verified before the staining by contrast-phase microscopy 120 hours after eCG injection. One blastocyst per group of ten embryos (which had the best morphology in phase-contrast microscope) was used for the staining.

A modified method of De la Fuente and King [11] was used for differential staining. Zona pellucida was removed by exposure to 0.1% pronase for

5-10 min (Sigma, USA) at 37°C. This was followed by 10-15 min of incubation in phosphate buffered saline (PBS, Sigma, USA) containing 10 μg/ml of calcium ionophore A23187 (Sigma, USA), 10 μg/ml of propidium iodide (Sigma, USA) and 1 μg/ml of Hoechst 33342 (Sigma, USA). The blastocysts were covered with glycerol and immediately evaluated under fluorescent microscope (Axioscop, Zeiss, Germany) using 345 nm ultraviolet light filter. The cells were counted using image analysis software (Microimage 4.0 Olympus, Japan; fig. 5A).

Identification of dead and living cells

The procedure was similar to differential staining, with the exception that the incubation mixture was deprived of the calcium ionophore. The dead cells were stained with propidium iodide, due to the cell membrane damage. The vital DNA-specific fluorochrome (Hoechst 33342) stained all the cells in the specimen. The blastocysts were also covered with glycerol and immediately evaluated under fluorescent microscope (Axioscop, Zeiss, Germany) at the wavelength of 345 nm (fig. 5B).

Identification of apoptosis – TUNEL

A TUNEL reaction {terminal deoxynucleotidyl transferase (TdT) – mediated dUTP Nick-End Labeling} was carried out using commercially available apoptosis detection system (Promega, USA). The reaction identified fluorescein-labeled DNA fragments in apoptotic cells of the blastocysts. Before staining, the embryos were treated with 2% formalin for 60 min then washed in PBS and next incubation in 0.2% solution of Triton X-100 for 5 min to perforate cell membrane. After another wash in PBS embryos were incubated for 60 min at 37°C in staining solution containing TdT-enzyme and fluorescein-12-dUTP. Immediately after the reaction was inhibited, the embryos were washed in PBS, transferred to DPBS and evaluated by laser scanning confocal microscopy equipped with difference-interference contrast (Fluoview 500, Olympus, Japan) at the wavelength of 488 nm ([61]; fig. 5C).

Statistical analysis

For each of the studied factors, at least 8 independent experiments were performed. Data are represented by median with upper and lower quartiles. Statistical analysis of total blastocysts counts, hatched blastocyst counts,

total blastocyst cell counts and inner cell mass cell counts of embryos cultured in studied media (tables) was conducted by means of the nonparametric analysis of variance (Anova). Frequency distribution of pre-implantation embryos cultured in studied media was compared by means of log-linear analysis (figures). Calculations were done using Statistica for Windows (StatSoft, Inc., Tulsa, OK., USA). Statistical significance was accepted at $p<0.05$.

RESULTS

Epidermal Growth Factor

More blastocysts survived in the media with EGF+H_2O_2 compared to the group with only H_2O_2. The embryos cultured in the EGF+H_2O_2 media had a significantly higher number of TBC and ICM cells than the embryos cultured in the media with H_2O_2. The most dynamic development and quality of embryos were found in the control group, followed by the EGF+H_2O_2 media. Cultures with H_2O_2 yielded the worst results (tab. 1, fig. 1).

