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THE cAMP ANALOGUE, dbcAMP CAN STIMULATE RABBIT REPRODUCTIVE FUNCTIONS. I. EFFECT ON OVARIAN FOLLICULOGENESIS, OVULATION AND EMBRYO PRODUCTION

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The aim of our study was to examine the influence of administration of N^6,2'-dibutyryladenosine 3'5'-cyclic monophosphate (dbcAMP), a cAMP agonist, on ovarian folliculogenesis and atresia, as well as on reproductive efficiency in rabbits, whose ovarian cycle and ovulation was induced by gonadotropins.

Ovarian cycle and ovulation of control rabbits were induced by 20 IU/kg PMSG followed by 35 IU/kg hCG administration. Experimental animals received PMSG and hCG together with dbcAMP (at 5, 25 or 50 μg/animal). After ovulation and insemination, the animals were sacrificed. Ovaries were weighted, histological sections of ovaries were prepared, and the presence of ovulated and not ovulated follicles and different stages of atresia was evaluated by light microscopy. The eggs were flushed from the oviducts after insemination and cultured up to blastocyst cell stage. Numbers of ovarian Corpora lutea, ovulated oocytes and oocyte-derived zygotes and embryos reaching hatched blastocyst stage were determined.

Administration of dbcAMP (at doses 25 or 50 μg/animal, but not at 5 μg/animal) was able to increase the proportion of follicles with cystic and luteinization-related atresia. Furthermore, dbcAMP (50 μg/animal, but not lower doses) increased the ovarian mass, number of Corpora lutea, number of harvested oocytes, zygotes and embryos at blastocyst stage derived from these zygotes after culture.

These data demonstrate that dbcAMP can stimulate rabbit ovarian follicle atresia, ovulation, oocyte, zygote and embryo yield and development. Furthermore, they confirm in the involvement of cyclic nucleotide-dependent intracellular mechanisms in the control of rabbit reproductive functions and potential practical usefulness of dbcAMP in improving animal reproduction and fertility.

Key words: cyclic nucleotide, dbcAMP, embryo development, fertility, ovarian follicullogenesis, ovulation, rabbit

INTRODUCTION

There is a growing body of evidence, that cyclic adenosine monophosphate (cAMP) is involved in the control of ovarian functions.

The cAMP/cAMP-dependent protein kinase (PKA) system plays an important role in the control of ovarian cell proliferation, apoptosis, secretory activity, oocyte maturation and in mediating the effect of hormonal stimulators on these processes (Makarevich and Sirotkin, 2000; Conti, 2002; Mehlmann, 2005; Sirotkin, 2005; Hunzicker-Dunn and Maizels, 2006). Furthermore, cAMP can mediate the effects of some hormonal stimulators on oviductal functions (Makarevich and Sirotkin, 1997; Orihuella et al., 2003). Temporal changes in cAMP activity, which can be regulated by pharmacological blockers of cAMP-specific phosphodiesterases (PDEs, catalysers of cAMP degradation) can control sexual maturation and ovarian cyclicity (Wang et al., 2007). Synthetic inhibitors of cAMP-specific PDEs were able to increase cAMP accumulation in ovarian cells, as well as number of ovulations, embryos and born pups in gonadotropin-stimulated rats (McKenna et al., 2005). Administration of IBMX, the inhibitor of cAMP- and cGMP-specific PDEs, stimulated rabbit ovarian follicullogenesis, affected ovarian and oviduct cell proliferation, apoptosis and release of hormones (Sirotkin et al., 2010b) and promoted ovulation and embryo production (Sirotkin et al., 2008, 2010a). These findings indicate, that pharmacological activators of cAMP/PKA could be promising promoters of ovarian functions, as well as enhancers or even alternative to classical hormonal stimulators of reproductive processes in agriculture, human and veterinary medicine and assisted reproduction. Nevertheless, the previous stimulation of reproductive processes *in vivo* via cAMP/PKA was achieved only by administration of PDE inhibitors, which effect on PKA is not specific (IBMX affects both cAMP and cGMP) and indirect (via inhibition of cyclic nucleotide degradation, but not via direct activation of cAMP and cGMP targets). The effect of direct PKA activator, stabile and cell-permeable cAMP analogue, N^6,2'-dibutyryladenosine 3'5'-cyclic monophosphate (dbcAMP; Posternak and Weiman, 1974), on rabbit ovarian steroidogenesis has been observed (Chrenek et al., 2010c), but its action on reproduction and fertility have not been previously studied yet.

