Summary of PhD thesis

EFFECT OF PACAP (PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE) ON REPRODUCTION

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Budapest, 2024.

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1. Introduction

The pituitary adenylate cyclase-activating polypeptide (PACAP) is the most conservative member of the secretin/glucagon/VIP peptide superfamily in terms of nucleotide and amino acid length and sequence identity. Its strong conservation suggests that PACAP is involved in important physiological processes. It plays a role in regulation of cell division in different neuronal and non-neuronal cell types. It controls cell cycle arrest, differentiation and is also involved in the protection of cells against apoptosis and in the induction of apoptosis.

PACAP is widely located in several brain regions and peripheral organs. It is widely expressed in the gastrointestinal tract, where it is involved, among other things, in the recovery from inflammatory conditions and neuronal damage, as well as in the regulation of intoxication and neoplastic processes. It is found in the pancreas, stimulating the release of insulin.

PACAP and its receptors have been described to be widely localized in the kidney and lower urinary tract, where it plays a role in urinary excretion, blood supply, hormone production and the regulation of inflammatory processes.

Its role in central cardiorespiratory regulation is not yet clearly understood. It is found in the heart and aorta. It also has receptors in blood vessels, including arteries, arterioles and capillaries. It has also been observed in the airways, from the smooth muscle bundles of the trachea, through the veins, to the bronchioles and plays a critical role in the regulation of neonatal respiration.

Over the past 30 years of PACAP research, it has been observed that the peptide affects fertility and reproduction on several levels. In primary pituitary cell culture, it has been observed to regulate gonadotropin production, both directly and indirectly. It enhances the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by pituitary cells stimulated with GnRH. Furthermore, the peptide and its receptors are widely and stage-specifically expressed in the gonads, and their presence has been demonstrated in several other reproductive organs. It has been found that gonadotropins induce the induction of PACAP mRNA in granulosa cells of preovulatory follicles, which suppresses follicular cell apoptosis and stimulates progesterone production in preovulatory follicles, thereby stimulating luteinization. GnRH regulates PACAP gene expression in a stage-dependent manner. While GnRH inhibited

PACAP mRNA levels in granulosa cells of immature follicles, it stimulated them in granulosa cells of preovulatory follicles. Therefore, PACAP expression is differentially regulated during the cycle and plays an important role in the regulation of preantral follicle growth and differentiation.

The lack of endogenous PACAP has a significant impact on fertility and reproduction. Studies in PACAP gene-deficient (KO) mice have shown lower reproductive rates. No differences were found in ovulation, ovarian histology or the presence of a seminal plug to confirm mating, but significant abnormalities were found in implantation and associated hormone levels and offspring mortality rates.

PACAP is found in high concentrations in the testes. It has stage-specific suppressive effects on the proliferation of immature Leydig cells and stimulates their testosterone secretion in a dose-dependent manner. Its presence in the acrosomes of epididymal spermatozoa has been demonstrated, suggesting a role for acrosomal PACAP in fertilization. Furthermore, a PACAP-specific receptor was observed in the outer layer of the cumulus cells, and its localization suggests that PACAP-mediated interaction between spermatozoa and cumulus cells

occurs at this site, indicating a role in fertilization during passage through the cumulus layer.

2. Aims of the study

The objectives of our study were as follows:

Study of the effect of endogenous PACAP

1. on the female cycle *(Experiment 1)*
2. on the development of preimplantation embryos and the potential for implantation *(Experiments 2 - 3)*
3. on sperm motility and morphology *(Experiment 4)*

In addition, we hypothesise that externally supplied PACAP may have a beneficial effect on the success of several assisted reproductive techniques, and therefore we aim to investigate this in the second half of our study.

Investigation of the effect of exogenous PACAP

1. during *in vitro* embryo culture *(Experiment 5)*
2. as a cell-protective substance during the vitrification process of the embryos *(Experiments 6 and 7)*
3. during cryopreservation of spermatozoa *(Experiment 8)*

3. Materials and methods

Animals used in the study

Due to the conservation of PACAP and the 100% identity of the human and mouse PACAP38 sequences, as well as the identity of the developmental stages and the cell lineages formed in preimplantation embryo development, the mouse was chosen as the model animal for the majority of our studies. The studies were conducted on 10- to 12-week-old BDF-1 mice, 10- to 12-week-old wild-type mice (PACAP with control) and PACAP KO mice of the CD1 mouse strain. The latter were provided by the Department of Anatomy, University of Pécs. The animals were housed in the experimental animal house of the Department of Obstetrics and Food Animal Medicine Clinic, University of Veterinary Medicine, Budapest, at a temperature of 21°C, a humidity of 65%, and a 12-hour dark/12-hour light schedule. The housing conditions of the animals comply with current legislation (PE/EA/1101-7/2017; PE/EA/1062-6/2021).

