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Growth factors protect *in vitro* cultured embryos from the consequences of oxidative stress

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Summary

The aim of the study was to evaluate the effect of insulin-like growth factors (IGF1 and IGF2), stem cell factor (SCF) and epidermal growth factor (EGF) on the development of embryos exposed to oxidative stress. C3B6F1 female mice were stimulated with 5 IU of pregnant mare serum gonadotropin and 5 IU of equine chorionic gonadotropin (eCG). Two-cell embryos were flushed out from the fallopian tubes 40 h after eCG administration and mating with DBA males. In each experiment embryos were divided into three groups and cultured in (1) control medium, (2) control medium with 0.1 mM hydrogen peroxide and (3) control medium with hydrogen peroxide and separately with IGF1, IGF2, SCF or EGF in concentrations of 1 ng/ml, 10 ng/ml and 100 ng/ml. Under phase-contrast microscopy, 8-cell and compacted embryos, and early, expanded, hatched and outgrown blastocysts were counted at 24 h. The total blastocyst (TB) and inner cell mass (ICM) cell numbers were established by differential staining. Blastocyst cell viability was examined under fluorescence microscopy. To detect apoptosis, TUNEL was performed and visualized under a laser scanning confocal microscope. Hydrogen peroxide decreased embryo growth, blastocyst rates, blastocyst cell viability as well as TB and ICM counts. The TUNEL reaction revealed significantly more apoptotic cells in oxidative stress conditions. Tested factors revealed a varying extent of protective activity against oxidative stress caused by hydrogen peroxide. In media containing hydrogen peroxide and one of the four tested factors (IGF1, IGF2, SCF or EGF) the embryos developed faster than in media with hydrogen peroxide alone. IGF1, IGF2 and EGF increased both TB and (or) ICM counts in embryos exposed to hydrogen peroxide. All tested factors reduced the number of apoptotic cells (TUNEL) in embryos exposed to hydrogen peroxide.

Keywords: Embryo, Growth factors, *In vitro* culture, Oxidative stress

Introduction

Culture of embryos to blastocyst stage in *in vitro* fertilization (IVF) programmes has become a procedure of increasing importance in recent years, though the utilization of this technique is still limited by the costs of culture media and the relatively low rates of embryos reaching the blastocyst stage (Desai *et al*., 2000; Gardner *et al*., 1998; Jones & Trounson, 1999). Embryos cultured *in vitro* are exposed to many potentially harmful factors and actually develop in suboptimal conditions which differ much from the environment of the female reproductive tract (Bavister, 2000). The typical external factors influencing *in vitro* embryo development are oscillations of temperature, pH and osmotic pressure. Also, the composition of the medium (the presence or lack of energy sources and growth promoting factors) and finally the presence of damaging substances such as free oxygen species (Moley *et al*., 1998) may play a role. The conditions within the oviduct and uterine cavity constitute the optimal environment with appropriate pH, temperature, electrolytes, and

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stable sources of nutrients and growth promoting agents (Brison & Schultz, 1998; Paria *et al*., 2000). A composition of the culture medium that mimics the natural environment, including growth factors, may be crucial for the success of IVF (Bavister, 2000; Desai *et al*., 2000).

Materials and methods

Experimental design

The aim of the study was to evaluate the effects of insulin-like growth factors (IGF1 and IGF2), epidermal growth factor (EGF) and stem cell factor (SCF) on mouse embryos exposed to oxidative stress. The following outcome measures were used to address this goal:

1. Numbers of embryos at different developmental stages in relation to time of culture evaluated by phase-contrast microscopy.
2. Inner cell mass (ICM) and total blastocyst (TB) cell number evaluated after 96 h of culture by fluorescence microscopy (differential staining).
3. Cell viability and screening for apoptosis in blastomeres evaluated after 96 h of culture by both fluorescence and confocal microscopy.

The experiments were approved by the local ethics committee.

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 equine chorionic gonadotropin (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 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 pyruvate (0.33 mM), sodium lactate (21.4 mM) and human serum albumin (4 g/l; HSA, Sigma, USA) – the medium most commonly used in human IVF laboratories. After an initial 6 h of incubation the embryos which passed the 2-cell block were transferred to:

1. control medium;
2. control medium with 0.1 mM hydrogen peroxide (Sigma, USA); or
3. control medium with 0.1 mM hydrogen peroxide enriched with 1 ng/ml, 10 ng/ml or 100 ng/ml of IGF1, IGF2, SCF or EGF (all Sigma, USA).

