Penicillin-Streptomycin in the Culture Medium during In Vitro Maturation (IVM) of Bovine Oocytes Affects Nuclear Maturation and Subsequent Embryo Development

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Abstract

Introduction: Standard concentrations of antibiotics in culture media are thought to have no detectable toxic effects on the cultured cells. However, since antibiotics are biologically active substances, the possibility that they interfere to some extent with cellular processes occurring in the cultured cells can not always be totally excluded. This study, therefore, was conducted to assess whether the presence of penicillin-streptomycin (pen-strep) during in vitro maturation (IVM) of bovine cumulus oocyte complexes (COCs) affect nuclear and cytoplasmic maturation and subsequent embryo development.

Materials and Methods: Bovine COCs were matured at 39oC in a humidified atmosphere with 5 % CO_2 in air for 24 h in: 1- culture medium M199 supplemented with 10 % FCS (Fetal calf serum), 0.05 IU/ml rhFSH (recombinant human FSH) and 100 units penicillin and 100 μg streptomycin/ ml. 2- culture medium M199 without FCS and rhFSH in the presence of pen-strep. Cultures without antibiotics served as control. Six series of experiments, each consisted of at least 3 replicates, were performed.

Results: In vitro maturation in the presence of pen-strep in culture medium supplemented with FCS and rhFSH significantly ($P<0.05$) increased the percentage of MII oocytes, however, when the COCs were divided, on the basis of apperance of the cumulus investment, into bright and dark groups, this effect was less obvious in both types of COCs, 76% vs 72% in bright COCs ($P=0.149$) or 83% vs 80% in dark COCs ($P=0.296$) in treated and control groups respectively. The percentage of oocytes with type III of cortical granules (CGs) distribution was not affected in the presence of pen-strep. The COCs expansion after IVM was not affected by the presence of antibiotics in culture medium. The subsequent embryo development of IVM/IVF produced ova, which were exposed to pen-strep during IVM, was significantly ($P<0.05$) decreased with respect to blastocyst formation by day 9. In vitro maturation in the presence of pen-strep in culture medium without FCS and rhFSH had no significant ($P=0.402$) effect on nuclear maturation.

Conclusion: The results indicate that the nuclear maturation of bovine oocytes was positively influenced by the presence of pen-strep during IVM when the culture media was supplemented with FCS and rhFSH. Moreover, despite of no notable effect of pen-strep on CGs distribution the subsequent embryo development was negatively influenced by the presence of pen-strep.

Key Words: IVM, rhFSH, Pen-strep, Nuclear maturation, Cortical granules

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Introduction

Standard concentrations of antibiotics in culture media are thought to have no detectable toxic effects on the cultured cells. However, since antibiotics are biologically active substances, the possibility that they interfere to some extent with cellular processes, occurring in the cultured cells, can not always be totally excluded (1).

Although the application of the combination of penicillin, belonging to the group of ß-lactam antibiotics and of streptomycin, belonging to the group of aminoglycosides, gives a wide-spectrum activity against microbial agents (2) and a minimum undesirable effects on eukaryotic cell, they may influence some part of cellular activities (3, 4). Several studies have been conducted to assess the risks of aminoglycoside and ß-lactam antibiotics on eukaryiotic cells (5, 6, 7, 8, 9). It was documented that in a time and concentration dependent manner they could affect some parts of cellular functions (10, 11, 12). It has been reported that antibiotics can adversely affect the growth rate of mammalian embryos by interfering with cell cleavage events or by blocking embryo development (13).

This study, therefore, was conducted to investigate whether the routine addition of penicillin-streptomycin to the culture medium during the in vitro maturation of bovine COCs affects the nuclear and cytoplasmic maturation of the oocytes and whether this has consequences for embryo development following fertilization.

