Summary

In this study the effects of endogenous and exogenous PACAP (Pituitary adenylate cyclase-activating polypeptide) were investigated. PACAP is a very important neuropeptide and functions as a hypothalamic hormone, neurotransmitter, neuromodulator, and neurotrophic factor. The effects of endogenous and exogenous PACAP were investigated on different areas and from different aspects such as: preimplantation embryo development, production of estradiol and progesterone (two very important sexual steroid hormones), follicular development, expression of HB-EGF (implantation marker), sperm parameters such as motility and morphology, embryo development and survival after cryopreservation / vitrification.

Összefoglalás

A kutatási program keretében az endogén és az exogén PACAP ((Pituitary adenylate cyclase-activating polypeptide) hatásait vizsgáltuk. A PACAP egy nagyon fontos neuropeptid, ami különböző funkciókat tölt be, illetve különböző formában tölti be feladatait (pl. hipotalamusz hormon, neurotranszmitter, neuromodulátor, neurotrof faktor). Az endogén és exogén PACAP hatásait különböző területeken és szempontokból tanulmányoztuk, mint például: preimplantációs stádiumban lévő embriók fejlődése, ösztradiol és progeszteron hormonok termelődése (a két legfontosabb reprodukciós szteroid hormon), follikulus fejlődés, HB-EGF expresszió (implantációs marker), spermiumok morfológiája és motilitása, krioprezerváció (vitrifikáció) után az embriók túlélése és tovább fejlődése.

1. Effects of endogenous PACAP

1.1. Effects of endogenous PACAP on preimplantation embryo development

In this part of the project the presence or absence of the PACAP on the preimplantation embryo development was assessed. Eight weeks old females of CD1 mice were used for embryo producing. Half of them have the *Adcyap1* locus (wild type, WT), other half lacked this gene (knock-out, KO). Following superovulation, the females were mated with males and the zygotes were obtained in the morning (5-8 hours after mating) and cultured in vitro for 96 hours. The vitality, developmental rate and potential were measured by counting the blastomers. Our results show that higher blastomer number can be counted in the blastocysts of PACAP KO mice than in the WT group ($p=<0.005$; KO: 58.17 ±14.9; WT: 40.40 ±7.4). Furthermore, the micronucleus rate, as a tool to measure the embryo quality was assessed. There was no significant difference in the blastocmer number between the KO and WT embryos if they reach only the early stages within 96 hours (KO: 29.4±6.8; WT: 17.5±5.9; $p=0.18$). The micronucleus rate was higher in early embryos than in the blastocysts (mid-, expanded- or hatched) ($p<0.005$).

However, if we analyze only the blastocysts, elevated micronucleus rate was detectable in the PACAP KO embryos than in the wild type ones (KO: 13±1.2; WT: 5.2±2.2; $p<0.05$).

1.2. Effects of endogenous PACAP in hormonal production

During the experiments, we analyzed if there are any difference in the progesterone and estrogen production between the PACAP WT and KO females. To achieve this, we had to collect samples day by day during 4 cycles per female. Since daily blood sampling in mice is highly laborous, requires special technical skills, and causes stress for the animal, we chose the non-invasive fecal ampling. Females were separeted and their bedding were changed every day. Fecal samples were collected every 24 hours at the same time (10:00). Following the hormonal extraction, estradiol and progesterone metabolites (E2Met and P4Met, respectively) were measured with radioimmunoassay. For the analysis, we calculated with the baseline and peak (or plateou, in the case of P4Met) values, and took the hormonal levels in the whole cycle into account. Our results show that, although the average P4Met level of KO females was significantly higher, neither the preovulatory E2Met peak, nor the P4Met plateou an baseline values differed between the two examinated groups.