The aim of our study was to examine the influence of administration of dbcAMP on ovarian folliculogenesis, atresia and luteogenesis, as well as on the reproductive efficiency (oocyte and zygote recovery end embryo yield and development) in rabbits, whose ovarian cycle and ovulation was induced by gonadotropins.

MATERIALS AND METHODS

Manipulations with animals and embryo production

Female New Zealand non-cycling rabbits (*Oryctolagus cuniculus, Leporidae, Lagomorpha*) 4 months of age, were bred and kept in individual cages under standard conditions at the local rabbit farm of Animal Production Research Centre, Nitra, Slovakia. Three days before mating, rabbits were treated with

pregnant mare serum gonadotropin (PMSG, Werfaser, Alvetra und Werfft AG., Vienna, Austria, 20 IU/kg) followed after 72 h by human chorionic gonadotropin (hCG, Werfachor, Alvetra und Werfft AG, 35 IU/kg). Control animals were treated only with these gonadotropins, whilst experimental females received gonadotropins together with dbcAMP (Life Science Institute, Bremen, Germany, at 5, 25 or 50 μg/animal). Gonadotropins and dbcAMP were dissolved in PBS immediately before injection. All substances (0.7 mL solution of gonadotropin with or without dbcAMP per animal) were injected intramuscularly. At 19-20h after artificial insemination with freshly diluted sperm, the females were killed by decapitation. The ovaries were collected, weighted, numbers of *Corpora lutea* in both ovaries were determined by visual inspection, and the ovaries were subjected to histological analysis (see below).

The pronuclear stage zygotes were flushed from the oviduct with PBS supplemented with 10% FCS (Gibco BRL/Invitrogen Corp., Carlsbad, CA, USA) and washed in CIM medium +10% FCS (Gibco BRL). The eggs were cultured in k–DMEM + 10% FBS (Gibco BRL) at 5% CO_2 and 39^{o}C up to blastocyst cell stage. Stages of embryogenesis were determined in different times of culture under a stereomicroscope at 40- or 100-fold magnification. Detailed description of animal and zygote treatment was published previously (Chrenek et al., 1999).

All experiments were carried out with the approval of a local ethical commission in accordance to Slovak and EU regulations concerning animal experiments.

Histological analysis

The ovaries were fixed 24 h in 10% formalin, washed 2 h in flowing water, dehydrated in 70%, 96% and 100% ethanol (2h each) and embedded in paraffin or Technovit 7100 (Heraeus Kulzer GmbH&CoKG, Wehrheim/Ts., Germany) according to instruction of manufacturer. Thereafter the ovaries were serially sectioned by using a standard rotation microtome at 5 or 10 μm thickness. The sections were subsequently dewaxed, rehydrated and stained with eosin-haematoxylin or basic fuchsin-toluidin blue (Edward Gurr Ltd., London, UK) and embedded to entelan (Merck, Darmstadt, Germany) according to Hayat (1993). Two representative sectional profiles from the stained ovaries were examined and photographed using an Jenaval (Carl Zeiss, Jena, Germany) light microscope. All primary, secondary and tertiary ovulated follicles, as well as not ovulated follicles at different stages of atresia were counted. The following stages of atresia were identified: (1) no or small atresia (no visible pathological changes or some disappearance of mitosis and appearance of nuclear vacuolisation), (2) obliteral atresia (degeneration of granulosa layer and dominance of fibrocytes in theca layer), (3) cystic atresia (disappearance of epithelial cells in both granulosa and theca layers) and (4) atresia associated with luteinization (nuclear pyknosis, light cytoplasm, cell hypertrophy and intensive multiplication of both granulosa and theca cells. Histological sections of healthy follicles and follicles at different stages of luteinization and atresia are shown in Fig.1.