Tab. 1. Total blastocyst counts (BC), hatched blastocyst counts (HBC), total blastocyst cell counts (TBC), inner cell mass cell counts (ICM) observed 96 h or 120 h after eCG administration in control, H_2O_2- treated and H_2O_2 + EGF-treated group. Median (lower – upper quartile)

Group	BC 96h	BC 120h	HBC 120h	TBC 120h	ICM 120h	n
Control	$25^{A,B}$ (25 – 33)	65^{A} (58 – 75)	25^{A} (0 – 63)	55^{A} (47 – 68)	15^{A} (12 – 20)	8
0.1 mM H_2O_2	0^{A} (0 – 5)	30^{A} (15 – 50)	$0^{A,C}$ (0 – 0)	$38^{*A,C}$ (36 – 41)	$8^{*A,C}$ (7 – 10)	8
0.1 mM H_2O_2+ EGF	20^{B} (0 – 20)	60 (40 – 65)	20^{C} (15 – 25)	52^{C} (51 – 57)	14^{C} (14 – 16)	8

$^{A}p<0.05$ – control vs. H_2O_2
$^{B}p<0.05$ – control vs. H_2O_2 + EGF
$^{C}p<0.05$ – H_2O_2 vs. H_2O_2 + EGF
*n=7

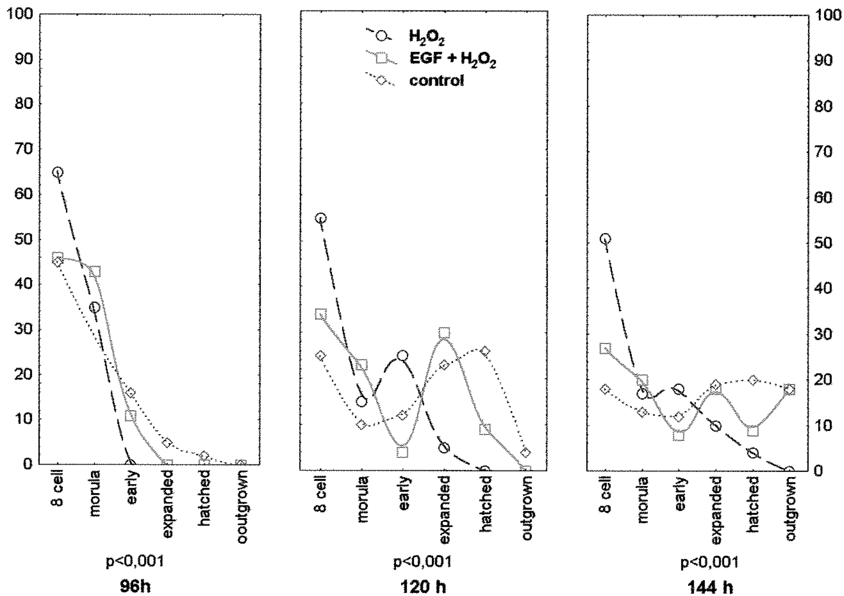

Fig. 1. Frequency distribution of pre-implantation embryos (No) cultured in the absence (control group) or presence of H_2O_2 (0.1 mM) or H_2O_2+EGF (10^{-7} g/ml) for 96, 120 and 144 h after eCG treatment (n=100 per group). Data represent counts of embryo stages with the fitted least squares curve.

Insulin-like growth factor I

IGF-I had a positive effect on cultured pre-implantation embryos. In media with IGF-I+H_2O_2, more hatched blastocyst were found compared to H_2O_2 alone. TBC and ICM cell numbers in IGF-I+H_2O_2 group were significantly higher than those found in the culture with H_2O_2. The control and IGF-I+H_2O_2 groups developed alike, while H_2O_2 alone impaired the process (tab. 2, fig. 2).

Tab. 2. Total blastocyst counts (BC), hatched blastocyst counts (HBC), total blastocyst cell counts (TBC), inner cell mass cell counts (ICM) observed 96 h and 120 h after eCG administration in control, H_2O_2- treated and H_2O_2 + IGF-I-treated group. Median (lower – upper quartile)

