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Short communication

EFFECT OF cAMP-RELATED SUBSTANCES ON SELECTED RABBIT REPRODUCTIVE PARAMETERS

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ABSTRACT

The aim of our study was to examine the effect of 3-isobutyl-1-methyl-xanthine (IBMX) and dibutyryl cyclic adenosine monophosphate (dbcAMP), stimulators of cAMP/protein kinase A-dependent intracellular signaling pathway, on reproductive function of gonadotropin-stimulated rabbit females. Ovarian cycle and ovulation in the control group were stimulated by PMSG (pregnant mare serum gonadotropin) 80IU/doe and after 72 hours with hCG (human chorionic gonadotropin) at 150 IU/doe. In the experimental group the females were stimulated with PMSG and hCG with the addition of either IBMX or dbcAMP at the dose of 50 μg/doe. In the first series of experiments, 18-19 hours after artificial insemination and ovulation the animals were killed, the pronuclear-stage eggs were flushed from the oviducts and cultured up to the blastocyst cell stage. Numbers of ovarian *corpora lutea*, ovulated oocytes, and oocyte-derived embryos reaching blastocyst stage were determined. In the second series of the experiments, all the animals were kept until parturition, when the conception rate and parturition rate, number of liveborn pups, stillborn pups and weaned pups were recorded. *In vitro* results show that both tested substances significantly increased female fertility parameters (number of *corpora lutea*, number of ovulated oocytes, number of oocyte-derived zygotes, embryos at morula stage and number of embryos at hatching blastocyst stage). The present result of *in vivo* studies show that both tested substances decreased female fertility parameters - conception rate, parturition rate and weaning rate compared to control, but they did not affect the stillborn pup rate. These observations confirm the involvement of cAMP/protein kinase A-dependent intracellular signaling pathway in control of rabbit reproduction

Key words: IBMX, dbcAMP, conception rate, parturition rate, liveborn pups, stillborn pups

INTRODUCTION

The involvement of cyclic nucleotides, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) and their targets – protein kinases A (PKA) and G (PKG), in control of reproductive processes is well documented. Both cAMP (Makarevich and Sirotkin, 2000; Conti, 2002; Mehlmann, 2005; Sirotkin, 2005; Hunzicker-Dunn and Maizels, 2006) and cGMP (Sirotkin *et al*., 2000; La Polt *et al*., 2003) play an important role in control of ovarian cell proliferation, apoptosis, secretory activity, oocyte maturation and in mediating the effect of hormonal stimulators on these processes. cAMP can mediate effect of some hormones on the oviductal functions too (Makarevich and Sirotkin, 1997; Orihuella *et al*., 2003; Sirotkin *et al.*,

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2010a). Both cyclic nucleotides are hydrolyzed by the enzymes - phosphodiesterases (PDEs). Mice, deficient in cAMP-specific phosphodiesterases - PDE3A and PDE4, have impaired differentiation of ovarian cells, their response to gonadotropin, oocyte maturation, ovulation and fertility (Conti, 2002; Park *et al.*, 2003; Masciarelli *et al.*, 2004). On the other hand, synthetic PDE4 inhibitors increased cAMP accumulation in ovarian cells, as well as number of ovulations, embryos and born pups in gonadotropin-stimulated rats (McKenna *et al*., 2005). Previous experiments performed on rabbits demonstrated the influence of IBMX, inhibitor of cAMP- and cGMP-specific PDEs on ovarian folliculogenesis, atresia, and steroid hormone release, as well as proliferation and apoptosis of ovarian and oviductal cells (Sirotkin *et al*., 2008). These findings suggest that PDEs and their targets – cyclic nucleotides and PKA or PKG could be potent regulators of reproductive processes in laboratory and farm rodents. dbcAMP is a synthetic analogue of cAMP and stimulator of cAMP-dependent protein kinase A. Analogues of cAMP can either decrease (Murphy and Dobias, 1999) or increase (Nakamura *et al*., 1995; Tano *et al*., 1997) the expression of FSH receptors and its mRNA, whilst PDE inhibitor can reduce expression of LH receptors (Peegel *et al*., 2005). dbcAMP stimulated ovarian progesterone production, but did not affect ovulation or maturation of follicular oocytes in the ovaries perfused in vitro (Hosoi *et al*.,1989). The additions of dbcAMP to culture medium promoted accumulation of both proliferation and apoptosis markers in cultured ovarian fragments. These data demonstrate that IBMX and dbcAMP can enhance the stimulatory effect of gonadotropins on the rabbit ovarian and oviductal functions and on rabbit fecundity via changes in ovarian hormone release, ovarian and oviductal cell proliferation and apoptosis (Sirotkin *et al.*, 2010b). Kolesárova et al. (2008) in their study shows the effects of dbcAMP on the release of steroid hormones and IGF-I by porcine granulosa cells. Moreover, it is not to be excluded, that PKA activators (PDE inhibitors or cAMP analogues) could be stimulators of reproduction in animal breeding, human and veterinary medicine. Therefore, further examination, confirmation and comparison of IBMX and dbcAMP effects on reproduction is required from both theoretical and practical viewpoints.