Related publication:

Török Dóra, Somoskői Bence, Reglődi Dóra, Tamás Andrea, Fülöp Balázs, Cseh Sándor. Hipofízis adenilát cikláz aktiváló polipeptid hatása nőstény egerek ciklusára és az embriófejlődésre - Előzetes eredmények

MAGYAR ÁLLATORVOSOK LAPJA 140: 3 pp. 181-187., 7 p. (2018)

1.3. Effects of endogenous PACAP on follicular development

Aim of this study was to assess the effects of endogenous PACAP on preantral follicle development, morphology and estradiol production. Preantral follicles were obtained from ovaries of 8-12 weeks old, WT or KO female CD1 mice by mechanical isolation. Follicles were cultured in vitro in Ultra-MEM medium, supplemented with 5% FBS and 10 mIU eCG, covered with Ovoil under 6.5% CO_2 and 37.5 °C. Follicular growth was examined on Day3 and Day6, and culture medium samples were collected on these days for radioimmunoassay. At the time of follicle collection, vaginal smears of females were made to check the cycle stage. Furthermore, immature oocytes were collected from late antral (preovulatory) follicles and were cultured for 24 hours to assess the maturation rate.

Both in the WT and KO groups, follicles showed intensive growth from Day2 to Day6 (a,b; $p<0.05$).

Genotype	**Day 2 (diameter µm ± SD)**	**Day 6 (diameter µm ± SD)**
PACAP WT	247.53± 36.13[a]	355.9±187.6[b]
PACAP KO	249.68 ± 37.9[a]	368±273.3[b]

Until the Day6, some of the follicles reached an extraordinary („expanded") size, which caused by the attachment and intensive proliferation of granulosa cells. In the KO group, 11.63% of follicles reached this stage and 21.27% of the follicles in the WT group.

To analyse the quality on Day 6, we classified the follicles as follows: good (G), poor (P) and degenerated (D). The table shows the rate of follicles in each categories:

	WT			KO		
	G	P	D	G	P	D
Day 2	74.5%	25.5%	0	65.1%	34.9%	0
Day 6	74.5%	6.4%	19.1%	72.1%	0	27.9%

Our results show slightly elevated rate of degenerated follicles in the KO group – however, significant difference was not found with chi-squared test.

We examined the in vitro maturation of oocytes collected from preovulatory follicles. Our overall results show that higher rate of oocytes reached the MII stage in the WT than in KO group (KO: 34.3±7.72%; WT: 42.86±9.11%). The cycle stage at the time of follicle collection had no effect on follicular development; both follicles of the follicular and luteal phase showed normal in vitro growth.

Estradiol levels of the culture media collected on Day 2 and 6 were as follows (nmol/l± SD):

Genotípus	**2.nap**	**6. nap**
PACAP WT	7.04±3[a]	8.32±4.89[a]
PACAP KO	7.24 ±4.1[a]	8.22±4.06[a]

Analysing the effect of cycle stage, we found difference only in the follicles collected from diestrus females: these follicles showed lower etradiol production on Day 6 than on Day 2. However, such difference was not detectable in the other cycle stages.

Based on our knowledge, effect of endogenous PACAP on follicular development was not studied before. Our results show that the PACAP does not play such an important role in the follicular development and hormonal production as it hipothesized before.

Planned publication:

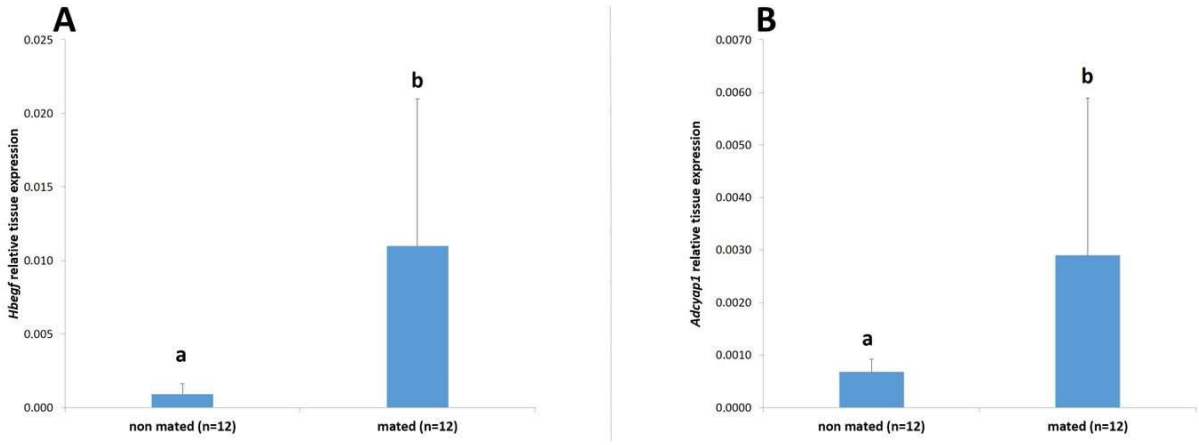
Török, D., Somoskői, B., Tamás, A., Reglődi, D., Cseh, S. New insights into the role of PACAP on preantral follicles.

Manuscript prepared for Reproductive Biology journal.

1.4. Effects of PACAP ont he expression of HB-EGF, an implantation marker

In this experiment, we analyzed the gene expression of an implantation marker, HB-EGF (coding ragion: *Hbegf*) in preimplantation embryos and uterus tissue samples of pregnant female mice.

Some of the females were not mated with males after superovulation, to assess the differences in gene expression of pregnant and non pregnant mice. Both the expression of *Hbegf* and *Adcyap1* were significantly higher in pregnant females than in non-pregnant ones (Figure 1; a,b[$p<0.001$]).

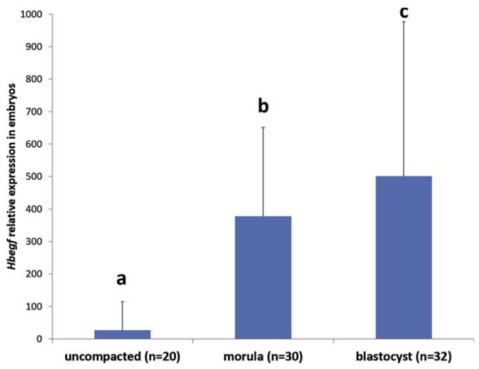


In the further part of the experiment, embryos were obtained ont he 4th day of pregnancy (Day4). Females were grouped based on the presence or absence of blastocysts in their uterine horn on the day of embryo collection (referred to as *non-blastocyst* and *blastocyst* females).

Based on this classification, both the *Hbegf* and *Adcyap1* mRNA-expression (of uterus tissue samples) were higher in females with blastocysts in their uterus on Day4.

Hbegf expression levels of collected embryos were also analyzed in this experiment. Results showed that the mRNA expression were is elevating in parrallel with the developmental stage:

	p-érték
uncompacted – blastocyst	<0.001
morula – blastocyst	0.0177
morula - uncompacted	<0.001



Possible correlation between Hbegf and Adcap1 expression was investigated. Correlation coefficients showed weak positive connection between PACAP and HB-EGF tissue expression levels ($R=0.31$; $p<0.005$). We found the same correlation between uterus tissue and embryonal HB-EGF relative expressions ($R=0.63$; $p<0.001$).

Adcyap1 expression was not detectable from any of the preimplantation embryos.

Related publication:

Somoskői B, Török D, Reglődi D, Tamás A, Fülöp BD, Cseh S. Possible effects of pituitary adenylate cyclase activating polypeptide (PACAP) on early embryo implantation marker HB-EGF in mouse. Reprod Biol. 2020 Mar;20(1):9-13

1.5. Effect of PACAP on different parameters of sperm

1.5.1. Effect of endogenous PACAP

At this stage, we examined sperm motility and morphology in WT and KO males. Sperm were obtained from 3-month-old individuals after cervical dislocation from the tail of epididymis. To analyze motility, we performed Computer-Aid Sperm Analysis (CASA) examining 4 categories: fast progressive, slow progressive, locally moving, and immotile. For morphological analysis, we prepared smears on slides and then stained with Spermac dye.