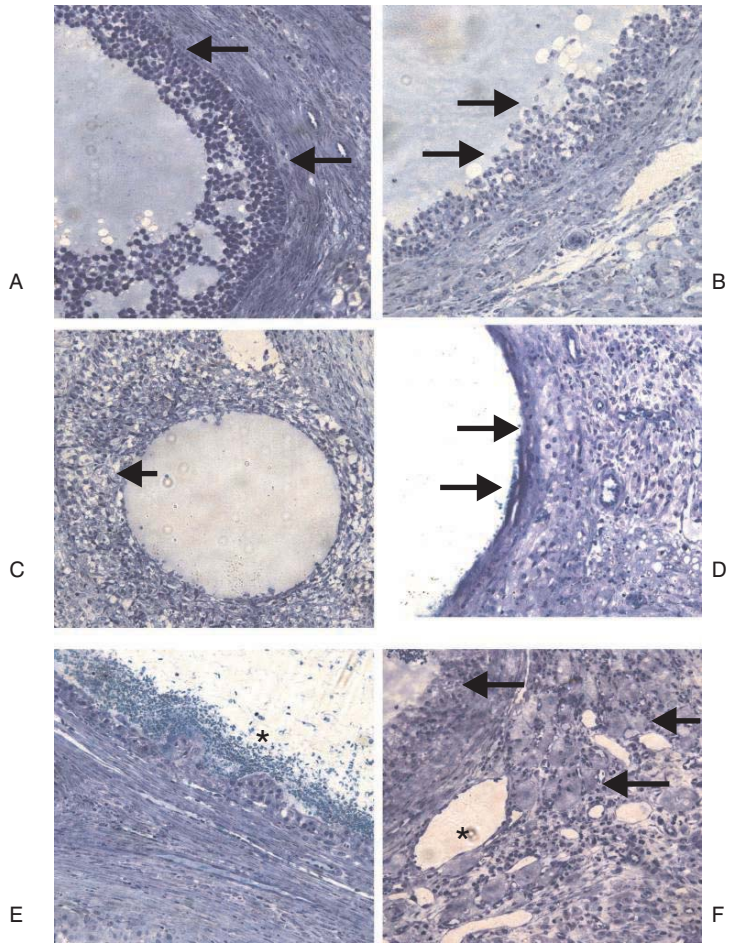


Figure 1. Histological sections of rabbit ovarian antral not ovulated follicles. Staining with eosin-haematoxylin. Magnification x 300.
A: Healthy follicle (control group) with intact granulosa and theca externa and theca interna cells (arrow).
B: First stages of atresia (control group). Interstitial cells (IC) are intact, some granulose cells contain pyknotic nuclei, some granulosa cells are present in follicular fluid (arrow).
C: Luteinization-associated atresia of follicle (treatment with dbcAMP, 50 μg/animal), hypertrophy of both granulose and theca cells is visible (arrow).
D: Cystic atresia (control group). No epithelial granulose cells in the follicular wall (arrow).
E: Hemorrhagic follicular cyst (asterisk). Some granulose cells with picnotic nuclei are visible in both follicular wall and follicular fluid. Ovary of rabbit treated with dbcAMP (50 μg/animal).
F: Hypertrophied luteinized granulosa and intestinal cells (IC) with pyknotic nuclei (arrows). The large capillaries in intestinal layer are visible (asterisk). The ovary of control animal.

Statistics

Each experiment was performed on 4 control animals and 4 animals treated with dbcAMP at each dose. Each animal from each group was killed, and ovaries and embryos harvested for subsequent analysis. Each experiment was performed 6 times. Histological data represent the data obtained by inspection of slices from 6 ovaries per experiment. The data shown are means of values obtained in 6 separate experiments performed in different days. Significant differences between the groups were evaluated by one-way ANOVA followed by paired t-test or chi-square test by using Sigma Plot 9.0 statistical software (Systat Software, GmbH, Erkrath, Germany). Differences at $p<0.05$ were considered as significant.

RESULTS

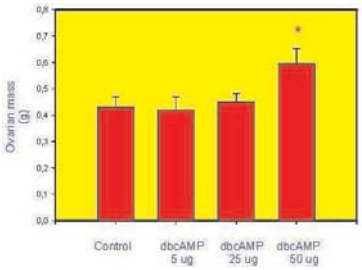
Administration of gonadotropins and subsequent mating of females induced development and ovulation of their ovarian follicles, formation of *Corpora luthea*, expulsion of oocytes into the oviduct, and subsequent embryo development *in vitro* (see below).

Histological examination of ovaries after ovulation induced with either with gonadotropins alone or gonadotropins in combination with dbcAMP revealed the presence of ovulated and non-ovulated follicles. Some non-ovulated follicles had signs of luteinization and atresia. Substantial part of ovarian follicles expressed signs of obliteral atresia, cystic atresia and atresia associated with luteinisation. Description of these morphological signs (reduced cell proliferation and appearance of nuclear vacuolization, pyknosis of nuclei, degeneration and disappearance of cells by obliteral and cystic atresia, degeneration the follicular wall, nuclear picnosis, light cytoplasm, cell hypertrophy and intensive multiplication of cells by luteinization and luteinization-associated atresia) is present in Materials and Methods and illustrated in Fig.1.