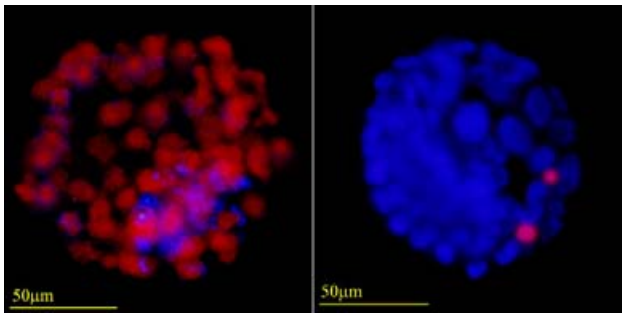

Figure 1 Staining methods used to evaluate embryo quality. Left panel: Differential staining (calcium ionophore, Hoechst 33342 and propidium iodide: blue, inner cell mass; red, trophoectoderm). Right panel: Staining of live and dead cells (Hoechst 33342 and propidium iodide: blue, live cells; red, dead cells). Control blastocyst (fluorescence microscopy).

The embryos from all groups were incubated (37 °C, 95% O_2, 5% CO_2) for a total 96 h, in groups of 10 in microdroplets of 50 µl covered with mineral oil (Sigma, USA).

The concentration of hydrogen peroxide which impaired embryo growth was established in separate preliminary experiments (see below).

Phase-contrast microscopy

The following stages of the embryos were observed at 24 h intervals: 8-cell embryos, compacted embryos, and early, expanded, hatched and outgrown blastocysts.

Fluorescence microscopy

Differential staining

Differential staining was used to determine the number of blastocyst cells and the ICM. The quality of blastocysts was verified before staining by phase-contrast microscopy. One blastocyst per group of 10 embryos (which had the best morphology in phase-contrast microscope) was used for the staining.

A modified method of De la Fuente & King (1997) was used for differential staining (Fig. 1). The 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 a fluorescence microscope (Axioscop, Zeiss, Germany) using a 345 nm ultraviolet light filter. The cells were counted by means of image analysis software (Microimage 4.0 Olympus, Japan).

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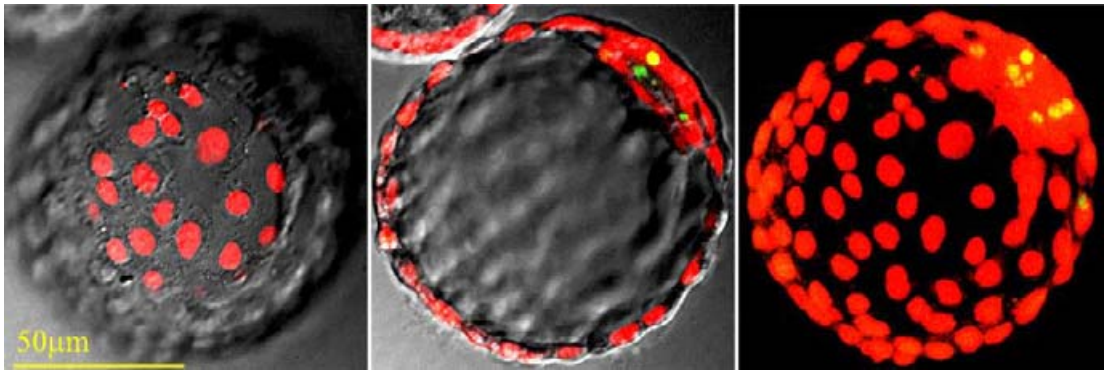

Figure 2 Embryos grown in media with 0.1 mM hydrogen peroxide and 10^{-7} g/ml SCF. TUNEL (fluorescein: green or yellow, apoptosis; additionally EthD-1: red, cell nuclei): a positive TUNEL reaction is seen in a few blastomeres, mainly of the inner cell mass. (1), (2) Sections through a blastocyst; (3) the same blastocyst after a three-dimensional reconstruction. Confocal microscopy with DIC.

Identification of dead and living cells
The procedure for the identification of living and dead cells 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 in the same conditions as above (Fig. 1).