Materials and Methods

* *Collection and culture of cumulus oocyte complexes*

Bovine ovaries were collected at a slaughterhouse in a thermos flask and transported to the laboratory within 1 h. The COCs were obtained by aspiration of 2-8 mm follicles and selected on the basis of presence of a multilayered compact cumulus investment (at least 3 layers). Selected COCs rinsed, once in HEPES buffered M-199 (Gibco BRL, Paisly, UK) and once again in maturation medium (M 199 + Earle ssalt + glutamine) (Gibco cat nr 31100-027) and then, in groups of 35, randomly allocated in each well of a 4-well culture plate (Nunc A/S, Roskilde, Denmark) containing 500 μl maturation medium, and cultured at 39°C in humidified atmosphere of 5 % CO_2 in air.

* *In vitro fertilization and embryo culture*

Both IVF and IVC (In vitro culture) took place at 39°C in a humidified atmosphere of 5% CO_2 in air. Frozen/thawed bovine spermatozoa used for IVF were centrifuged over a percoll gradient for 30 min at 700 x g at 27°C. The sperm sample was collected by removing the gradient except for the last 150 μl containing the sperm pellet. Thirty-five COCs were transferred to 0.43 ml of fertilization medium (Fert-Talp), as described by Parrish et al. (14), without glucose and 100 units penicillin and 100 μg streptomycin/ml instead of gentamycin. Twenty microliter of sperm suspension (final concentration 0.5 x 10^6 spermatozoa/ml), 20 μL heparin (final concentration 10 μg/ml), and 20 μL PHE (consisting of 20 mM D-penicillamine, 10 mM hypotaurine, 1 mM epinephrine), were added. After 18-22 h of incubation, the oocytes were freed from cumulus cells by vortexing for 3 min and all the zygotes were placed in culture medium. Following IVF groups of 35 oocytes were randomly placed in a co-culture system of 0.5 ml of M199 containing 10 % FCS on a monolayer of BRL (Buffalo rat liver) cells in each well of a 4-well culture plate. On the fourth and eighth day of culture, embryos were transferred to fresh co-culture wells.

* *BRL-cell Culture*

Buffalo rat liver cells, separated from the BRL cell line from the American Type Culture Collection (ATCC) (15) were cultured routinely in a 1:1 mixture of Ham's F12 medium and Dulbecco's Modified Eagle's medium (Gibco) supplemented with 7.5 % FCS fetal Calf Serum (Gibco) and antibiotics. These cells differ from those currently available from ATCC in that they exhibit contact inhibition of growth.

* *Assessment of nuclear maturation and cumulus expansion*

After culture the nuclear status of the oocytes was determined by DAPI-staining as described by Mori

et al. (16). Briefly COCs were denuded by vortexing for 3-6 min and then fixed for 15-30 min in 2.5 % w/v glutaraldehyde, washed with PBS, stained with 2.5 % w/v 4,6-diamino-2-phenyl-indole (DAPI) for 2-3 min, washed with PBS, and then mounted on slides. The nuclear status of the stained oocyte was assessed under a fluorescence microscope. Oocytes in which diffuse or slightly condensed chromatin could be identified, were classified as being in the germinal vesicle (GV) stage. Oocytes that possessed clumped or strongly condensed chromatin that formed an irregular network of individual bivalents (prometaphase) or a metaphase plate but no polar body were classified as being in the Metaphase I (MI) stage, and oocytes with either a polar body or two shiny chromatin spots were classified as being in the Metaphase II (MII) stage of the maturation process. Cumulus expansion was assessed by measurement of the diameter of the COCs, using a calibrated stage micrometer, at the end of the incubation (24 h). Diameter measurement for each COCs was done in two perpendicular directions.