Group	BC 96h	BC 120h	HBC 120h	TBC 120h	ICM 120h	n
Control	23^{A} (0 – 33)	50^{A} (33 – 54)	33^{A} (25 – 35)	52^{A} (50 – 57)	16^{A} (13 – 16)	8
0.1 mM H_2O_2	$0^{A,C}$ (0 – 0)	$0^{A,C}$ (0 – 20)	$0^{A,C}$ (0 – 0)	$36^{*A,C}$ (36 – 43)	$8^{*A,C}$ (7 – 10)	8
0.1 mM H_2O_2+ IGF-I	21^{C} (0 – 30)	41^{C} (38 – 62)	21^{C} (20 – 27)	55^{C} (51 – 57)	15^{C} (14 – 16)	8

$^{A}p<0.05$ – control vs. H_2O_2
$^{B}p<0.05$ – control vs. H_2O_2 + IGF-I
$^{C}p<0.05$ – H_2O_2 vs. H_2O_2 + IGF-I
*n=4

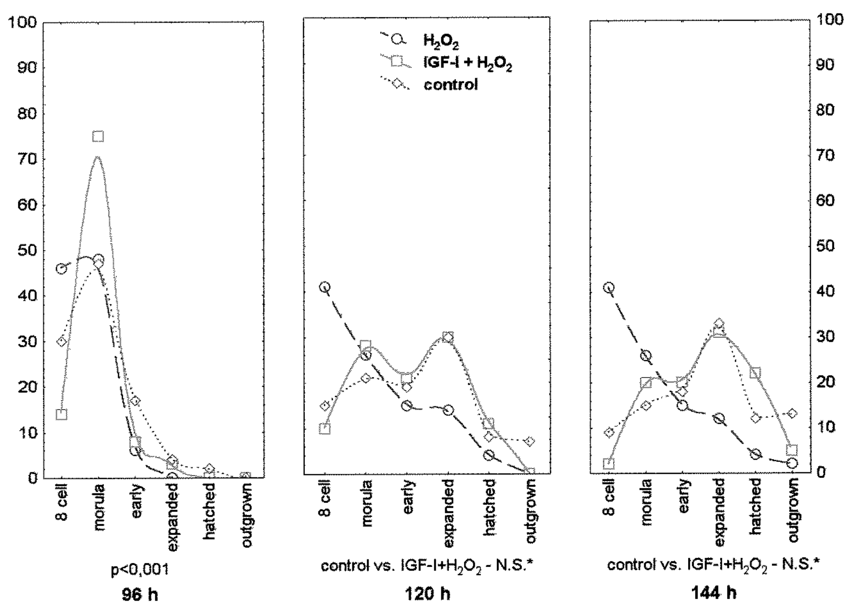

Fig. 2. Frequency distribution of preimplantation embryos (No) cultured in the absence (control group) or presence of H_2O_2 (0.1 mM) or H_2O_2+IGF-I (10^{-7} g/ml) for 96, 120 and 144 h after eCG treatment (n=100 per group). Data represent counts of embryo stages with the fitted least squares curve.* remaining interactions: $p<0.001$

Insulin-like growth factor II

As in the previous group, IGF-II also improved culture conditions deteriorated by H_2O_2. This was pronounced by a higher percentage of blastocysts in IGF-II+H_2O_2 media compared to H_2O_2 alone. The development of control and IGF-II+H_2O_2 embryos did not differ. TBC and ICM cell numbers were significantly higher in control and IGF-II+H_2O_2 media when compared to H_2O_2 (tab. 3, fig. 3).

Tab. 3. Total blastocyst counts (BC), hatched blastocyst counts (HBC), total blastocyst cell counts (TBC), inner cell mass cell counts (ICM) observed 96 h or 120 h after eCG administration in control, H_2O_2- treated and H_2O_2 + IGF-II-treated group. Median (lower – upper quartile)

