EFFECT OF db-cAMP ON EMBRYONIC DEVELOPMENT OF BOVINE OOCYTES FERTILIZED WITH SEX SORTED SEMEN.

Efecto del db-cAMP Sobre el Desarrollo Embrionario de Ovocitos Bovinos Fecundados con Semen Sexado.

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ABSTRACT

The objective of this study was to improve sexed bovine embryo production with sorted sperm in chemically defined conditions by supplementing the IVF medium with db-cAMP. Cumulus-oocyte complexes (COCs) were matured for 18 h in supplemented TCM-199 and fertilized with X- or Y-bearing sperm in the presence of heparin (10 µg/ml), db-cAMP (1 µM) or no treatment (control). Presumptive zygotes were cultured 54 h in g-SOF. From 72 to 144 h post- insemination (hpi) embryos were cultured in c-SOF+NEA and from 144 to 192 hpi embryos were placed in maturation medium without hormones. No significant differences were found among treatments for Y-sperm when compared to controls. A significant (P<0.01) improvement in the proportion of cleaved oocytes was found for X-sperm treated with db-cAMP (70.83%) compared to the Y-sperm inseminated oocytes treated wit db-cAMP (46.37%). Treatment with db-cAMP enabled a better (P<0.05) blastocyst formation rate (19.29%) compared to control (8.47%) and heparin (10.44%). Treatment of db-cAMP significantly increased the rate of blastocysts in X-sperm inseminated oocytes (30.77%) compared to Y-sperm inseminated oocytes treated the same (9.68%) and compared to X- and Y-sperm treated with heparin (5.88% and 15.15%, respectively) and not treated (9.68% and 7.14%, respectively, P<0.05). These results suggest that db-cAMP may prove to be an effective treatment of sorted sperm for *in vitro* production of female bovine embryos under chemically defined conditions.

Key words: Sorted sperm, db-cAMP, *in vitro* bovine embryos.

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RESUMEN

El objetivo de este estudio fue mejorar la producción de embriones bovinos con semen sexado bajo condiciones químicamente definidas mediante la suplementación del medio de fecundación con db-cAMP. Los complejos ovocitos cumulus (COCs) fueron madurados por 18 horas en TCM-199 suplementado y fueron fecundados con espermatozoides X o Y en presencia de heparina (10 g/ml), db-cAMP (1 µM) o sin tratamiento alguno (control). Los presuntivos cigotos fueron cultivados por 54 horas en g-SOF. Desde las 72 a las 144 horas post-inseminación (hpi) los embriones se cultivaron en c-SOF+NEA y desde 144 a 192 hpi fueron colocados en medio de maduración pero sin hormonas. No se observaron diferencias entre tratamientos para los oocitos fecundados con espermatozoides Y cuando se compararon a los controles. Se observó una mejora significativa (P<0,01) en la proporción de ovocitos que se dividieron cuando fueron fecundados con espermatozoides X tratados con db-cAMP (70,83%) en comparación con los fecundados con espermatozoides Y tratados con db-cAMP (46,37%). El tratamiento con db-cAMP fue capaz de inducir una mayor (P<0,05) tasa de formación de blastocistos (19.29%) en comparación a los tratamientos control (8,47%) y heparina (10,44%). El tratamiento con db-cAMP incrementó (P<0,05) la tasa de embriones de cuatro células alcanzando el estadio de blastocisto cuando los oocitos fueron fecundados con espermatozoides X (30,77%) en comparación a cuando la fecundación se realizó con espermatozoides Y (9,68%) y en comparación a cuando se llevó a cabo con espermatozoides X o Y en presencia de heparina (5,88% y 15,15%, respectivamente) o en el tratamiento control (9,68% y 7,14%, respectiva-

mente). Estos resultados sugieren, que el db-cAMP puede ser un tratamiento efectivo para el semen sexado, a fin de incrementar la producción *in vitro* de embriones bovinos hembra bajo condiciones químicamente definidas.

Palabras clave: Semen sexado, db-cAMP, embriones bovinos *in vitro.*

INTRODUCTION

Based on the differential content of DNA present in the sex chromosomes it is technically possible to separate semen ejaculates into X- and Y- rich fractions using a flow cytometer/cell sorting device [13,17,32]. Sperm sexing by flow cytometry is expensive, and use of sexed sperm for artificial insemination (AI) is limited by sperm number that can be sorted, 5-15 millions per hour [32]. In vitro fertilization (IVF) promises to become one of the most viable avenues for efficient use of sexed semen [3,32]. Nonetheless, up to date technology of sexed semen in combination with IVF has yielded inferior fertilization and development rates after using fresh sexed semen compared to unsexed control sperm [2]. A lower pregnancy rate (41%) was obtained using ram sexed semen when compared with pregnancy rates of ewes inseminated with non-sorted frozen-thawed semen (59%, P<0.001) [14].

