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Effect of pituitary adenylate cyclase-activating polypeptide supplementation, applied during or after vitrification on mouse embryo

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RESEARCH ARTICLE



ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide with widespread occurrence and diverse functions. It occurs in high levels in the gonads suggesting a potential central role in reproduction. The aim of our study was to assess the effect of PACAP treatment during embryo vitrification on the developmental rate and the expression of the heparin-binding EGF-like growth factor gene (*Hbegf*). Mouse embryos, obtained from superovulated females were allocated into the four treatment groups. In EM1 and EM2, the embryos were prepared for vitrification in an Equilibration Solution that was supplemented with 1 or 2 μ M PACAP1-38, respectively. The embryos in groups CM1 and CM2 were not treated prior to vitrification but were cultured in a medium supplemented with 1 or 2 μ M PACAP1-38 after thawing. The Vitrified Control group consisted of embryos vitrified and thawed then cultured without PACAP1-38 treatment. A non-vitrified, non-treated Fresh Control group was also used. After 24 h of culture, the developmental rate of the embryos, as well as the relative expression level of the *Hbegf* gene, as determined by qPCR, were compared among groups. Higher developmental rate and *Hbegf* gene expression level were found in the embryos treated with a higher concentration of PACAP. These results indicate that PACAP treatment has a beneficial effect on the survival and development of vitrified/thawed mouse embryos.

KEYWORDS

PACAP, embryo, vitrification, cryoprotectant, HB-EGF

INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide belonging to the vasoactive intestinal peptide (VIP)/secretin/glucagon superfamily (Shioda and Naka-machi, 2015), of which it is a highly conserved member. The amino acid sequences of PACAP in all the different vertebrates share 84–100% identity, suggesting that PACAP is involved in important physiological processes (Isaac and Sherwood, 2008). The peptide plays roles in vasodilation, immunomodulation, cytoprotection and regulation of gastrointestinal, cardiovascular, respiratory and reproductive processes (Vaudry et al., 2009). The lack of PACAP in knockout animals leads to several biochemical and pathophysiological alterations, including increased cellular stress, early aging and pathological rhythmic functions (Reglodi et al., 2018a).

PACAP has several functions in the reproductive system. Among others, it plays a role in spermatogenesis (Reglodi et al., 2018b), in uterine innervation (Podlasz and Wasowicz, 2021) and acts as a hormonal regulator of the ovarian cycle (Winters and Moore, 2020).

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In the ovary, PACAP mRNA, protein and PAC1 receptors are found in the follicular and corpus luteum granulosa and theca cells (Isaac and Sherwood, 2008). In the regulation of oocyte maturation, PACAP might cooperate with other factors, including the epidermal growth factor (EGF)-like factors amphiregulin, epiregulin and beta-cellulin (Canipari et al., 2016). Watanabe et al. (2016) have found that PACAP promotes fertilization. Furthermore, in cumulus-oocyte complexes, pretreated with PACAP at different concentrations ranging from 10 nM to 1 μ M, fertilization rates have increased in a dose-dependent manner (Tanii et al., 2011). In a study on PACAP and its role in embryo implantation in mice, it has been found that PACAP may have an important function at the beginning of gestation, specifically in implantation. PACAP null females, compared to wild-type female mice, have been found to have lower implantation rates, three times lower serum prolactin levels, lower serum progesterone levels and reduced live births (Isaac and Sherwood, 2008). In addition, PACAP may have a role in the secretion of placental hormones. In choriocarcinoma cells, PACAP has been found to cause a 12-fold increase in cAMP secretion. cAMP, in turn, stimulates the expression of the glycoprotein hormone alpha subunit in the placenta, which is a subunit present in the pituitary hormones LH, FSH and TSH, as well as human chorionic gonadotropin (Reglodi et al., 2012; Horvath et al., 2016).