No significant differences were found in motility data.

After morphological examination, the following results were obtained:

	p-value	WT mean [%] ± sd	KO mean [%] ± sd
normal	0.096	69.92 ± 10.51	54.17 ± 28.85
proximal cytoplasmic droplet	0.065	5.08 ± 5.09	11.75 ± 10.54
dystal cytoplasmic droplet	0.073	1.92 ± 2.13	0.6 ± 1.98
broken	0.21	11.08 ± 5.07	25.08 ± 36.11
coiled	0.77	5.54 ± 7.51	4.75 ± 6.03
microcephaly	0.18	2 ± 2.23	0.92 ± 1.62
macrocephaly	0.68	1.08 ± 1.11	0.83 ± 1.69
acrosome absent	0.14	1.68 ± 1.88	0.75 ± 1.05

Statistical analyzes did not show a significant difference within each parameter between the two groups, however, normal morphology and the presence of proximal cytoplasmic droplets suggest difference.

1.5.2. Effect of exogenous PACAP

In the experiment, the above-mentioned test methods were applied to WT, KO, and heterozygous (HZ) males. Motility analysis (CASA) was supplemented with PACAP1-38 treatment at a concentration of 1 nM. The samples were incubated for 5 min at 37.5 ° C in the mentioned solution before the CASA analysis. The morphological result - similarly to motility - did not show a significant difference between the three groups (69.6 ± 3.1%, 68.8 ± 8.1 and 58.8 ± 6.7 in WT, HZ and KO males). The added PACAP1-38 did not increase motility values. The results of the above two studies suggest that decreased fertility in PACAP KO males is not related to motility or morphological parameters.

Related publication:

Reglodi, D, Cseh, S, Somoskoi, B, Fulop, B D, Szentleleky, E, Szegeczki, V, Kovacs, A, Varga, A, Kiss, P, Hashimoto, H, Tamas, A, Bardosi, A, Manavalan, S, Bako, E, Zakany, R, & Juhasz, T. (2018). Disturbed spermatogenic signaling in pituitary adenylate cyclase activating polypeptide-deficient mice, Reproduction, 155(2), 127-137.

2. Effects of exogenous PACAP, application possibilities during embryo vitrification

2.1. Effect of PACAP treatment on developmental rate

At this stage, we examined whether culturing in medium containing PACAP affects the rate of embryo development; what proportion of embryos reach the blastocyst stage during 96 hours of *in vitro* culture?

Zygotes were obtained from 8–12-week-old females of BDF1 mouse strain after superovulation treatment (7.5 IU eCG + 7.5 IU hCG) and natural mating. After obtaining, the zygotes were placed in medium (Vitrolife G1 medium; 37.5 ° C, 6.5% CO2). In addition to the control group (factory medium), 2 types of supplements were used: 2 PM PACAP1-38 and solvent control (PBS; 36 µl PBS + 363 µ G1).

The rate of progression in none of the treatment groups showed difference from the values in the control group. (table - BL = blastocyst; non-BL = morula or less developed forms)

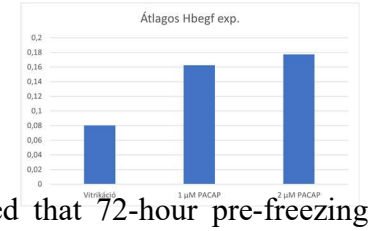
stage	**control**		**PACAP**		**PBS**	
	72h	96h	72h	96h	72h	96h
BL%	51.72	90.91[a]	48.39	84.78[a]	51.85	95.35[a]
non-BL%	48.28	9.09	51.61	15.22	48.15	4.65

2.2. Application of PACAP treatment before vitrification

At this stage of the experiments, we sought to answer the question of how the addition of PACAP1-38 to the embryo culture medium affects the expression levels of the *Hbegf gene* encoding the HB-EGF implantation marker. Studies were performed on embryos from 8–12-week-old BDF1 female mice. After obtaining, zygotes were placed in medium and cultured *in vitro* for 72 hours. At this stage, 3 treatment groups were tested: control (Vitrolife G1 medium), 1 µM PACAP1-38 and 2 µM l PACAP1-38.