Confocal microscopy

Identification of apoptosis: TUNEL
A TUNEL reaction (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick-End Labeling) was carried out (Promega, USA). The reaction identified fluorescein-labelled DNA fragments in apoptotic cells of embryos. Before staining, the embryos were treated with 2% formalin for 60 min, washed in PBS and incubated in 0.2% Triton X-100 for 5 min to perforate the 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. After the reaction was inhibited, all embryo nuclei were labelled with 4 μM EthD-1. Thereafter the embryos were washed in PBS, transferred to DPBS and evaluated by a laser scanning confocal microscope equipped with difference-interference contrast (Fluoview 300, Olympus, Japan) at the wavelength of 488 nm (Reynaud *et al.*, 2001; Byrne *et al.*, 1999) (Fig. 2).

Statistical analysis

Statistical analysis of blastocyst rates, TB and ICM cell counts of embryos cultured in the different media used was done by means of nonparametric analysis of variance (ANOVA).

The frequency distributions of preimplantation embryos cultured in the different media was compared by means of log-linear analysis. Calculations were done using Statistica for Windows (StatSoft, Tulsa, OK). Statistical significance was accepted at $p < 0.05$.

Results

Cultures of embryos in media with hydrogen peroxide

Hydrogen peroxide in the concentration range from 0.01 mM to 1 mM decreased the blastocyst rates and significantly reduced TB and ICM numbers. In media with 1 mM of hydrogen peroxide only a few embryos reached the blastocyst stage, which made statistical analysis imprudent (Fig. 3).

Cultures of embryos in media with hydrogen peroxide and growth factors

There were no significant effects of any of the tested growth factors at concentrations of 1 ng/ml and 10 ng/ml on *in vitro* growth of embryos exposed to 0.1 mM hydrogen peroxide.

All tested growth factors (EGF, IGF1, IGF2 and SCF) at the concentration of 100 ng/ml had a positive effect on embryos cultured with 0.1 mM hydrogen peroxide. The highest blastocyst rate and quality of embryos as measured by TB and ICM were found in control group, followed by the group with hydrogen peroxide supplemented with a growth factor and finally in the group with hydrogen peroxide alone (Fig. 2). In the case of SCF only an increase in the TB cell count was found, with no impact of growth factor on the ICM cell counts (Fig. 4).

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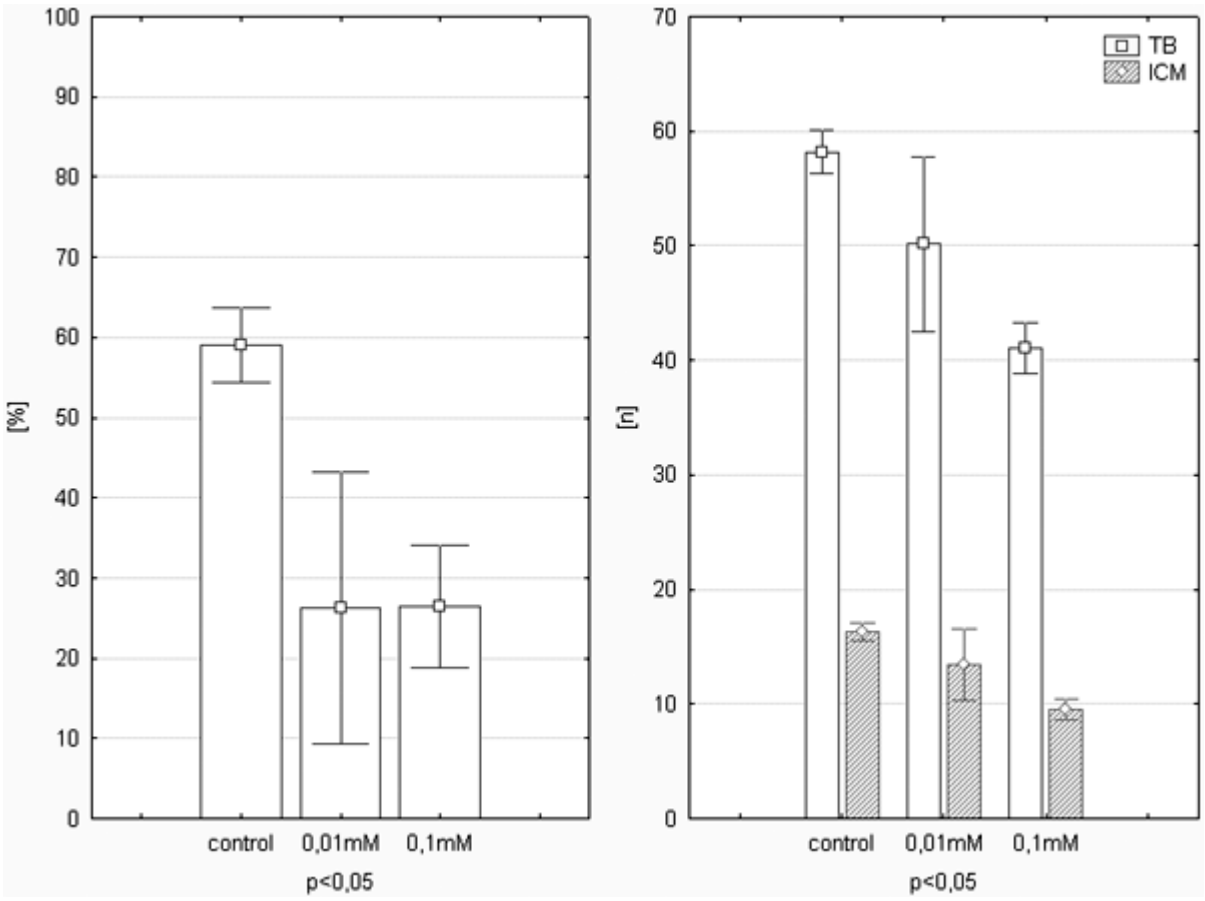