In order to assess the efficacy of exogenous PACAP in deep cryopreservation of spermatozoa, due to the higher volume and less invasive intervention, and the justification for gene conservation in endangered breeds,

we utilised sperm from the epididymis of dogs, for which the necessary samples were obtained from the shelter dog neutering programme of the clinic of the Department of Obstetrics.

Methods

Experiment 1: Cycle diagnostics were performed to investigate the effect on the female cycle. Radioimmunoassay technique was used to measure daily levels of estradiol and progesterone using faeces samples from CD1 wild-type and PACAP KO mice.

Experiment 2*:* The impact of endogenous PACAP on the development of preimplantation embryos was investigated in CD1 mouse strain wild-type and PACAP KO females, following superovulation (7.5 IU eCG intraperitoneally and 7.5 IU hCG after 48 h) and mating. After 96 hours of *in vitro* culture, the micronucleus ratio (chromosome fragments) of blastocyst-developed embryos was determined by SYBR14 fluorescent staining. Its high presence was found to be associated with a reduced probability of successful implantation and developmental arrest.

Experiment 3: To investigate the effect of endogenous PACAP on implantation and embryo

development, the expression levels of *Adcyap1* (gene encoding PACAP) and *Hbegf* (gene encoding heparin binding epidermal growth factor-like growth factor) were measured with qPCR in uterine tissue of pseudopregnant and pregnant BDF1 females and blastocyst embryos. Thus, we investigated whether the level of uterine tissue expression depends on the presence of embryos (pseudopregnant vs. pregnant female), as well as the developmental level of embryos with each uterine tissue expression and the correlation between the expression of the two genes tested.

Experiment 4: To investigate the effect of endogenous PACAP on male reproduction, the motility of spermatozoa obtained from the epididymis of CD1 wild-type and PACAP KO mice was measured using CASA and morphological differences were determined by Spermac staining.

Experiment 5: The effect of exogenous PACAP in *in vitro* culture on the development of preimplantation embryos was investigated using BDF1 mouse strain. After superovulation and mating, the obtained embryos were cultured in G-1 ^{TM} PLUS media supplemented with 0 μM [control group], 4 μM, 6 μM, 8 μM and 10 μM PACAP.

After 96 h, the quality of the embryos (micronucleus ratio) was determined using SYBR14 fluorescent stain.

Experiment 6: To investigate the use of PACAP during vitrification, embryos from BDF1 superovulated females were vitrified and further development was determined after 24 h *in vitro* culture following thawing. 1 μM and 2 μM PACAP supplementation was used in two different steps. On the one hand, during vitrification preparation, equilibration medium (EM) was supplemented with 1 or 2 μM PACAP (EM1, EM2) and then cultured in culture medium (TM) for 24 h after thawing without supplementation. On the other hand, embryos were vitrified without PACAP treatment and cultured *in vitro* in culture medium supplemented with 1 or 2 μM PACAP for 24 h after thawing (TM1, TM2). For the control group, embryos were vitrified and *in vitro* cultured without supplementation.

Experiment 7: To investigate the effect of vitrification on the implantation potential after vitrification, the expression levels of *Hbegf* were measured by qPCR in vitrified and cultured embryos as described in the previous experiment. A fresh control group was added to our study.

Experiment 8: The use of exogenous PACAP during cryopreservation of spermatozoa was investigated using canine epididymal spermatozoa due to the higher volume and less invasive procedure. The sperm-rich solution was divided into 4 parts and 0 µM (control group), 2 µM, 4 µM, 8 µM PACAP was added to the solution according to the treatment group and cryopreservation was performed according to the manufacturer's protocol. CASA was used for sperm motility analysis and Spermac stain was used for morphological analysis.

4. Results and discussion

Studies of the effects of endogenous PACAP

In our study of the estrus cycle in females, we found no significant difference between estradiol and progesterone production in wild-type and PACAP KO mice, which is consistent with the observation in vaginal smears reported by Isaac and Sherwood in 2008. In their study, they also found a reduced number of implanted embryos in PACAP KO females. In our case, we observed a higher micronucleus ratio in the gene-deficient embryos as a consequence of PACAP on preimplantation embryo development. Since its increased presence was associated with poor implantation ability, our observation may support and explain Isaac and Sherwood's hypothesis that PACAP may play a role in implantation processes.

Gene expression measurements on day 4 of pregnancy showed significantly higher relative expression of *Adcyap1* and *Hbegf* in the uterine tissue of pregnant females with embryos. Literature data suggest that progesterone, which rises steadily during early pregnancy, increases PACAP concentrations. Since no significant difference in serum progesterone levels was

found in pregnant and pseudopregnant mice, our results suggest that higher *Adcyap1* levels are not limited to progesterone-induced stimulatory effects, but that the presence of embryos may also influence endometrial *Adcyap1* levels. For PACAP, we also found a significant relationship between endometrial expression and embryo development. For a 0.001 increase in expression in female uterine tissue, embryos with blastocyst development were 3.92 times more likely to occur, suggesting feto-maternal communication.