Mean relative *Hbegf gene expression* levels were as follows:

	Mean *Hbegf gene exp.*
Vitrification	0.080383571
1 µM PACAP	0.162360808
2 µM PACAP	0.177440107



Based on the above data, it can be assumed that 72-hour pre-freezing PACAP treatment improves the chances of blastocyst implantation. The data are preliminary results and are evaluated on an ongoing basis.

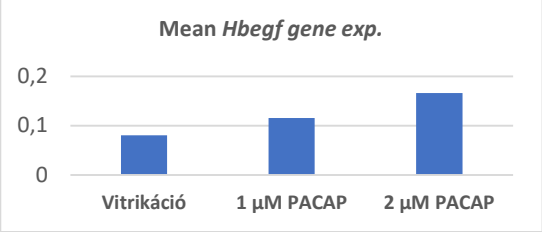
2.3. Application of PACAP treatment during vitrification

At this stage of the experiments, we examined the effect of the addition of PACAP1-38 to vitrification solution on the expression levels of the *Hbegf* gene encoding the HB-EGF implantation marker. Studies were performed on embryos from 8–12-week-old BDF1 female mice. After obtaining, zygotes were placed in medium (Vitrolife G1 medium, without supplementation) and cultured *in vitro* for 72 hours. Then the embryos were vitrified. After thawing, they were again placed in G1 medium for 24 h. At the end of the experiment, the survival rate was recorded and *Hbegf expression* levels in each treatment group were measured by qPCR. The following vitrification protocol was used for cryopreservation (different treatments are also shown in the table):

	Composition of solutions			
	1.	2.	3.	4.
Vitrification	M199+20% FBS	M199+20% FBS	M199+20% FBS+7.5% ethylene glycol +7.5% DMSO **Incubation for 3 minutes**	M199+20% FBS+15% ethylene glycol +15% DMSO+1 M sucrose
1 µM PACAP	M199+20% FBS	M199+20% FBS	M199+20% FBS +7.5% ethylene glycol +7.5% DMSO + 1 µM PACAP **Incubation for 3 minutes**	M199+20% FBS +15% ethylene glycol +15% DMSO +1 M sucrose
2 µM PACAP	M199+20% FBS	M199+20% FBS	M199+20% FBS +7.5% ethylene glycol +7.5% DMSO + 2 µM PACAP **Incubation for 3 minutes**	M199+20% FBS +15% ethylene glycol +15% DMSO +1 M sucrose

Survival after thawing was 69.87% in the control without supplementation vitrification group. Of the treated groups, 65.42% of embryos incubated in medium containing 1 µM PACAP reexpanded or developed. In the group treated with 2 µM PACAP, we found a significant increase, a survival rate of 90.23%.

	Mean *Hbegf gene exp.*
Vitrification	0.080383571



1 µM PACAP	0.115045521
2 µM PACAP	0.16594048

Based on the above results, it can be concluded that PACAP treatment increases the *expression level of Hbegf* in blastocyst-stage mouse embryos, both before and during freezing. Higher gene expression makes greater probability of implantation. To confirm this in vivo (embryo implantation), we are currently investigating.

TSA treatment and other investigations:

At the time of submitting the application, the work plan included an investigation into the effect of TSA, the assessment of how the compound could be used in embryo vitrification. Preliminary tests showed no positive or negative effect. We have thus focused on the widest possible examination of PACAP. Due to the unfortunate effects of the COVID-19 pandemic, we were not able to start the embryo transfer work planned for the last year, but we managed to start it by the end of 2020, with a low sample size so far. These experiments are still ongoing.