* Assessment of cortical granules distribution

After in vitro maturation, cumulus cells were removed from the oocytes by vortexing for 3 min. Cortical granules distribution was assessed by immunofluorescence method as described by Yoshida et al. (17); cells were permeabilized in 0.1% Titon-X-100 (v/v) in PBS for 5 min at 39°C . The oocytes were then fixed in 2% (v/v) paraformaldehyde in PBS for 30 min at room temperature. After fixation, the oocytes were washed 3 times in PBS and then incubated in a blocking solution containing 0.1 M glycine, 0.01% Triton-X -100 (w/v), 1% (w/v) powdered milk, 0.5% (w/v) BSA and 0.02 % (w/v) sodium azide, Kim et al. (18) at 39°C for 1 h. Subsequently oocytes were incubated in 100 μg/ ml FITC conjugated lectin (peanut agglutinin; PNA) (FITC- PNA) (EY Laboratories, Inc., San Mateo, California) in PBS at 39°C for 30 min. Following washing with PBS (3 times), oocytes were stained with 2.5 % w/v DAPI at room temperature. The oocytes were mounted under a coverslip and evaluated under an epifluorescence microscope and according to the observed distributional pattern of the CGs, the oocytes were classified in three types: I) Large aggregates of CGs distributed over the entire cytoplasm, II) CGs localized in the cortical cytoplasm and distributed as individual particles as well as small aggregates, III) CGs more or less evenly distributed in the cortical cytoplasm aligning the oolemma (17).

* Experiments

Six series of experiments were performed. Each experiment consisted of at least three replicates.

I) COCs were matured in vitro in medium M-199 supplemented with 10% FCS and 0.05 IU/ml recombinant hFSH (Organon International, Oss, The Netherlands), in the presence of 100 units penicillin and 100 μg streptomycin/ml (Gibco). The percentage of oocytes in different nuclear maturational stages was determined after 24 h of incubation. Cultures without antibiotics served as controls.

II) COCs were matured in vitro in medium M-199 without FCS and without rhFSH in the presence of pen-strep, and then the nuclear status of oocytes was assessed after 24 h of incubation. Cultures without antibiotics served as controls.

III) Two groups of bright and dark COCs (the classification of COCs in two groups was based on the compactness and clarity of the cumulus investment; de Wit et al. (9) were matured in vitro in medium M-199 supplemented with FCS and rhFSH, in the presence of pen-strep. Then the nuclear status of oocytes was assessed after 24 h of incubation. Cultures without antibiotics served as controls.

IV) Pattern of cortical granules distribution in MII (Metaphase II) oocytes after 24 h of incubation of COCs, in medium M-199 supplemented with FCS and rhFSH, in the presence of pen-strep was investigated. Cultures without antibiotics served as controls

V) Cumulus expansion of COCs after 24 h of incubation in medium M-199 supplemented with FCS and rhFSH, in the presence of pen-strep was evaluated. Cultures without antibiotics served as controls

VI) COCs were matured in medium M-199 supplemented with FCS and rhFSH, in the presence of pen-strep, in vitro fertilized and the presumptive

zygotes were cultured on a monolayer of BRL-cells as described. Cultures without antibiotics served as controls.

* *Assessment of embryo development*

Embryos were scored morphologically, and the efficiency of the culture systems was evaluated as (I) the percentage of cleaved embryos 4 days after fertilization, (II) the percentage of blastocysts on day 9 expressed on the basis of the number of oocytes at the onset of culture and (III) the percentage of hatched blastocysts on day 11 expressed on the basis of the total number of blastocysts on day 9.

* *Statistical analysis*

The results were analysed by the Chi-square test and P< 0.05 was considered significant.

Results

The effect of addition of pen-strep to M-199 supplemented with FCS and rhFSH, during in vitro maturation of bovine oocytes is shown in table 1. As shown the percentage of MII oocytes was significantly increased in treated group, which supplemented with pen-strep in comparison to the control group.

Table 1: The effect of addition of pen-strep to M199 supplemented with FCS and rhFSH on the nuclear maturation of bovine oocytes cultured for 24 h

Experimental groups	Tutal number of oocytes	*Nuclear stage (%)*		
		GV	MI	MII
Control	471	7(1)	81(17)	383(81)[a]
Pen-strep	438	8(2)	52(12)	378(86)[b]

a, b; (P<0.05)

As depicted in table 2, the effect of the presence or absence of pen-strep in maturation media, M-199 without FCS and without rhFSH, on nuclear maturation was evaluated after 24 h of incubation. As shown the percentage of MII stage oocytes was not influenced by antibiotic supplementation (P= 0.402).

The effect of pen-strep supplementation during IVM in maturation media, M-199 supplemented with FCS and rhFSH, in two categories of bright and dark COCs, on nuclear maturation was evaluated after 24 h of incubation (tables 3).