Micromorphometric analysis of ovarian slices showed the influence of dbcAMP treatment on the occurrence of some forms of follicular atresia of different classes in the ovary (Table 1). Administration of dbcAMP tended to increase percentage of ovulated follicles and decrease the proportion of non-ovulated follicles, but these changes were statistically not significant. Injections of dbcAMP did not substantially affect the proportion of non-ovulated follicles without expressed atresia or with obliteral atresia. On the other hand, ovaries of rabbits treated with dbcAMP at doses 25 and 50 μg/animal, but not 5 μg/animal, had higher proportion of follicles with signs of cystic atresia. Moreover, administration of dbcAMP at the dose of 25 μg/animal, but not at other doses, resulted in increased percentage of follicles with atresia associated with luteinization.

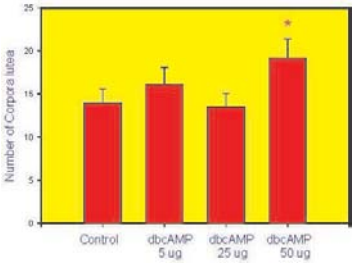
Macromorphometric analysis of ovaries and subsequent evaluation of oocyte and embryo yield and development demonstrated, that administration of dbcAMP at doses 50, but not 5 or 25 μg/animal significantly increased the ovarian mass (Fig. 2a) number of *Corpora luthea* (Fig. 2b) in the ovaries of rabbits treated with gonadotropins. Furthermore, dbcAMP, when administrated at this dose, but

a)



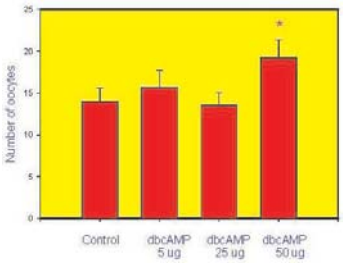
Effect of dbcAMP administration on the ovarian mass in rabbit plasma
*significant ($p<0.05$) differences with control (no dbcAMP)

b)



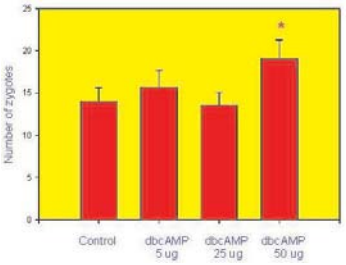
Effect of dbcAMP administration on the number of Corpora lutea in rabbits
*significant ($p<0.05$) differences with control (no dbcAMP)

c)



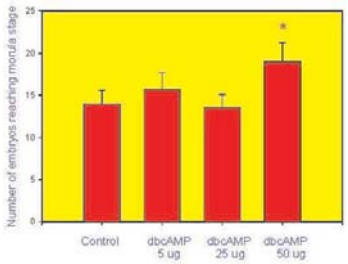
Effect of dbcAMP administration on the number of oocytes in rabbits
*significant ($p<0.05$) differences with control (no dbcAMP)

d)



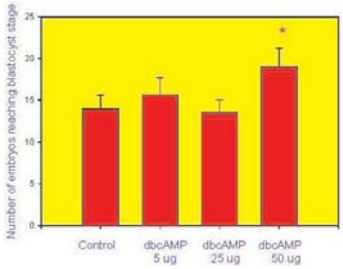
Effect of dbcAMP administration on the number of zygotes in rabbits
*significant ($p<0.05$) differences with control (no dbcAMP)

e)



Effect of dbcAMP administration on the number of embryos reaching morula stage in rabbits
*significant ($p<0.05$) differences with control (no dbcAMP)

f)



Effect of dbcAMP administration on the number of embryos reaching hatched blastocist stage in rabbits
*significant ($p<0.05$) differences with control (no dbcAMP)

Figure 2. Effects of dbcAMP treatment (5, 25 or 50 μg/animal) on reproductive parameters in rabbit females: ovarian mass (a), number of *Corpora lutea,* (b), number of harvested oocytes (c), zygotes (d), embryos reaching morula stage (e) and embryos reaching blastocyst stage (f). Values are means±S.E.M., * – significant ($p<0.05$) differences with control (no dbcAMP treatment)

not at lower doses, significantly increased the number of harvested ovulated oocytes (Fig. 2c), zygotes (Fig. 2d), embryos reaching the morula stage (Fig. 2e) and later hatching the blastocyst stage (Fig.2f) derived from these zygotes after culture.