Figure 3 Blastocyst rates and cell counts following the preliminary embryo culture in media with tested levels of hydrogen peroxide. TB, total cell count of the blastocyst; ICM, cell count of the inner cell mass. Values are means (with 95% CI).

The blastocysts in media with hydrogen peroxide and growth factors had a cell viability which usually exceeded 90%. Also the TUNEL reaction was only sporadically positive, mainly among the ICM cells (Fig. 2). When all embryo stages were taken into account, the most dynamic growth pattern was found in the control media, followed by the media with hydrogen peroxide and growth factor supplementation, and finally in media with hydrogen peroxide alone. In the case of media supplemented with hydrogen peroxide and IGF1 or hydrogen peroxide and IGF2, the frequency distribution of embryos reached the distribution of control groups, indicating the same growth rate (Fig. 5).

Discussion

Current clinical and laboratory strategies in IVF focus on the optimum preparation of blastocysts for implantation (Gardner *et al*., 1996; Kim *et al*., 1999; Paria *et al*., 2000). Blastocyst transfer is associated with 40–60% implantation rates compared with 10–20% for the transfer of the 4- to 8-cell embryos (Gardner *et al*., 1998; Jones & Trounson, 1999). However, the culture of preimplantation embryos requires a strict definition of culture media.

Conditions of *in vitro* culture should be comparable to the *in vivo* environment. In *in vitro* culture embryos are exposed to the negative influence of environmental factors (temperature, components of the culture media and contamination) capable of inducing apoptosis and leading to the death of the embryo (Goto *et al*., 1993; Nasr Esfahani *et al*., 1992). Some positive effects of EGF, IGF1 and IGF2 on *in vitro* embryo development have already been reported; however, the conclusions of these studies very often were based on basic embryological data. Other studies provide evidence that some growth factors can suppress apoptosis and enhance embryo viability (Brison & Schultz, 1998; Hardy, 1999; Herrler *et al*., 1998; Pampfer, 2000).

Fallopian tube mucosa and endometrium are sources of cytokines and growth factors which are necessary for the proper development of embryos (Barmat *et al*., 1997; Kurachi *et al*., 1994; Sunder & Lenton, 2000). Epidermal growth factor (EGF), and insulin-like growth factor I (IGF1) and II (IGF2), have been reported to be mitogens inducing a positive effect on preimplantation