Table 2. The effect of addition of pen-strep to M199 without FCS and rhFSH on the nuclear maturation of bovine oocytes cultured for 24

Experimental groups	Tutal number of oocytes	*Nuclear stage (%)*		
		GV	MI	MII
Control	513	39(7)	142(28)	2332(65)
Pen-strep	551	29(5)	152(28)	370(67)

As shown the percentage of MII stage oocytes in dark COCs after 24 h of incubation, was significantly (P<0.05) higher than the corresponding percentage in bright COCs. There was no significant difference in percentage of MII oocytes in the presence or absence of antibiotic in both types of COCs, however, there was a trend toward the pen-strep supplemented groups, 76% vs 72% in bright COCs (P=0.149) and 83% vs 80% in dark COCs (P=0.296) in treated and control groups, respectively.

Table 3. The effect of addition of pen-strep to M199 supplemented with FCS and rhFSH on the nuclear maturation of bovine oocytes from bright and dark COCs cultured for 24h

Types of COCs	Experimental groups	Total number of COCs	Nuclear stage(%)		
			GV	MI	MII
Bright	Control	558	2	154(27)	402(72)
	Treated	638	3	152(24)	483(76)
Dark	Control	345	0	70(20)	275(80)
	Treated	399	0	69(17)	330(83)

The pattern of CGs distribution in MII stage oocytes after 24 h of incubation in the presence or absence of pen-strep was shown in table 4. The percentage of type III of CGs distribution showed no notable difference in the presence or absence of pen-strep in maturation media, M-199 supplemented with FCS and rhFSH.

Table 4: The effect of addition of pen-strep to M199 supplemented with FCS and rhFSH on cortical granules distribution in bovine oocytes cultured for 24 h

Experimental groups	Tutal number of oocytes	Cortical granules distribution (%)		
		TypeI	TypeII	TypeIII
Control	292	6(2)	199(68)	87(30)
Treated	292	18(6)	182(62)	92(31)

Table 5. The effect of addition of pen-strep to M199 supplemented with FCS and rhFSH on the embryo development following IVF of bovine oocytes matured for 24 h

Experimental Group	No. of oocytes	No. of cleaved embryos (%)	No. of blastocysts Total 9(%)	No of blastocysts & heatched blastocysts Total day 11(%)	
Control	537	360(67)	135(25)[a]	143(27)	91(64)
Treated	541	357(66)	107(20)[b]	129(24)	82(64)

a, b; ($P<0.05$)

As indicated in table 5 when pen-strep was applied during IVM (24 h exposure), there was no significant difference ($P= 0.715$) in cleavage rate by day 4 of culture. Regarding to blastocysts formation by day 9, it was significantly ($P<0.05$) retarded by exposure of IVM/IVF produced ova to pen-strep during IVM. By the day 11, there was no significant difference in the percentage of hatched blastocyst formation.

The comparison of COCs expansion after 24 h of incubation in the presence or absence of pen-strep showed no significant difference between experimental groups (table 6).

Table 6. The effect of addition of pen-strep to M199 supplemented with FCS and rhFSH on the cumulus expansion of bovine COCs cultured for 24h

Condition of Culture	no. COCs	COC diameter (μm) Mean±SD
Pen-strep	150	32.75±6.98
Control	130	32.90±7.71

Discussion

Antibiotics are common to all media used in tissue culture, in order to avoid microbial contamination. Supplementation with antibiotics is routinely carried out using concentration determined in pioneering studies which evaluated their toxicity in cell culture systems (4, 19). These experiments selected penicillin and streptomycin as the most useful and generally safe drugs. The concentration recommended for cell culture is, 100 IU/ml and 100 μg/ml for penicillin and streptomycin, respectively. It is the same concentration, which is usually used in culture of bovine oocytes and embryos without regard to their possible effects, however small, on cell metabolism (20), and embryo morphology or its growth rate.