In a recent study, we have shown positive effects of PACAP on mouse preimplantation embryo development (Török et al., 2018). Our results have revealed a connection between endometrial PACAP levels and embryo development. Furthermore, our group has found positive correlation between the mRNA levels of *Adcyap1* (the gene coding for PACAP) and *Hbegf* (the gene coding for the heparin binding EGF-like factor) (Somoskői et al., 2020). Since *Hbegf* reaches its expression peak 5–8 days after the ovulation in uterus epithelial cells, and it is also expressed by the embryonic trophoblast before the implantation (Hamatani et al., 2004), these results suggest that PACAP, together with HB-EGF, plays an important role in implantation (Somoskői et al., 2020). Previously, it has been shown that, the supplementation of the cryopreservation solution with PACAP in case of small-bowel autotransplantation led to amelioration of the small intestinal oxidative stress and structural lesions during the cold preservation/reperfusion injury (Ferencz et al., 2009). These results raise the possibility that PACAP might be protective in embryo vitrification. The aim of our present study was to assess the effect of PACAP treatment, applied prior to vitrification or after thawing, on mouse embryo developmental and implantation potential, estimated by measuring the relative expression level of the *Hbegf* gene.

MATERIALS AND METHODS

Animal housing, mating and embryo collection

Procedures with animals were performed following good veterinary practice established for animal welfare according to Hungarian national laws in force. The protocol of the

animal experiment was approved by the Food Chain Safety and Animal Health Directorate of Pest County's Government Office (PE/EA/1062-6/2021). Eight-week-old BDF1 (National Institute of Oncology, Budapest, Hungary) mice were kept under a 12 h light/12 h dark schedule at a temperature of 21 °C with 30% relative humidity in the air. Feed and drinking water were available *ad libitum*. Female mice were superovulated by i.p. injection of 7.5 IU equine serum gonadotropin (Folligon, Intervet, Germany) followed by 7.5 IU human chorionic gonadotropin i.p. (Veterin corion, Alvetra und Werfft, Austria) after 48 h. Then the females were placed together with mature males overnight.

Embryos were collected on Day 1 (E1.0) from the ampulla and washed in PBS + 20% FBS (Fetal bovine serum, Sigma-Aldrich, Canada), then they were cultured *in vitro* in G-1™ PLUS medium (Vitrolife, Sweden) for 72 h (37.5 °C, 6.5% CO₂, maximum humidity).

We obtained embryos from 23 females, of which a total of 347 embryos were vitrified after 72 h of *in vitro* culture. RNA extraction was performed on 204 embryos (30 per group) included Fresh Control group. Embryos were randomly allocated into the groups.

Treatment groups

Six groups were established. In the Fresh Control group, the embryos were cultured without any treatment and after Day 3 of culture they were placed in culture medium (CM) G-2™ PLUS (Vitrolife, Sweden) for 24 h. The Vitrified Control group contained embryos that were vitrified on Day 3 of culture using the vitrification protocol detailed below. The embryos were then thawed and cultured *in vitro* in CM without any treatment for 24 h. There were four PACAP-treated groups. In two of these (EM 1 and EM 2), the treatment was carried out during the preparation for vitrification, when the Equilibration Medium was supplemented with 1 or 2 μ M PACAP1-38. The full-length 38-amino acid peptide (PACAP-38) was synthesized at the Department of Medical Chemistry of the University of Szeged (Hungary) as described previously (Figueiredo et al., 2022). After thawing, the embryos were cultured for 24 h in CM without any supplementation. The embryos in the other two groups were treated only after thawing by supplementing the CM with 1 or 2 μ M PACAP1-38 (groups CM 1 and CM 2, respectively). The treatment groups are summarized in Table 1.

Table 1. Summary of treatments in the experimental groups

Group name	Vitrification	PACAP1-38 in EM	PACAP1-38 in CM
Fresh Control	–	–	–
Vitrified Control	✓	–	–
EM 1	✓	1 μ M	–
EM 2	✓	2 μ M	–
CM 1	✓	–	1 μ M
CM 2	✓	–	2 μ M

EM: equilibration medium; CM: culture medium



Vitrification

After 72 h of *in vitro* culture, the developmental stage and quality of embryos were examined under stereomicroscope and evaluated based on their morphology characteristics. Only grade 1 blastocysts were vitrified. First, the embryos were washed in Holding Medium i.e. HEPES-modified Medium 199 (Thermo Fisher Scientific, Waltham, MA, USA) + 20% foetal bovine serum (FBS), then incubated in EM (Equilibration Medium: Holding Medium + 7.5% ethylene glycol (EG) + 7.5% dimethyl sulfoxide (DMSO) (both Sigma-Aldrich, Canada) at room temperature for 3 min. Then, embryos were transferred into VM (Vitrification Medium: HEPES-modified Medium 199 + 1M sucrose + 20% FBS + 16.5% EG + 16.5% DMSO) for 20 s, collected into an open pulled straw and plunged into liquid nitrogen. Vitrified samples were stored for one week in liquid nitrogen.