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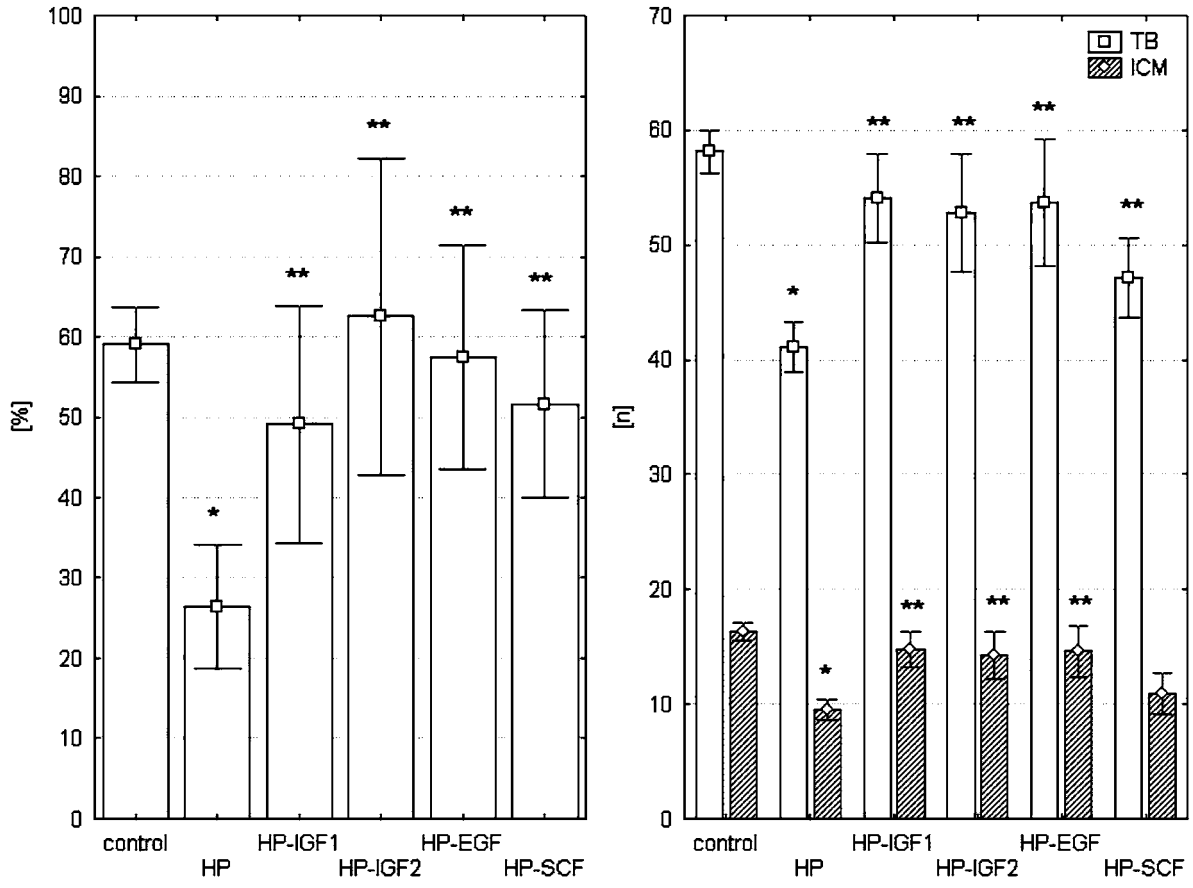

$^{*}p < 0.05$ versus control; $^{**}p < 0.05$ versus hydrogen peroxide (HP).

Figure 4 Blastocyst rates and cell counts following embryo culture in media with 0.1 mM hydrogen peroxide with 100 ng/ml of tested cytokines. Left panels: Blastocyst rates. Right panels: Cell counts. TB, total cell count of the blastocyst; ICM, cell count of the inner cell mass; HP, hydrogen peroxide. Values are means (with 95% CI).

development, and stimulating metabolism and growth of embryos. They increase the proliferation of embryo cells, including both the cells forming the ICM and the trophectoderm. Moreover, these factors are involved in the compaction and formation of the blastocyst, activate transport systems responsible for the uptake of glucose, enhance endocytosis and probably protein turnover, and influence the processes of replication, translation and degradation of proteins (Goldman & Gonen, 1998; Herrler *et al*., 1998; Kurzawa *et al*., 2001; Pantaleon *et al*., 2003; Pantaleon & Kaye, 1996). The other growth factor studied in our experiment is SCF. The c-kit (SCF receptor) mRNA has been reported to be expressed within mouse as well as human preimplantation embryos (Arceci *et al*., 1992, Sharkey *et al*., 1995). On the other hand, SCF is a proven survival factor for several types of cells including primordial germ cells (PGC) and haemopoietic progenitor and precursor cells (Ashman, 1999; Smith *et al*., 2001; Matsui *et al*., 1991; Godin *et al*., 1991). However, the effect of SCF on preimplantation development has not been well investigated yet.