Group	BC 96h	BC 120h	HBC 120h	TBC 120h	ICM 120h	n
Control	$23^{A,B}$ (15 – 26)	59^{A} (33 – 80)	20^{A} (17 – 50)	55^{A} (52 – 56)	16^{A} (14 – 17)	8
0.1 mM H_2O_2	0^{A} (0 – 0)	$10^{A,C}$ (0 – 28)	$0^{A,C}$ (0 – 0)	$43^{*A,C}$ (36 –51)	$10^{*A,C}$ (7 – 13)	8
0.1 mM H_2O_2+ IGF-II	11^{B} (0 – 35)	63^{C} (40 – 80)	20^{C} (0 – 23)	53^{C} (51 – 57)	14^{C} (14 – 16)	8

$^{A}p<0.05$ – control vs. H_2O_2
$^{B}p<0.05$ – control vs. H_2O_2 + IGF-II
$^{C}p<0.05$ – H_2O_2 vs. H_2O_2 + IGF-II
*n=4

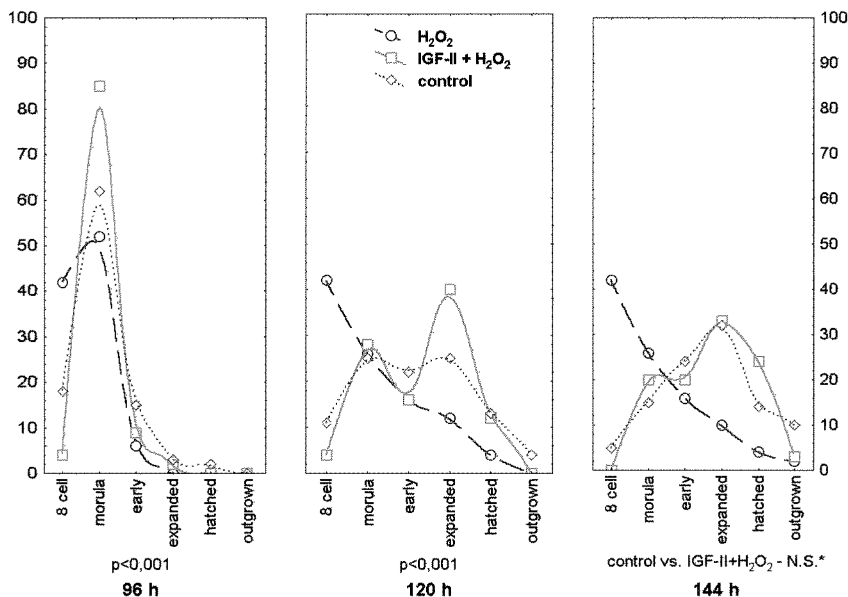

Fig. 3. Frequency distribution of preimplantation embryos (No) cultured in the absence (control group) or presence of H_2O_2 (0.1 mM) or H_2O_2+IGF-II (10^{-7} g/ml) for 96, 120 and 144 h after eCG treatment (n=100 per group). Data represent counts of embryo stages with the fitted least squares curve.* remaining interactions: $p<0.001$

Insulin, transferrin and selenium

The mixture of insulin, transferrin and selenium also had a positive effect on cultured pre-implantation embryos. In media with ITS+H_2O_2, the numbers of hatched blastocysts were similar to those of the control group and significantly higher than in the group with only H_2O_2. Similarly, embryos from the same groups had more cells in ICM. Embryos developed less dynamically in ITS+H_2O_2 media than in the controls, but significantly better than in the H_2O_2 group (tab. 4, fig. 4).

Tab. 4. Total blastocyst counts (BC), hatched blastocyst counts (HBC), total blastocyst cell counts (TBC), inner cell mass cell counts (ICM), observed 96 h or 120 h after eCG administration in control, H_2O_2- treated and H_2O_2 + ITS (insulin+transferring+selenium)-treated group. Median (lower – upper quartile)