Thawing and *in vitro* culture

According to the thawing protocol, embryos were washed in Holding Medium + 50% sucrose medium (SM) (HEPES-modified Medium 199 + 1M sucrose + 20% FBS) for 5 min, then the amount of SM was gradually reduced (Holding Medium + 25% SM) during further washes for another 5 min. Finally, the embryos were collected in Holding Medium. After the thawing, we cultured the embryos in CM for 24 h. After 24 h in culture, we recorded the developmental rate (number of re-expanded and further developed embryos divided by the total number of vitrified embryos expressed as a percentage) and determined the *Hbegf* gene expression by qPCR in each of the embryos, individually.

RNA extraction

The total RNA from the embryos was extracted using the column-based Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. In-column DNase treatment was applied to remove the residual DNA.

cDNA preparation and quantitative PCR

Reverse transcription of the RNA samples was carried out with oligoDT using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) using the manufacturer's instructions and primers. The PCR analysis was performed with FastStart Essential DNA Green Master (Roche Diagnostics, Basel, Switzerland). The mRNA expression levels of *Hbegf* were normalized to that of a house-keeping enzyme, namely the glyceraldehyde

3-phosphate dehydrogenase (*Gapdh*). Table 2 shows the nucleotide sequences of forward and reverse primers for both genes (*Gapdh* and *Hbegf*). The *Gapdh* primers have been published previously, whereas the primers for the *Hbegf* gene were designed using the SnapGene software (GSL Biotech LLC). The qPCR was carried out in a Roche LightCycler Nano real-time PCR system (Roche Diagnostics, Basel, Switzerland). The cycling conditions consisted of an initial denaturation and enzyme activation step at 95 °C for 2 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s and extension steps at 72 °C for 30 s. To evaluate the results, we normalized the obtained Threshold Cycle (Ct) values. For this, we used the following formula: $2^{\Delta Ct}$, where $\Delta Ct = Ct_{\text{housekeeping gene}} - Ct_{\text{tested gene}}$. Thus, the expression level of the *Hbegf* gene was determined relative to that of the *Gapdh* gene.

Statistical analysis

The data were analyzed with R v3.0.0 software (R Development Core Team). Difference of developmental rate in controls and treated groups were analyzed with Chi-squared test. Effect of PACAP on *Hbegf* expression levels was analyzed with Kruskal-Wallis rank sum test, with *post hoc* pairwise comparison using Wilcoxon rank sum test (p-adjustment method by Benjamini and Hochberg, 1995). Differences at a probability value of $P < 0.05$ were considered significant.

RESULTS

Effect of PACAP treatment on developmental rate of mouse embryos

As shown in Fig. 1, the developmental rate in group EM 2, was 90.9% (50/55), significantly higher compared to Vitrified Control group (68.8%, 44/64, $P = 0.0031$). It was also significantly higher than that in groups EM 1 (69.6%, 39/56, $P < 0.0001$), CM 1 (54.9%, 56/102, $P < 0.0001$) and CM 2 (68.6%, 48/70, $P = 0.0026$). No significant difference was found between the groups treated with PACAP after vitrification (CM 1 and CM 2).

Effect of PACAP treatment on the relative expression level of *Hbegf* gene

In the amount of specific mRNA levels, there was no significant difference between the Fresh and the Vitrified Control groups. No significant difference was seen in the EM 1 group either. We observed significantly higher relative

Table 2. Sequences of the PCR primers used for the detection of the genes of the enzyme glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and the heparin-binding EGF-like growth factor (*Hbegf*)

Gene	Forward	Reverse	Reference
Mouse <i>Gapdh</i>	GCTACACTGAGGACCAGGTTGT	CTCCTGTTATTATGGGGGTCTG	Xu et al. (2016)
<i>Hbegf</i>	CTGAAGGTTCTATAGCTCAGGTCCT	GAGAGACCCATGCCTCAGGAAATAC	Designed in house