Preimplantation embryo development *in vitro* is still a subject of great interest, mostly because of its association with assisted reproduction programmes (Bavister, 2000; Desai *et al*., 1999; Kim *et al*., 1999; Martin, 1998). Many reports concerning this problem have focused on factors influencing the quality of the cultured embryos, which is important in terms of the efficacy of IVF-ET. Nevertheless, IVF-ET still remains a relatively ineffective procedure, with pregnancy rates limited by low implantation rates of transferred embryos. Both clinical and laboratory strategies in IVF should focus on the culture of the most suitable blastocysts for implantation. However, current results of blastocyst culture are far removed from embryological expectations.

Since the metabolism of human embryos is to certain degree comparable to the metabolism of mouse embryos (Quinn & Horstman, 1998; Weiss *et al*., 1992), the latter were used in our study. IVF media for human embryo culture or embryo manipulation are usually tested on mouse embryos. For this reason in our experiments EBSS was used as the basic culture

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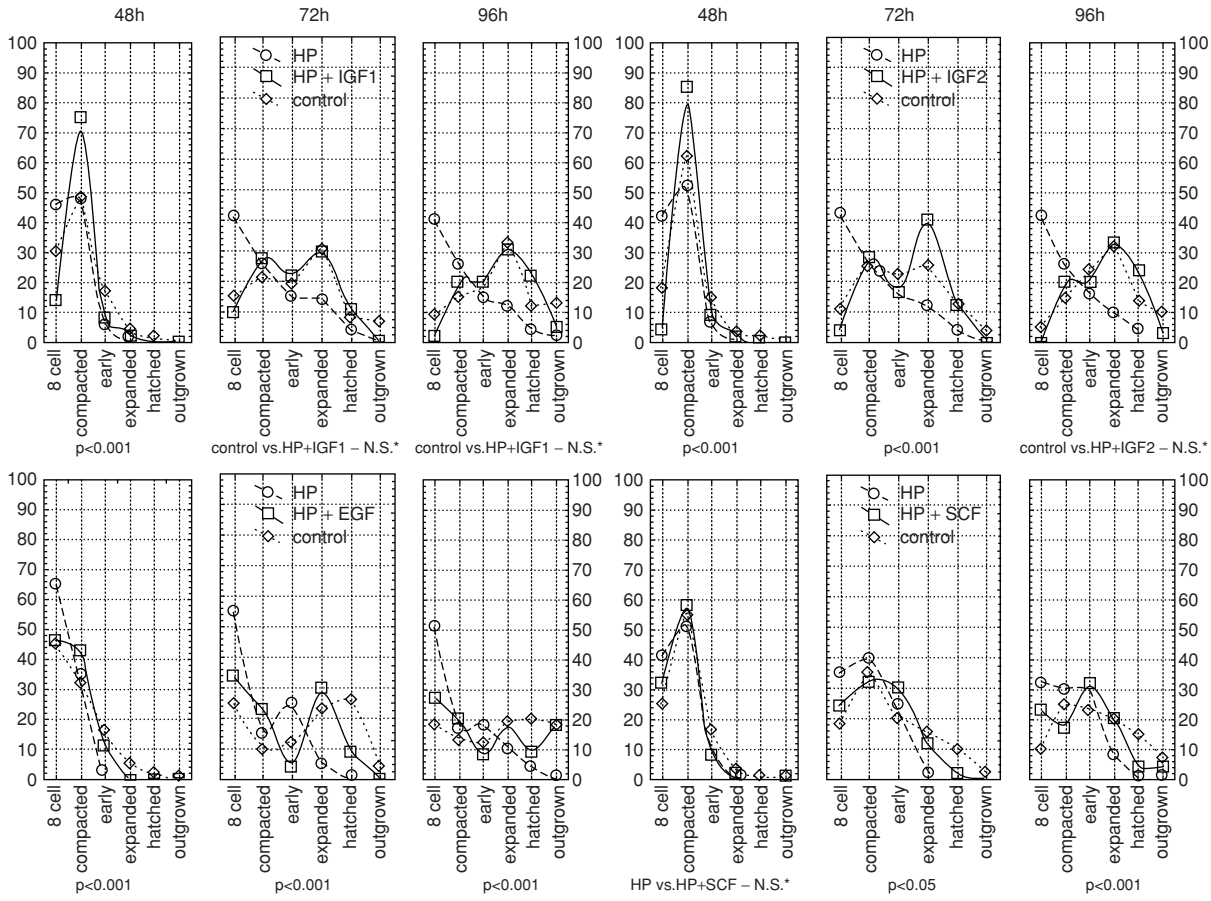