An explanation for this lower fertility is the lower motility and acrosomal integrity that results after semen sexing procedures [14,31], in addition to the possible DNA damage [22]. Flow-cytometric sorting has been reported to compromise the viability and membrane integrity of sperm [26,27]. It has been suggested that fertility could be improved with the use of semen additives [33].

Dibutyryl cyclic AMP (db-cAMP) is well known as a sperm activator [9,15,23,25]. db-cAMP significantly stimulates sperm motility and metabolism in the bull [8,9,14,22], human [1], and primates [25]. In the bull, boar and stallion, db-cAMP stimulates sperm capacitation [4,6,29]. In bulls, this effect was accomplished by inducing protein tyrosine phosphorylations [7]. In cynomolgus macaques (*Macaca fascicularis*), db-cAMP increased the number of sperm bound to the zona pellucida and sperm motility increased only when combined with caffeine although, treatment of sperm with db-cAMP only increased the percentage of acrosome-reacted sperm on the zona pellucida [36]. The aim of this study was to evaluate the effect of db-cAMP during in vitro fertilization on bovine embryo production with sex sorted sperm under chemically defined conditions.

MATERIALS AND METHODS

In vitro maturation

Bovine ovaries collected at a slaughterhouse immediately after the sacrifice of the cows and were transported to the IVF Laboratory without added medium in thermos (28-32°C) reaching the laboratory within 3h. Immediately after arriving at the laboratory, cumulus oocyte complexes (COCs) were harvested from follicles with surface diameters less than 6 mm using a 10 ml syringe and 1½"x18G needles. Oocytes surrounded by at least two complete compact layers of cumulus cells were utilized without any further selection of COCs. They were washed twice with maturation medium and cultured in groups of 20-22 COCs in 100 µL drops of maturation medium covered with light mineral oil (S894-00, J.T. Baker, Phillipsburg. NJ, USA). The maturation medium was TCM-199 (M-3769, Sigma Chemical Co., St Louis, MO, USA) supplemented with 50 µg/ml sodium pyruvate (Sigma Chemical Co., St Louis, MO, USA), 2.2 µg/mL NaHCO3 (Sigma Chemical Co., St Louis, MO, USA), 1 mg/ml PVA (Sigma Chemical Co., St Louis, MO, USA), 0.25 mM glutamine (Sigma Chemical Co., St Louis, MO, USA), 0.1 mM cystine (Sigma Chemical Co., St Louis, MO, USA), 0.1 mM cysteine (Sigma Chemical Co., St Louis, MO, USA), 10 mM hydroxymethylpiperazine ethanesulfonic acid (HEPES, Sigma Chemical Co., St Louis, MO, USA), 50 µg/mL gentamicin sulphate (Sigma Chemical Co., St Louis, MO, USA) plus 0.1 UI/mL of rhFSH (1.7 UI/µg, Ares Advanced Technology Inc., Randolph, MA, USA) and 5 ng/mL of rhIGF-I (Promega, Madison, WI, USA). Incubation of COCs took place under saturated moist atmosphere of 5% $C0_2$, 5% O_2 and 90% N_2 in a modular incubator chamber (Billups-Rothenberg Inc., Del Mar, CA, USA) at 38.5°C for 24 h. This atmosphere, reagents and these physical conditions were employed for subsequent in vitro embryo cultures.

In vitro fertilization

Refrigerated flow-cytometric sorted semen from one Angus bull was used. X- and Y-rich fractions were centrifuged for 10 min at 2500 rpm. The resulting pellet was diluted in 5 mL of modified defined medium (mDM) [12,18,19] and then centrifuged at 1400 rpm for 5 min. After centrifugation, the pellet was recovered from the bottom of the tubes by aspirating 200 µl of medium. Before insemination an aliquot of semen was taken to evaluate sperm motility and concentration in order to adjust the volume to be added into the insemination drop. The volume needed (12-14 µl) to achieve a concentration of 2 x 10^6 motile spermatozoa per mL was added to each 86-88 µl drop (to complete 100µl) of mDM prepared with one of the following three treatments: control (no treatment), heparin (10 µg/ml) or db-cAMP (1 µM). Matured oocytes were added to each of these drops, and the gametes were coincubated for 18 h, under the same conditions of temperature, moisture and gas atmosphere described previously.