The aim of our study was to examine the influence of administration of IBMX (an inhibitor of cAMP and cGMP phosphodiesterases) and dbcAMP (analogue of cAMP, direct PKA activator) on reproductive efficiency and fertility in female rabbits whose ovarian cycle and ovulation were induced by gonadotropins.

MATERIALS AND METHODS

Manipulations with animals

New Zealand White rabbits, 3.5 month of age and older were bred in individual cages at the local rabbit farm of Animal Production Research Centre Nitra. Female rabbits in the control group were treated 3 days before mating with pregnant mare serum gonadotropin (PMSG, Werfaser, Alvetra und Werfft AG, Vienna, Austria, at dose 80 IU/doe) followed after 72 hours by human chorionic gonadotropin (hCG, Werfachor, Alvetra und Werfft AG, Vienna, Austria at dose 150 IU/doe). Gonadotropins were dissolved in phosphate-buffered solution (PBS). Control animals were intramuscularly injected only with these gonadotropins (in 0.5 ml saline per doe), whilst experimental females received gonadotropins with either IBMX (Sigma, St. Louis, MI, USA, 50 µg/doe in 0.5 ml PBS) or dbcAMP (Fluka, Heidelberg, Germany, 50 µg/doe in 0.5 ml PBS). Immediately prior to administration of hCG females were inseminated by freshly diluted sperm (0.5 ml/female). In the first series of experiments the animals were killed 19-20 hours after artificial insemination and ovulation. The pronuclear-stage eggs were flushed from the oviducts with PBS and washed in CIM medium + 10% FBS (fetal bovine serum, Gibco BRL/Invitrogen Corp., Carlsbad, CA, USA). The eggs were cultured in k-DMEM (knock out dulbecco modified eagle´s medium) + 10% FBS at 5% CO_2 39°C up to the blastocyst cell stage. Stages of embryogenesis were determined at different time points of culture under a stereomicroscope at 40- or 100-fold magnification. In the second series of experiment, all the animals were kept until parturition, when the pregnancy rate, parturition rate, number of liveborn pups and number of stillborn pups were recorded. On the 35th day after parturition number of weaned pups was recorded.

Statistics

In vitro experiments were performed on 30 control animals and 2 experimental animals in the IBMX group and 9 experimental animals in the dbcAMP group. *In vivo* experiments were performed on 15 control animals, 12 experimental animals per each of the IBMX and dbcAMP group. Average number of liveborn pups, stillborn pups, weaned pups, *corpora lutea*, ovulated oocytes, zygotes, morula, hatching blastocyst per doe was statistically evaluated using t-test or chi-square test in SigmaPlot (Systat Software Inc., Germany). Chi-square ($\chi 2$) test was used for the evaluation of conception rate and parturition rate. Results are expressed in % or as means ± SEM.

RESULTS AND DISCUSSION

In vitro experiments

Treatment of females with gonadotropins and subsequent artificial insemination resulted in development and ovulation of ovarian follicles, formation of *corpora lutea*, expulsion of oocytes into the oviduct, and subsequent embryo development *in vitro*. The IBMX and dbcAMP treatment was able to influence all these processes. Injections of IBMX and dbcAMP increased number of *corpora lutea* (IBMX 35.00±4.33, dbcAMP 38.34±2.20 vs. control 21.97±0.82 CL/doe), number of harvested zygotes (IBMX 30.00±2.88, dbcAMP 38.12±2.21 vs. control 21.20±0.82/doe), number of embryos at morula and hatching blastocyst stage (IBMX 30.00±2.88, dbcAMP 38.00±2.23 vs. control 21.20±0.82/doe, Table 1). The differences between the experimental groups and control group were statistically significant. These results are in accordance with results of previous study of Sirotkin *et al*. (2008; 2010c; 2010d), where IBMX injections at doses of 5, 25 or 50 μg/doe significantly increased the number of ovulations/*corpora lutea*, harvested zygotes, and embryos derived from these zygotes. These data confirm previous report of McKenna et al. (2005) that PDE inhibitors can increase the number of ovulations, embryos and born pups in gonadotropin-stimulated rats. Our results do not correspond to the report of Hosoi *et al.* (1989) where dbcAMP stimulated ovarian progesterone production, but did not affect ovulation or maturation of follicular oocytes in ovaries perfused *in vitro*. The stimulation of ovaries by exogenous gonadotropins can increase ovulation rate and number of ovulated oocytes, but decrease quality and developmental potential of harvested oocytes (Hillier, 2001; Craig *et al*., 2007). In our experiments, administration of dbcAMP was able not only to increase the number of harvested oocytes, but also number of fertilized oocytes (zygotes) and subsequent embryos reaching morula and hatching blastocyst stage after culture.