Figure 5 Frequency distribution of embryos cultured in control medium, medium with 0.1 mM hydrogen peroxide (HP) and 0.1 mM HP supplemented with 100 ng/ml EGF, IGF1, IGF2 or SCF in relation to time (96, 120 and 144 h following eCG). Data represent counts of embryo stages with the fitted least squares curve. The experiment was done on 300 embryos to test the effect of one factor.

medium. According to the manufacturers' information supplied with commercially available EBSS-based media, more than 80% of cultured control mouse embryos reached the blastocyst stage. Such good results suggest that testing of new potential media components should be carried out in suboptimal conditions. Thus in our experiments detrimental conditions were created to evaluate the influence of the growth factors. To this end we used several different methods of embryo assessment including basic embryological evaluation under phase-contrast microscopy, differential staining, as well as staining for cell viability and apoptosis. An experimental model was established to determine whether the tested growth factors could compensate for the unfavourable culture milieu of oxidative stress created by the addition of hydrogen peroxide.

Hydrogen peroxide is a donor of free oxygen species that may be spontaneously generated in routine *in vitro* embryo cultures (Goto *et al*., 1993; Nasr Esfahani *et al*., 1992; Pampfer, 2000). Extensive evidence has been reported that hydrogen peroxide either directly induces, or acts as a second messenger to induce, apoptosis in a variety of cells systems (Butke & Sandstrom, 1994; Hockenbery *et al*., 1993). At low concentration exogenous hydrogen peroxide induced an adaptive response of cells by promotion of synthesis of antioxidant enzymes and heat shock proteins. Hydrogen peroxide can cause either apoptosis or necrosis in cells exposed to it (Kim *et al*., 2000). Most cell types protect themselves against oxidative stress by activation of catalase, glutathione peroxidase and glutathione. However, in the mouse embryo the onset of catalase synthesis is at the blastocyst stage (Harvey *et al*., 1995) and they are protected against oxidative stress by hypotaurine secreted by oviduct epithelial cells. On the other hand, glutathione and its peroxidase are synthesized by oocytes and also by cumulus cells during oogenesis (Guerin *et al*., 2002). Thus, preimplantation embryos developing *in vitro* are deprived of the protective mechanisms mentioned above.

It has previously been demonstrated that the addition of antioxidant solutions to *in vitro* culture

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systems for embryos stimulates development (Orsi & Leese, 2001). Several studies have demonstrated a beneficial effect of either antioxidants or metal ion chelators such as EDTA in culture media for mammalian embryos (Nasr Esfahani *et al*., 1992; Orsi & Leese, 2001), indicating that reactive oxygen species such as hydroxyl radicals damage the embryos.

Although oxidation impairs both nuclei and cytoplasm, the latter is more sensitive than nuclei to oxidative stress. It is suggested that cytoplasmic components, most likely mitochondria, play a central role in mediating both development and apoptotic cell death induced by oxidative stress in mouse zygotes (Liu, & Keefe, 2000).

In our study we treated the embryos with 0.1 mM hydrogen peroxide. The concentration of hydrogen peroxide was established in separate preliminary experiments. In a higher concentration of hydrogen peroxide (1 mM) embryo mortality reached nearly 100%. The apoptotic effect of hydrogen peroxide was visible at the concentration range from 0.01 mM to 0.1 mM. The effect on cells viability is dose-dependent, and in higher doses caused necrosis of embryo cells. These results are similar to the findings of Liu & Keefe (2000) as well as those of Mystkowska *et al*. (2001) and Takahashi *et al*. (2003).

The positive effect of antioxidants is well known, but the beneficial influence of growth factors on embryo development in an unfavourable milieu created by oxidative stress has not been well documented or explained. There are a number of hypotheses for the mechanisms by which the IGF1 survival pathway achieves the inhibition of apoptosis (e.g. transcriptional activation of Bcl-2, inhibition of caspases, upregulation of DNA repair enzymes) but the anti-apoptotic effect on the embryo cells is very poorly known. IGF1 and IGF2 showed an anti-apoptotic effect on mouse embryo cells when the apoptosis was induced by TNFα (Byrne *et al*., 2002a). IGF1 may play a role as an apoptotic survival factor in the early human embryo (Spanos *et al*., 2000).