In vitro culture

After the insemination interval, i.e. 18 hpi, loosely attached cumulus cells were removed by gentle pipetting of the oocytes. Presumptive zygotes were washed thoroughly before they were placed in culture drops. Presumptive zygotes were cultured in groups of 20 in 50 mL of culture drops. Embryo cul-

tures were done using three sequential media as follows: From 18 to 72 hpi, presumptive zygotes were cultured in synthetic oviductal fluid (SOF) modified by addition of 0.1 mM non-essential amino acids (M-7145, Sigma Chemical Co., St Louis, MO, USA), 0.5 mM glutamine (Sigma Chemical Co., St Louis, MO, USA), 0.4 mM threonine (Sigma Chemical Co., St Louis, MO, USA) and 3 mg/ml PVA (Sigma Chemical Co., St Louis, MO, USA) [18]. At 72 hpi, embryos of at least four cells were freed from any remaining cumulus cells and were cultured in SOF containing citrate and without glutamine, i.e. c-SOF + NEA [12,18,19] plus 0.4 mM threonine. After 144 hpi, the basic media was the same as described above for oocyte maturation medium but without hormones.

Statistical analysis

Proportions of oocytes reaching the four-cell stage and four-cell stage embryos reaching the blastocyst stage according to treatment and X- or Y- chromosome-bearing sperm fertilization were analyzed by Chi-square (Statistical Analysis System, SAS) [30].

RESULTS AND DISCUSSION

The presence of db-cAMP during in vitro fertilization did not significantly improve the rate of oocytes cleaving (58.86%, 83/141) compared to heparin (46.25%, 74/160) and control (49.54%, 54/109, P>0.05). db-cAMP during fertilization using Y-chromosome-bearing sperm (Y-sperm) did not affect the percentage of resulting four-cell embryos (46.37%, 32/69) in comparison with Y-sperm treated with heparin (43.20%, 35/81) or Y-sperm controls (43.13%, 22/51), P>0.05 (see FIG. 1). In contrast, a significant (P<0.01) improvement in the percentage of oocytes reaching the four-cell stage was observed in the group of oocytes fertilized with X-chromosome-bearing sperm (X-sperm) treated with db-cAMP (70.83%, 51/72) in comparison to X-sperm treated with heparin (49.36%, 39/79) and controls (55.17%, 32/58). A significant difference between X- and Y-sperm treated with db-cAMP was also observed (P<0.01).

The blastocyst formation rate was affected by the presence of db-cAMP during in vitro fertilization (19.29%, 11/57) compared to the heparin (10.44%, 7/67) and control (8.47%, 5/59) groups (P<0.05). The proportion of four-cell embryos reaching the blastocyst stage was significantly higher (P<0.05) when oocytes were inseminated with X-sperm in the presence of db-cAMP (30.77%, 8/26) compared to Y-sperm in the presence of db-cAMP (9.68%, 3/31) and to X- and Y-sperm fertilization groups in the presence of heparin (5.88%, 2/34 and 15.15%, 5/33 respectively) or control (9.68%, 3/31 and 7.14%, 2/28 respectively). No effects of treatments were observed on the resulting blastocyst formation rates when oocytes were fertilized with Y-sperm (see FIG. 2).

Exogenous cyclic nucleotides or inhibition of cyclic nucleotide phosphodiesterase have been reported to stimulate sperm motility and fertilization of oocytes in vitro [5,23]. Stimulation of sperm motility is dependent on protein phosphorilation, a process activated by c-AMP-dependent protein kinase A (PKA) [16,36]. PKA activity has been shown to be increased during capacitation [36]. In the mouse sperm, adenylate cyclase activity (which is involved in the production of cyclic adenosine monophosphate, cAMP) increased in a time-dependent manner simultaneously with a decrease in phosphodiesterase activity [5,28,34].

Dibutyryl cyclic AMP improves the developmental competence of bovine oocytes maintained in meiotic arrest with cycloheximide but not in its absence [10]. In this study, fertilization in the presence of db-cAMP significantly increased the

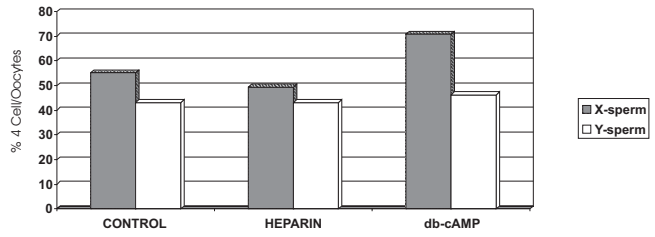

FIGURE 1. PROPORTION OF IN VITRO INSEMINATED OOCYTES REACHING AT LEAST THE FOUR-CELL STAGE BY 72 HPI AFTER USE OF CELL-SORTED X- AND Y-BEARING SPERMATOZOA TREATED WITH HEPARIN, db-cAMP OR NO TREATMENT (CONTROL). [a, b] DIFFERENT (P<0.01) / PROPORCIÓN DE OVOCITOS INSEMINADOS IN VITRO QUE ALCANZARON EL ESTADIO DE EMBRIONES DE CUATRO CÉLULAS, 72 HPI LUEGO DEL USO DE SEMEN SEXADO X O Y, EN PRESENCIA DE HEPARINA, db-cAMP O CONTROL (SIN TRATAMIENTO). [a, b] DIFERENTES (P<0,01).