Group	BC 96h	BC 120h	HBC 120h	TBC 120h	ICM 120h	n
Control	25^{A} (25 – 33)	65^{A} (58 – 75)	25^{A} (0 – 63)	55^{A} (47 – 68)	15^{A} (12 – 20)	8
0.1 mM H_2O_2	0^{A} (0 – 5)	$30^{A,C}$ (15 – 50)	$0^{A,C}$ (0 – 0)	$40^{*A,C}$ (36 – 45)	$9^{*A,C}$ (8 – 11)	8
0.1 mM H_2O_2+ insulin+trans- ferrin+selenium	20 (0 – 20)	70^{C} (40 – 85)	20^{C} (20 – 45)	57^{C} (52 – 59)	16^{C} (14 – 17)	8

$^{A}p<0.05$ – control vs. H_2O_2
$^{B}p<0.05$ – control vs. H_2O_2 + ITS
$^{C}p<0.05$ – H_2O_2 vs. H_2O_2 + ITS
*n=7

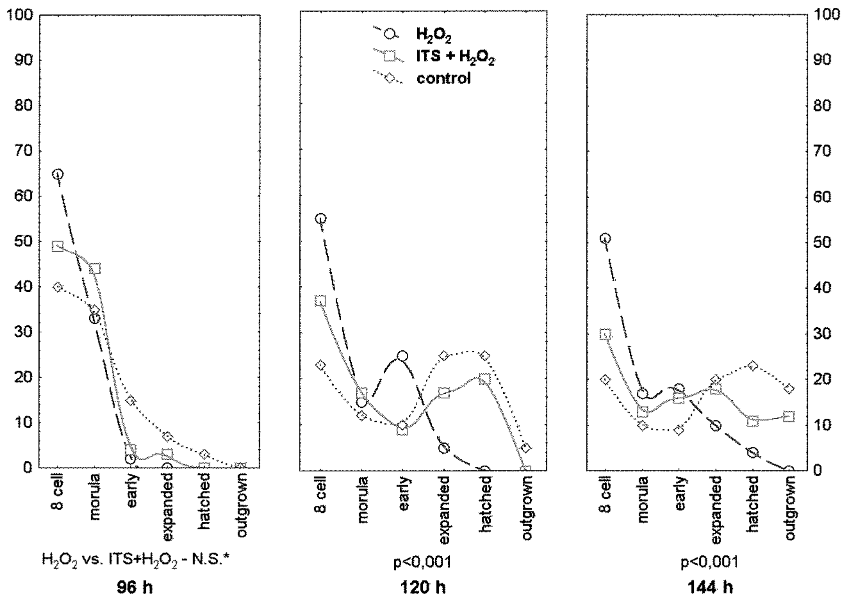

Fig. 4. Frequency distribution of preimplantation embryos (No) cultured in the absence (control group) or presence of H_2O_2 (0.1 mM) or H_2O_2+ITS (insulin+transferrin+selenium: details in the text) for 96, 120 and 144 h after eCG treatment (n=100 per group). Data represent counts of embryo stages with the fitted least squares curve.* remaining interactions: $p<0.001$

Staining for dead and living cells

In medium with only hydrogen peroxide there were numerous dead cells observed. Supplementation with EGF, IGF-I or IGF-II improved the viability of the embryos as only a few non-viable cells were noticed. The same was the case in the control group.

TUNEL

The positive TUNEL reactions were only sporadically found in embryos cultured with hydrogen peroxide supplemented with EGF, IGF-I or IGF-II. A similar observation was made in the control group. In contrast, the TUNEL reaction was observed in the majority of the cells the hydrogen peroxide group (fig. 5C).

DISCUSSION

There are numerous reports on *in vitro* pre-implantation embryo development [1, 4, 5, 10, 12, 13, 15, 19, 29, 30, 40, 41, 42, 44, 46, 49, 53, 58, 60, 63, 65, 70, 76]. In many cases, the studies were focused on factors influencing the quality of the cultured embryos, which is important in terms of efficacy of IVF-ET. IVF-ET still remains a relatively ineffective procedure with pregnancy rates limited by low implantation rates of transferred embryos. Both clinical and laboratory strategy in IVF should focus on the culture of the most suitable blastocysts for implantation. However, current results of blastocyst culture are far away from embryological expectations.