Table 1. State of rabbit ovarian follicles after ovulation induced by gonadotropins with and without dbcAMP given at different doses

Stage of follicullogenesis	Control (no dbcAMP treatment) n/%	dbcAMP treatments at dose		
		5 μg/animal n/%	25 μg/animal n/%	50 μg/animal n/%
All examined follicles	380/100	166/100	159/100	278/100
Follicles after ovulation	116/30,5	64/38,5	51/32,1	102/36,7
Not ovulated follicles total	264/69,5	102/61,4	108/68,6	176/63,3
Not ovulated follicles, no or small atresia	76/28,8	26/25,5	25/23,1	45/25,6
Not ovulated follicles, expressed obliteral atresia	51/19,3	21/20,6	19/17,6	34/19,3
Not ovulated follicles, cystic atresia	23/8,7	9/8,8	21/19,4*	29/16,5*
Not ovulated follicles, atresia associated with luteinisation	39/14,8	17/16,7	26/24,1*	36/20,4

Legend:
n – number of follicles at particular stage of folliculogenesis presented in ovaries;
% – percentage of follicles at particular stage of folliculogenesis versus total number of follicles presented in ovaries;
* – significant ($p<0.05$) differences with control (no dbcAMP treatment).

DISCUSSION

The obtained results demonstrate the ability of dbcAMP addition to affect ovarian functions of rabbits, which reproduction was stimulated with gonadotropins. It was shown, that administration of dbcAMP can potentially increase the percentage of ovulated follicles, although this increase determined by histological analysis was statistically insignificant. Furthermore, this analysis demonstrated, that dbcAMP treatment significantly promoted the occurrence of cystic and luteinization-associated atresia in non-ovulated follicles after gonadotropin treatment. These changes suggest, that dbcAMP can potentially promote ovarian follicular luteinization and atresia. The dbcAMP-induced ovarian cell luteinisation associated with increased proliferation and hypertrophy of cells within the ovarian follicles observed in our experiments are in line with the ability of cyclic nucleotides to promote ovarian cell proliferation, as reported previously (Cheadle et al., 2008, Viegas et al., 2008). The dbcAMP-induced atresia expressed in our experiments, correspond previous reports of promotion (Amsterdam et al.,

2006), but not on suppression (Vigas et al., 2008) of ovarian cell apoptosis. These effects of dbcAMP are similar to the effects of gonadotropins, known promoters of ovarian follicles ovulation, luteinization and atresia (Hillier, 2001).

Macromorphometric analysis of ovaries and subsequent evaluation of oocyte and embryo yield and development demonstrated, that administration of dbcAMP can significantly increase the ovarian mass, number of *Corpora luthea* and ovulated oocytes. These effects of dbcAMP are in line with the ability of PDE inhibitors to increase the number of ovulations and Corpora lutea in rats (McKenna et al., 2005) and rabbits (Sirotkin et al., 2008; 2010a) as reported previously. These data confirm the previous data on involvement of cAMP/PKA-dependent intracellular mechanisms in the stimulation of ovarian functions. Furthermore, the present observations represent the first *in vivo* demonstration, that dbcAMP can promote ovarian growth, ovulation and oocyte production. Since these effects are similar to known effects of gonadotropins (Hillier, 2001), it suggest that dbcAMP can have gonadotropin-like action on rabbit ovarian cells functions. Moreover, the stimulatory effects of dbcAMP was expressed in rabbits treated with gonadotropins. The additive stimulatory action of dbcAMP suggests, that this molecule can increase the action of gonadotropins on ovarian functions.

It is well-known, that stimulation of ovaries by exogenous gonadotropins can increase ovulation rate and number of ovulated oocytes, but decrease the quality and developmental potential of harvested oocytes (Hillier, 2000; Craig et al., 2007). In our experiments, administration of dbcAMP was able not only significantly to increase the number of harvested oocytes but also the number of fertilized oocytes (zygotes) and subsequent embryos reaching stages of morula and hatching blastocysts after culture. Therefore, the stimulatory action of dbcAMP on rabbit ovarian functions was not associated with negative effects on quality of harvested oocytes and their ability to be fertilized and to develop to embryos up to preimplantation (hatched blastocyst) stage.