Table 1: Effect of IBMX and dbcAMP on female reproductive traits *in vitro*

Group	Number of ♀ n	Corpora lutea/♀ average per 1 ♀	Zygotes/♀ average per 1 ♀	Morula/♀ average per 1 ♀	Hatching blastocyst/♀ average per 1 ♀
Control	30	21.97±0.82^{a}	21.20±0.82^{a}	21.20±0.82^{a}	21.20±0.82^{a}
IBMX	2	35.00±4.33^{b}	30.00±2.88^{b}	30.00±2.88^{b}	30.00±2.88^{b}
dbcAMP	9	38.34±2.20^{b}	38.12±2.21^{c}	38.00±2.23^{c}	38.00±2.23^{c}

Values with different superscripts within columns are significantly different at $p<0.05$; (t-test)

In vivo experiments

Performed *in vivo* experiments showed that both tested substances decreased female fertility parameters - conception rate, parturition rate and weaning rate, but not stillborn pup rate compared to control. Conception rate was 33.3 % in the IBMX group, 58.3 % in the dbcAMP group versus 73.0 % in the control group. Parturition rate was 25.0 % in the IBMX group, 50.0 % in the dbcAMP versus 66.7 % in the control group (Table 2). The data were statistically significant. These data do not correspond to the report of Sirotkin *et al.* (2010b), where administration of IBMX at doses of 500 μg/animal or 50 μg/doe to nulliparous young animals (4.5 month of age) significantly increased their pregnancy rate, birth rate, litter size and litter weight. In multiparous old animals (2 years of age), IBMX at a dose of 50 μg/doe, but not 500 μg/doe, significantly increased their pregnancy rate and litter size, but not the birth rate, number of pups per female, or litter weight.

In our experiments, positive influence of IBMX (7.7±3.3 liveborn pups/doe) and dbcAMP (8.7±1.2 liveborn pups/doe) versus control (6.4±1.4 liveborn pups/doe) on liveborn pup rate was observed, although this difference was not statistically significant. In the parameter „stillborn pups" the IBMX group resulted in an average of 0.0±0.0 stillborn pups/doe, in the dbcAMP group in an average of 0.0±0.0 stillborn pups/doe too. In comparison, in the control group of does resulted in an average of 3.1±1.4 stillborn pups/doe and this difference was statistically significant. In the parameter „weaned pups" the IBMX group resulted in an average of 3.3±0.9 weaned pups/doe, in the dbcAMP group in an average of 5.5±0.7 weaned pups/doe versus control 7.1±0.8 weaned pups/doe (Table 2). These differences were statistically significant. Furthermore, performed *in vitro* and *in vivo* results showed that dbcAMP is more potent regulator of reproductive functions than IBMX. This could be due to the fact that IBMX is a nonspecific inhibitor of cAMP and cGMP phosphodiesterases, whilst dbcAMP is specifically targeting PKA. Mechanisms of IBMX and dbcAMP effects require further studies. Nevertheless the ability of these substances to affect production of porcine

(Kolesárová *et al*., 2008) and rabbit (Sirotkin *et al*., 2010e) steroids, IGF-I and proliferation-related substances suggest that the effects observed in our experiments could be due to changes in ovarian steroidogenesis and cell proliferation.

The differences between effects of IBMX and dbcAMP observed in our *in-vitro* and *in-vivo* experiments could be explained by different experimental models, time of material collection or physiological state of animals used. To confirm a pattern of action of these substances on rabbit female fertility and to understand the variability in these effects further studies are required. Nevertheless, our data suggest a potential usefulness of both dbcAMP and IBMX for the improvement of animal reproduction efficiency.

Table 2: Effect of IBMX and dbcAMP on female reproductive traits *in vivo*

Group	Inseminated ♀ n	Fertilized ♀/ conceptional rate n/% #	Kindled ♀/ parturition rate n/% #	liveborn pups(n)/ average per 1 ♀ ‡	stillborn pups(n)/ average per 1 ♀ ‡	weaned pups(n)/ average per 1 ♀ ‡
Control	15	11/73.0[a]	10/66.7[a]	(64)/6.4±1.4	(31)/3.1±1.4[a]	(71)/7.1±0.8[a]
IBMX	12	4/33.3[b]	3/25.0[b]	(23)/7.7±3.3	(0)/0.0±0.0[b]	(10)/3.3±0.9[b]
dbcAMP	12	7/58.3[c]	6/50.0[c]	(52)/8.7±1.2	(0)/0.0±0.0[b]	(33)/5.5±0.7[c]

Values with different superscripts within columns are significantly different at $p<0.05$; (# - Chi square test, ‡ - t-test)

CONCLUSIONS

The results of our *in vitro* experiments show that both IBMX and dbcAMP significantly increased female fertility parameters (number of *corpora lutea*, number of oocyte-derived zygotes, embryos at morula stage and number of embryos at hatching blastocyst stage) and the data were statistically significant. The present *in vivo* results show that both tested substances decreased female fertility parameters - conception rate, parturition rate, and weaning rate compared to control, but they did not affect the stillborn pup rate. IBMX and dbcAMP slightly increased the liveborn pup rate, but this increase was not statistically significant. These observations confirm the involvement of cAMP/protein kinase A-dependent intracellular signaling pathway in control of rabbit reproduction.

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