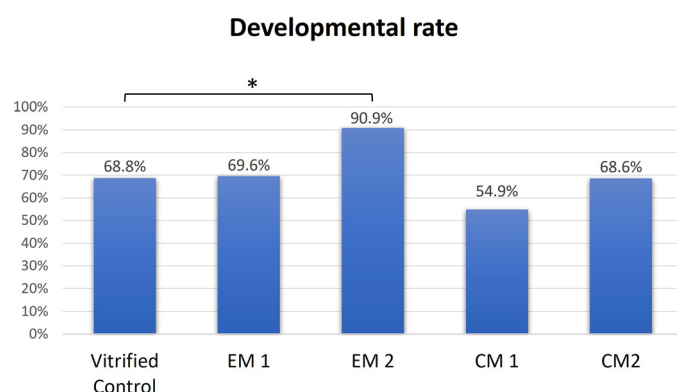


Fig. 1. Developmental rate of mouse embryos in the different treatment groups after vitrification and 24-h post-thaw *in vitro* culture. *Statistically significant difference ($P = 0.0031$)

Hbegf expression levels in embryos treated with higher dose (2 μ M) of PACAP during the preparation for vitrification (EM 2) compared to the embryos in the Fresh Control group ($P = 0.01265$). The relative expression level of *Hbegf* was significantly lower in embryos treated with PACAP during the *in vitro* culture after the vitrification (CM 1, CM 2) compared to the other groups, but there was no difference between the two PACAP doses (Fig. 2).

DISCUSSION

Our results revealed a higher rate of development in the group of mouse embryos treated with a higher dose of PACAP during vitrification. Cryopreservation induces oxidative stress in cells due to the harmful effect of the cryoprotective agents (CPAs). Ahn et al. (2002) have observed physical and chemical changes in frozen-thawed, two-cell mouse embryos, such as the destruction of the

cell membrane integrity, redistribution of actin fibers, mitochondrial depolarizations and increased production of reactive oxygen species (ROS), which may trigger the apoptotic cascade leading to a decrease in the survival rate and in the developmental rate of the embryos. Several other studies have revealed that oxidative stress is associated with early developmental impairment and embryo fragmentation and induces apoptosis in oocytes and early embryos (Takahashi et al., 2000; Dennerly, 2007; Takahashi, 2012). Dhali et al. (2007) have found lower blastocyst ratios in vitrified, as well as in non-vitrified but cryoprotectant-treated embryos compared to the control group. This suggests that the toxicity of CPAs and/or the dehydration and osmotic events significantly damage the embryos.

We found a significantly higher rate of post-thaw development in embryos vitrified using EM containing 2 μ M of PACAP compared to the Vitrified Control group. However, if PACAP was used after thawing during the *in vitro* culturing (CM 1, CM 2), no significant difference was found in the developmental rate compared to the Vitrified Control embryos. It can be assumed that the presence of PACAP during the 3-min incubation in EM primed the embryos to better withstand the damaging effects of cryopreservation and even brought about protective effects during the subsequent 24-h post-thaw *in vitro* culture. In contrast, the presence of PACAP in the CM resulted in no advantage. Our observation suggests that PACAP treatment has a beneficial effect on mouse embryo survival as it can reduce the harmful effects of the cryopreservation/vitrification process. This hypothesis is supported by several studies indicating that PACAP has a significant antiapoptotic effect as well as providing protection against oxidative stress and toxins (Lee et al., 1999; Morelli et al., 2008).

Vitrification can alter or modify gene expression and transcriptional activities. It has been observed that vitrification increases the expression levels of apoptotic genes. Majidi Gharenaz et al. (2016) has found a significantly higher *Bax* pro-apoptotic and significantly lower *Bcl-2* anti-apoptotic gene expression in re-vitrified embryos compared to fresh embryos. However, no significant difference has been observed between re-vitrified and vitrified groups. These results can be related to the experiences of the previously mentioned study, in which a strong relationship has been found between the compromised developmental competence and altered transcriptional activities of *Bax* and *Bcl-2* genes in the vitrified embryos (Dhali et al., 2007). Furthermore, Majidi Gharenaz et al. (2016) have observed a significantly lower level of *ErbB4* gene expression in re-vitrified embryos compared to fresh embryos, and similar levels have been found in vitrified embryos as in re-vitrified embryos. Interaction between *ErbB4* and HB-EGF mediates the attachment of blastocyst to the endometrium (Paria et al., 1993; Lim et al., 2006; Davidson and Coward, 2016). Signalling by HB-EGF back to the embryo, in turn, activates the program of trophoblast differentiation required for adhesive functions during subsequent attachment and invasion (Lim et al., 2006; Davidson and Coward, 2016). Connected with the expression of *Hbegf* in embryos