Comparison of effects of dbcAMP given at different doses on both ovarian histological indexes and reproductive parameters showed, that low dbcAMP dose (5 μg/animal) was not efficient at all. dbcAMP, when administrated at medium dose (25 μg/animal), was able to increase cystic and luteinization-associated atresia of ovarian follicles detected by histological analysis, but these changes did not result in significant changes in rabbit reproductive parameters. It suggests, that medium (25 μg/animal) dose of dbcAMP can potentially affect ovarian functions and promote gonadotropin-induced atresia, but not stimulatory gonadoitropin effects on ovarian functions. Finally, the dbcAMP, when administrated at high (50 μg/animal) dose, promoted cystic, but not luteinization-associated atresia, as well as increased all reproductive indexes. It demonstrates, that this dose of dbcAMP is high enough to promote not (or not only) gonadotropin-induced follicular atresia, but also follicular development up to ovulation and yield of oocytes and embryos of good quality. This suggests that dbcAMP can control different reproductive events relatively independently and that different ovarian targets have different sensitivity to the dbcAMP action.

From practical viewpoints, this is the first demonstration of the beneficial influence of the cAMP agonist on farm animal reproduction on stimulation of

ovarian follicullogenesis and increased number of ovulations/C*orpora lutea*, harvested oocytes, zygotes and embryos without loss of their quality. Practical application of this molecule and other promoters of cAMP/PKA-dependent intracellular mechanisms as additional or alternative stimulator of reproduction in assisted reproduction, animal production, human and veterinary medicine should not to be excluded. Nevertheless, prior to practical application of synthetic cAMP analogues in control of animal and human reproduction and fertility, some key questions should be addressed. In our experiments, dbcAMP given at different doses affected different reproductive parameters, and only one dose (50 μg/animal) was really efficient. The optimal dose for animals of different ages from both physiological and economical viewpoints, should be chosen. Mechanisms of dbcAMP action, including hormonal mechanisms, should be elucidated. The cAMP-related substances should be tested on other species (especially on large farm animals and human). Possible side-effects should be carefully examined in large-scale and long-term studies. Nevertheless, the present observations are the first demonstration, that cAMP analogue, in addition to gonadotropins, could be successfully used for stimulation of rabbit ovarian development, ovulation, oocytes and embryos production.

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ANALOG cAMP-a, dbcAMP MOŽE DA STIMULIŠE REPRODUKTIVNE FUNKCIJE KUNIĆA. I - UTICAJ NA FOLIKULOGENEZU U JAJNICIMA, OVULACIJU I NASTANAK EMBRIONA

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i SIROTKIN AV

SADRŽAJ

Cilj naših ispitivanja je bio da se ispita uticaj aplikacije N^6,2'-dibutiril-adenozin 3'5'-cikličnog monofosfata (dbcAMP), agoniste cAMP-a, na folikulogenezu i atreziju u jajnicima, kao i na reproduktivnu efikasnost ženki kunića, kod kojih su ovarijalni ciklus i ovulacija indukovani gonadotropinom.

Ovarijalni ciklus i ovulacija kontrolnih ženki kunića su indukovani sa 20 IU/kg PMSG, i nešto kasnije aplikovanjem 35 IU/kg hCG. Ženke eksperimentalne grupe su dobijale PMSG i hCG zajedno sa dbcAMP-om (5,25 ili 50 μg/životinji). Posle ovulacije i inseminacije, životinje su žrtvovane, a zatim je merena masa jajnika. Svetlosnom mikroskopijom je vršena histološka analiza i određivano je prisustvo ovuliranih i neovuliranih folikula i kao i folikula sa različitim stepenom atrezije. Jajne ćelije su posle inseminacije ispirane iz jajovoda i uzgajane do faze blastocite. Utvrđivan je broj *corpora lutea* na jajnicima, broj ovuliranih oocita i zigota izdvojenih iz oocita kao i broj embriona koji su dostigli fazu blastocita

Aplikacija dbcAMP-a u dozama 25 or 50 μg/životinji (ali ne i 5 μg/ životinji) je dovodila do povećanja udela folikula sa cističnom atrezijom i atrezijom povezanom sa luteinizacijom. Dodatno je dbcAMP u dozi od 50 μg/ životinja (ali ne i u nižim dozama) povećavao masu jajnika, broj *corpora lutea* i broj dobijenih oocita, zigota i embriona u stadijumu blastocista izolovanih od ovih zigota posle kultivacije.

Ovi podaci ukazuju da dbcAMP može da stimuliše atreziju folikula jajnika kod ženki kunića, ovulaciju, prinos i razvoj oocita, zigota i embriona. Dalje, oni potvrđuju da su intracelularni mehanizmi zavisni od cikličnih nukleotida uključeni u kontrolu reproduktivnih funkcija ženki kunića i da dbcAMP-a ima potencijalnu praktičnu vrednost u poboljšanju reprodukcije i plodnosti farmskih životinja.