The positive effect of EGF on embryos cultured in an unfavourable milieu is difficult to explain. As yet the possible anti-apoptotic action of EGF on embryo cells stressed with hydrogen peroxide has not been established by other authors. The embryos treated with hydrogen peroxide growing in the presence of IGF1, IGF2, EGF and SCF had better morphology, faster development and better blastocyst morphology than those growing without these factors. The concentration of IGF 1, IGF2, SCF and EGF was established in preliminary studies. The positive effect of these factors on embryo development was detected in the range from 1 ng/ml to 100 ng/ml. In this paper we documented a positive effect of these factors at a concentration of 100 ng/ml on embryos treated with hydrogen peroxide.

The beneficial effect of the tested growth factors was also documented by differential staining. Embryos exposed to oxidative stress that were cultured with IGF1, IGF2, EGF or SCF had more cells, including those present in the ICM (except SCF, which improved only TB cell counts). As to the numbers of cells, particularly that of the ICM, which are good, sensitive markers of embryo quality, it may be postulated that the tested growth factors minimize the negative effects produced by reactive oxygen species upon the cultured embryos. The differential staining method with calcium ionophore that we used, as proposed by De la Fuente & King (1997), is easy and fast. Since the cellular effects of the ionophore are dynamic, blastocyst assessment is possible using a strictly controlled incubation time.

Another parameter such as viability should be applied to make the hypothesis of an 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 IGF1, IGF2, SCF or EGF the TUNEL reaction was only sporadically positive, compared with 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 in their cells. In the staining for cell viability, the embryos cultured in control media and those cultured in media containing the tested factors only sporadically revealed non-viable cells, in contrast to cultures without growth factors. On the other hand, in embryos cultured with hydrogen peroxide alone, significantly more dead cells were detected.

The utilization of several independent methods enables a more precise evaluation of embryo quality. Most previous studies have been based on the assessment of a single embryological parameter (Desai *et al*., 2000; Herrler *et al*., 1998; Narula *et al*., 1996). In a few experiments the number of blastocyst cells and ICM cell number (differential staining), apoptosis and viability of embryos were assessed, but most often separately (Herrler *et al*., 1998; Pampfer, 2000; Spanos *et al*., 2000; Stojkovic *et al*., 1998; Van Soom *et al*., 1997).

Simple embryological data such as blastulation and hatching rates following culture in the tested media, although uncomplicated, are not satisfactory parameters for evaluating embryo quality. Nevertheless, this study also confirmed better blastulation, hatching and dynamics of embryo growth in media containing IGF1, IGF2, EGF or SCF. The tested factors seemed to minimize the consequences of oxidative stress. Similar effects but in standard conditions were also reported previously (Brison & Schultz, 1998; Goldman & Gonen, 1998; Morales *et al*., 1997; Spanos *et al*., 2000).

The results suggest that supplementation of media with IGFs, EGF or SCF has a positive effect on the development of embryos cultured in unfavourable conditions. It is well documented that the addition of IGF1, IGF2 or EGF to the culture medium increases the number of blastocyst cells and ICM cells and the dynamics of embryo development. The factors investigated exerted an embryotrophic influence also in normal, optimal culture conditions (Grupen *et al*., 1997; Herrler *et al*., 1998; Kowalik *et al*., 1999, Lighten *et al*., 1998). However, there are also conflicting reports. Chi *et al*. (2000), for instance, reported previously the unfavourable influence of IGF1 on blastocyst development, suggesting that long-lasting IGF1 stimulation might reduce the sensitivity of its receptors. There is also no consensus concerning the effect of EGF, although the majority of reports suggest it has positive effects (Kim *et al*., 1999; Terada *et al*., 1997). EGF seems to stimulate metabolism (increased structural proteins and nutrient uptake) and the growth of the embryo (Martin *et al*., 1998). The effect of SCF in this area has not been well studied. Our results document that IGF1, IGF2, EGF and SCF have a potential to compensate for or prevent the consequences of oxidative stress *in vitro*. Although the mechanism is unclear, the tested factors showed the ability to prevent the apoptosis of embryo cells and improved embryo development. Similar conclusions were also proposed by Hardy (1999) and Spanos *et al*. (2000). To a certain degree, apoptosis is a physiological process responsible for formation of the blastocyst cavity (Pierce, 1989). It is also a result of an adverse influence of the external *in vivo* and *in vitro* environment. Preimplantation embryo development depends on a balance between growth-promoting and growth-inhibiting factors. The disruption of the balance can result in apoptosis and subsequent embryo demise.

The addition of growth factors 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 media enriched with these agents may be better prepared for implantation. This may subsequently improve outcomes of assisted reproduction.

Acknowledgements

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