Since the metabolism of human embryos is similar to the metabolism of mouse embryos [59, 75], the latter embryos were used in our study. This also explains why mouse embryos are so widely used in biological and medical laboratories [75]. This study is one of the first to comprehensively evaluate the effects of growth factors on the *in vitro* culture outcomes by means of joint criteria involving basic embryological data, differential staining as well as staining for cells viability and apoptosis. An experimental model was established to evaluate whether the tested growth factors may compensate for the unfavorable culture milieu of oxidative stress created by the addition of hydrogen peroxide. Hydrogen peroxide is a donor of free oxygen species, also generated in routine *in vitro* embryo cultures [23, 52, 55]. The use of several independent methods enables a more precise evaluation of embryo

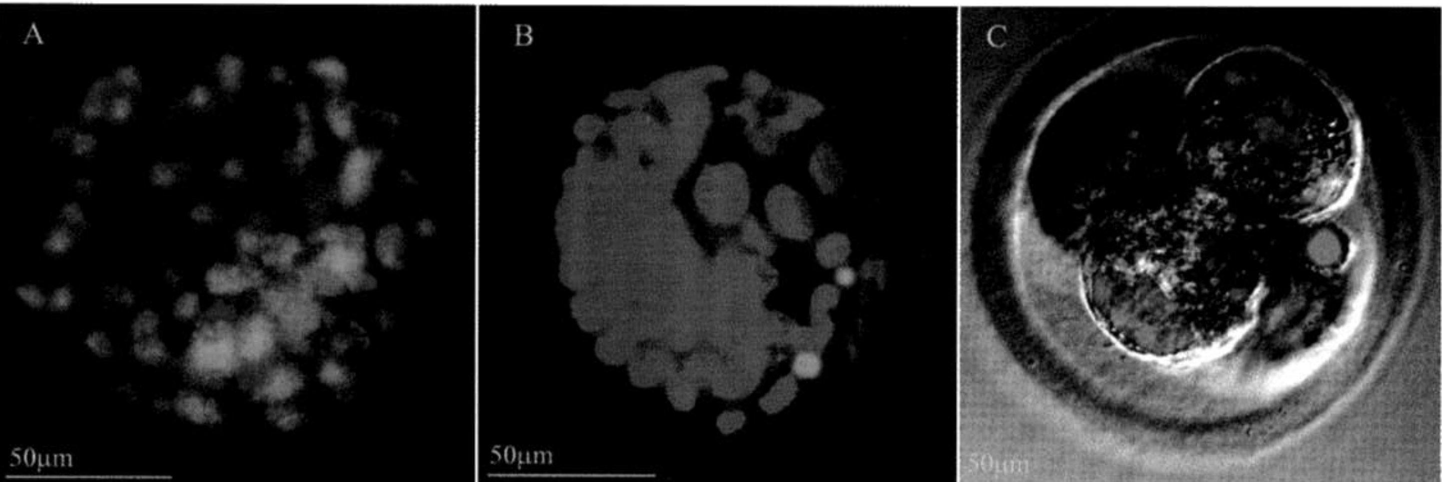

Fig. 5. Staining methods used to evaluate embyos quality. A/ Differential staining with calcium ionophore, Hoechst 33342 and propidium iodide: blue – inner cell mass; red – trophoectoderm; a control blastocyst (fluorescence microscope). B/ Staining of live and dead cells with Hoechst 33342 and propidium iodide: blue – live cells; red – dead cells; a control blastocyst (fluorescence microscope). C. Staining for apoptosis (TUNEL): green (fluorescein) – apoptotic cells. A four cell embryo cultured with 0.1 mM H_2O_2 (laser scanning confocal microscope with difference-interference contrast).

quality. So far, most of the studies were based on the assessment of a single embryological parameter [14, 29, 30, 45, 51]. In fewer experiments the number of blastocyst cells and ICM (differential staining), apoptosis and viability of embryos were assessed – but often separately [11, 26, 27, 28, 30, 32, 55, 57, 66, 67, 71, 72].