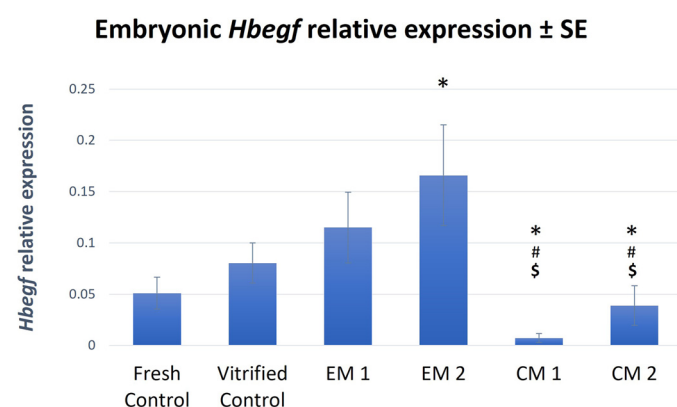


Fig. 2. Means (\pm SE) of the relative expression levels of *Hbegf* gene of embryos in each treatment group as compared to that of the gene of house-keeping enzyme glyceraldehyde 3-phosphate dehydrogenase. *, #, \$ mark significant differences ($P < 0.05$) compared to Fresh Control, Vitrified Control and during vitrification PACAP-treated groups, respectively



treated with high dose of PACAP during vitrification, Majidi Gharenaz et al. (2016) have found opposite result, since unlike us, they have observed low expression levels. Gazor et al. (2018) have found a negative effect of cryopreservation on epidermal growth factor receptor (*EGFR*) gene expression. This has been confirmed also by Riesco and Robles (2013). These data suggest that gene expression might change during the vitrification and thawing procedure. Their explanation is that cryopreservation affects the stability of mRNA and therefore some of them are susceptible to degradation. The regulation of some mRNAs involve translation inhibition. Reduction and even elimination of some transcripts as a result of cryopreservation, have also been observed as reported by García-Herrero et al. (2011). In the view of the fact that we found higher level of *Hbegf* expression in embryos treated with 2 μ M PACAP during vitrification compared to the Fresh Control group suggests that exogenous PACAP might protect the embryo from the previously mentioned damaging effect of cryopreservation. Shaw et al. (2012) have studied gene expression in fresh and frozen-thawed human preimplantation embryos. They have found significantly lower gene expression levels after thawing. Similarly, we also experienced significantly lower *Hbegf* expression levels in the groups of CM 1 and CM 2. Our data indicate that if the PACAP supplementation in the CM is used after freezing-thawing, PACAP is no longer able to exert the protective effect after the vitrification. Further studies are needed to find the explanation to this phenomenon.

It has been reported, that *Hbegf*, expressed by the implantation-competent blastocyst induces *Hbegf* expression in the uterine endometrium in a paracrine manner (Hamatani et al., 2004). The synthesized HB-EGF improves development of the embryo to the hatching blastocyst stage, promotes trophoblast outgrowth and regulates trophoblast activity during implantation (Lim et al., 2006). Furthermore, HB-EGF induces the uterine expression of *Ptgs2*, the gene of the prostaglandin-endoperoxide synthase. Lim et al. (1997) have found defective implantation and decidualization in *Ptgs2* KO mice. This suggests that the chance of implantation can be predicted from the expression level of this gene. Higher HB-EGF levels have been associated with higher implantation rates (Lim et al., 2006). Based on our research, higher expression levels were found in PACAP-treated embryos compared to fresh embryos, indicating that PACAP treatment prior to and during vitrification has a beneficial effect on *Hbegf* gene expression, thus on the probability of implantation in a dose-dependent manner.

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