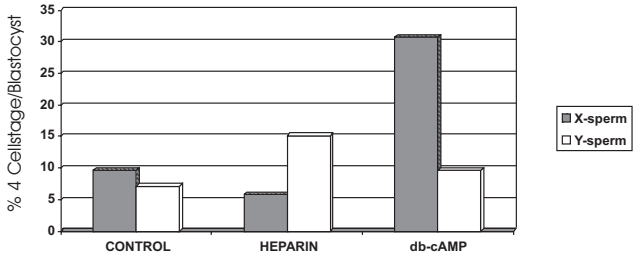

FIGURE 2. PROPORTION OF FOUR-CELL EMBRYOS REACHING THE BLASTOCYST STAGE (192 HPI) AFTER IVF TREATMENTS WITH HEPARIN, db-cAMP OR NO TREATMENT (CONTROL). [A,B] DIFFERENT (P<0.05) BETWEEN X- AND Y-BEARING SPERM INSEMINATED GROUPS / PROPORCIÓN DE EMBRIONES DE CUATRO CÉLULAS ALCANZANDO EL ESTADIO DE BLASTOCISTO (192 HPI) LUEGO DE LA FECUNDACIÓN IN VITRO CON HEPARINA, db-cAMP O SIN TRATAMIENTO (CONTROL). [a,b] DIFERENCIAS (P<0,05) ENTRE GRUPOS INSEMINADOS CON ESPERMATOZOIDES X O Y.

proportion of oocytes reaching the four-cell stage when oocytes were fertilized with X-sperm. This effect could possibly be explained by intrinsic physiological differences or different gene expression patterns between X- and Y-bearing sperm cells [11,39] and according to Madrid-Bury et al., [24], differences in capacitation between X- and Y- sperm are possible.

Alternatively, the maturation state of the oocyte might be modified by elevated intracellular concentrations of cAMP that could alter the way in which oocytes favour to be fertilized by either X- or Y-bearing sperm cells. Earlier work suggested a difference in oocyte interaction (according to maturation stage) with X- and Y-bearing spermatozoa [11]. Maturation of pig oocytes in the presence of db-cAMP increased the synchronization of meiotic maturation resulting in high developmental competence measured in terms of monospermic fertilization [35]. Probably, db-cAMP acts through several and unknown mechanisms both in sperm cells and oocytes in such a way that favors oocyte fertilization by the X-sperm.

Recently, the duration of gamete interaction has been associated to alterations in the sex-ratio of in vitro produced bovine embryos [20], with a short interaction increasing the proportion of male embryos. Nonetheless, in the present study, both oocytes and X- or Y-sperm cells were coincubated during the same time interval (18 h). When bovine spermatozoa were pre-incubated prior to in vitro fertilization, the sex-ratio was altered in favor of the female sex [21]. This suggests that the X-sperm cells have a greater longevity. In addition, X-bearing sperm may have a greater ability to complete the process of fertilization due to the greater longevity in motility, higher progression and hyperactivation shown by the X-bearing sperm after 24 h of incubation when compared to Y-bearing sperm [38]. This greater potential for fertilization could be increased by the presence of db-cAMP.

No beneficial effects on cleavage and blastocyst formation rate were seen after treatment of sexed sperm with heparin compared to control, and this supports earlier work demonstrating initiation of sperm capacitation due to cell sorting procedure normally used in sperm sexing [27]. These results suggest that heparin and db-cAMP not only act by different mechanisms to activate the sperm but also possibly act by sex sperm-dependent mechanisms.

CONCLUSIONS

db-cAMP during in vitro fertilization may differentially affect the development of ova fertilized by X- and Y-bearing spermatozoa as was observed in this study by the increased blastocyst development of cleaved ova fertilized by X-bearing spermatozoa. These results suggest that db-cAMP may prove to be an effective treatment of sex-sorted spermatozoa for production of female bovine embryos in vitro under chemically defined media conditions. More studies are necessary to further add clarity to these and other findings.

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