The endometrium signals back to the embryo via HB-EGF to activate trophoblast differentiation. This process is necessary for subsequent attachment and penetration and only occurs when sufficiently developed embryos (blastocysts) are present. Our results on the relationship between tissue and embryonic *Hbegf* expression also support this theory, as we found a weak positive relationship between *Hbegf* in uterine tissue and relative *Hbegf* expression in embryos, and low levels of *Hbegf* mRNA in non-compact embryos, whereas morulae and especially blastocysts expressed high levels of *Hbegf* mRNA.

The correlation coefficients also show a weak positive relationship between relative tissue expression of

Adcyap1 and *Hbegf*, suggesting a role for PACAP in the peri-implantation period, whereas the correlation coefficient between relative tissue *Adcyap1* and embryo *Hbegf* mRNA levels is not significantly different from zero, suggesting that PACAP has no or limited effect on embryo HB-EGF production during this period.

Our studies on PACAP KO and wild-type males showed no significant differences in either sperm motility or morphology.

Effect of exogenous PACAP

When exogenous PACAP was applied during *in vitro* culture of embryos, DNA fragmentation was significantly reduced in the 8 μM PACAP-treated group compared to the control. In a 1999 study on follicles by Lee et al, PACAP treatment inhibited apoptotic DNA fragmentation in a dose-dependent manner. Our study supports these results and we have observed that this phenomenon is also observed in embryos.

When applied during cryopreservation, the group treated with 2 μM PACAP (EM2) showed a significantly higher rate of development 24 h after thawing compared to vitrified controls, and higher *Hbegf* expression was observed compared to fresh embryos. However, when

PACAP was used during *in vitro* culture after thawing (TM 1, TM 2), no significant difference in developmental rate was found compared to vitrified control embryos. Suggesting that the presence of PACAP during incubation in EM prepared the embryos to better withstand the adverse effects of cryopreservation and even exerted a protective effect during subsequent *in vitro* culture 24 h after thawing. It has been observed in the literature that gene expression changes may occur during vitrification and thawing because cryopreservation affects the stability of mRNAs, making some of them susceptible to degradation. In our study, we found higher *Hbegf* expression levels in embryos treated with 2 μM PACAP during vitrification, suggesting that exogenous PACAP may protect the embryo from the damaging effects of cryopreservation. Higher expression levels compared to fresh embryos suggest that PACAP treatment during the vitrification protocol has a dose-dependent beneficial effect on *Hbegf* gene expression.

Frozen sperm will inevitably have some cryo-damage, mainly due to the formation of ice crystals. The most obvious effect of cryopreservation is a reduction in sperm motility. This is thought to be the result of an increase in membrane permeability, leading to

extracellular diffusion of many molecules, including ATP, which is necessary for motility and which spermatozoa are no longer able to produce after cold shock. When PACAP was applied during cryopreservation of spermatozoa, there was no significant difference in motility of fresh spermatozoa in the groups treated with 4 μM and 8 μM PACAP, whereas a significant decrease was observed in the control and 2 μM groups. Our results show that PACAP is able to protect spermatozoa against cryodamage in a dose-dependent manner.

Summary

Our studies have shown that endogenous PACAP plays a cytoprotective role against DNA fragmentation during preimplantation embryo development, has a positive effect on preimplantation embryo quality and, in the absence of PACAP, increases the percentage of micronuclei, which reduces implantation ability. We also found a positive association between uterine tissue expression and embryo development. The use of exogenous PACAP in preimplantation embryo culture improves embryo quality, reduces DNA fragmentation and its use as a cytoprotectant in both embryos and sperm dose-dependently increases survival after

cryopreservation. These new findings may help in the future development of various steps in assisted reproduction.

5. Overview of the new scientific results

1. Using PACAP KO mice, we objectively demonstrated that PACAP does not affect the estrous cycle in female mice by measuring the levels of steroid hormone metabolites (estradiol and progesterone).

2. Endogenous PACAP has a cytoprotective role against DNA fragmentation during preimplantation embryo development, with positive effects on preimplantation embryo quality. In the absence of PACAP, the micronucleus ratio increases.

3. Our experiments were the first to measure the expression of PACAP *(Adcyap1)* in preimplantation embryos and were the first to show a link between uterine tissue PACAP and an implantation-associated growth factor, HB-EGF, indicating that PACAP may play a role in supporting implantation during the peri-implantation period. It also has a stimulatory effect on the development of preimplantation embryos. Moreover, its expression in uterine

tissue in early pregnancy is dependent on the presence of embryos and is not limited to the stimulatory effect of progesterone.

4. Our results provide the first evidence that the use of exogenous PACAP in preimplantation embryo culture improves embryo quality and reduces DNA fragmentation.

5. In our studies, PACAP was used for the first time in embryo vitrification and sperm cryopreservation. It increases post-cryopreservation survival in both embryos and sperm in a dose-dependent manner.

6. Publications in the topic of the dissertation

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