Simple embryological data like blastulation and hatching rates following culture in the tested media, although uncomplicated, are not satisfactory parameters to evaluate embryo quality. Nevertheless, this study also confirmed better blastulation, hatching and dynamics of embryo growth in media containing IGF-I, IGF-II, EGF or ITS. The tested factors seemed to minimize the consequences of oxidative stress. Similar results of experiments performed in standard conditions were also published in some previous reports [4, 7, 27, 50, 54, 55, 66].

The beneficial effect of the tested growth factors as well as ITS was also documented by differential staining. The embryos exposed to oxidative stress, cultured with IGF-I, IGF-II, EGF or ITS had more cells including those present in ICM. As to the number of cells, particularly that of ICM, which are good, sensitive markers of embryo quality, it may be postulated that the tested growth factors and ITS minimize negative effects produced by reactive oxygen species upon the cultured embryos. The applied differential staining method with calcium ionophore proposed by De la Fuente and King is easy and fast [11]. Since the cellular effects of the ionophore are dynamic, blastocyst assessment is possible using a strictly set incubation time. Another parameter like viability should be applied to make the hypothesis of anti-oxidative action of the tested growth factors more convincing.

Hydrogen peroxide may induce apoptosis and necrosis of cells and the effect is dose-dependent. However, in embryos cultured with hydrogen peroxide and IGF-I, IGF-II or EGF the TUNEL reaction was only sporadically positive, compared to the frequent positive reaction seen in embryos exposed to hydrogen peroxide alone. Even if the embryos treated with hydrogen peroxide appeared to have a normal morphology, the TUNEL reaction was often positive. The evaluation of the embryos cultured in control media as well as those containing the tested factors only sporadically revealed non-viable cells, which contrasted to cultures without growth factors. In embryos cultured with only hydrogen peroxide alone significantly more dead cells were detected.

The results suggest that the supplementation of the media with IGF-I, IGF-II, EGF or ITS has a positive effect on the development of embryos cultured in unfavorable conditions. It is well documented that the addition

of IGF-I, IGF-II, EGF or ITS to the culture medium increases the number of blastocyst cells and ICM and the dynamics of embryo development. The factors investigated exerted embryotrophic influence also in normal, optimal culture conditions [25, 29, 30, 37, 43]. However, there are also conflicting reports. Chi et al. [8], for instance, reported previously the unfavorable influence of IGF-I on blastocyst development, suggesting that long-lasting IGF-I stimulation might reduce the sensitivity of its receptors. There is also no consensus concerning the effect of EGF [21], although the majority of reports suggest its positive effects. EGF seems to stimulate metabolism (increased structural proteins and nutrients uptake) and the growth of the embryo [44, 61, 73]. Our results document that IGF-I, IGF-II, EGF and ITS have a potential to compensate or prevent consequences of oxidative stress *in vitro*. Although the mechanism is unclear, the tested factors showed the ability to prevent the apoptosis of embryo cells leading to improved embryo development. Similar conclusions were also proposed by Hardy and Spanos [27, 66]. To a certain degree, apoptosis is a physiological process responsible for formation of the blastocyst cavity [57]. It is also a result of an adverse influence of external *in vivo* and *in vitro* environment. Pre-implantation embryo development depends on a balance between growth promoting and inhibiting factors; the disruption of the balance can result in apoptosis and subsequent embryo demise.

The addition of growth factors or antioxidants to the culture medium may have practical implications. It was confirmed that the factors evaluated in the study prevent apoptosis and cell degeneration. It seems that embryos cultured in the media enriched with these agents may be better prepared for implantation. This may subsequently improve outcomes of assisted reproduction.

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