According to the results presented in this study, addition of antibiotics (pen-strep) during IVM of bovine COCs to maturation medium, M-199 supplemented with rhFSH and FCS, has a positive effect on the rate of nuclear maturation of oocytes and this effect became statistically significant ($P< 0.05$) if the period of incubation was extended to 24 h. When the exposure time to antibiotics, during IVM, decreased to 16 h no significant effect on nuclear maturation was observed (21). Interestingly, when rhFSH and FCS were removed from culture media the positive effect of pen-strep on nuclear maturation was omitted. Hence, it might be concluded that pen-strep, probably, potentiates the effects of rhFSH and FCS on resumption of nuclear meiosis.

Many studies in vivo point to interaction between aminglycosides and membranes and/or enzymes associated with membranes (9, 22, 23, 24, 25, 26). It has been known that these antibiotic can interfere with signal transduction mechanisms and influence agonist binding of G-protein coupled receptors (27, 28).

It is indicated that the effect of FSH on resumption of meiosis induced indirectly via cumulus cell, which is known to be mediated via cAMP signal transduction pathway (29). Therefore, it might be proposed that pen-strep potentiate the effects of rhFSH, probably, via a G-protein coupled mechanism. Since, we could not see the positive effect of pen-strep on cumulus cells expansion, the therefore, this question that how the pen-strep could affect the process of nuclear maturation remaines to be elucidate. It was also indicated that penicillin and streptomycin could interfere with ion channel (30, 31, 32, 33, 34) and some other receptor (35).

The effects of pen-strep on two different types, Bright & dark, of COCs on nuclear maturation showed no significant difference between treated and control groups. However, as depicted there was a trend toward the treated groups. The overall percentage of

MII oocytes in dark COCs after 24 h of incubation was significantly (P<0.05) higher than that of bright COCs. These results are in agreement with the results of Leibfried and First (36), de Wit et al. (37) and Laurincik et al. (38, 39) which have shown that the type of COCs before culture reflects the ability of the oocyte to mature in vitro. Overall the resumption of meiosis in oocytes derived form dark COCs was accelerated and at all time points the maturational stages of oocytes drived from dark COCs seemed to be a few hours ahead of bright COCs (37).

Inspite of the positive effect of pen-strep on nuclear maturation, the cytoplasmic maturation has not been affected, the pattern of cortical granules ditribution of type III showed no notable difference in the presence or absence of antibiotics. The pattern of cortical granules distribution is a reliable indicator for evaluation of the developmental competence of bovine oocytes (17).

Addition of pen-strep during IVM had an adverse effect on the rate of embryo development. The cleavage rate had not been affected by day 4, though, the rate of blastocyst formation at day 9 of embryo culture was significantly (P< 0.05) retarded by exposure of IVM/IVF produced ova to antibiotic during IVM.

As indicated, if we consider CGs distribution as a reliable indicator of cytoplasmic maturation, it had not been affected by the presence of pen-strep while the subsequent embryo development had been influenced negatively. Therefore, it might be concluded that besides CGs distribution, other indicators such as the pattern of mitochondrial distribution (40) or rearrangement of other organelles and microvilli should be considered (41) for proper assessment of the process of cytoplasmic maturation.

The inhibitory effect of antibiotics on the proliferation of eukaryotic cells cultured in vitro may occur at different stages of cell metabolism. It has been recently, documented that the protein synthesis inhibitors may exert its effect on triggering or blocking the mechanisms of programmed cell death (42) and it is indicated that, faster cleaving embryos have been clearly demonstrated to be more capable of implantation in animal species (43) and it has been also shown a strong correlation between chromosomal abnormalities and delayed embryo development (44, 45).

Among the several mechanisms of toxicity of beta-Lactam antibiotics, toxicity to mitochondrial anionic substrate carriers, with a secondary reduction of mitochondrial respiration with those substrate is common (12, 46).

It has been postulated that the aminoglycosid antibiotics may interact electrostaticaly with membrane anionic phospholipids and disrupt membrane structure and function, these antibiotics also alter membrane permeability and promote membrane aggregation and disrupt the function of other membranes and organelles including mitochondria (47). This group of antibiotics can inhibit the (Na^{+}-K^{+})-ATPase activity of membrane and may possibly disrupt the balance of cellular electrolytes, leading to a cellular dysfunction (48).

Assessment of the COCs expansion as an indicator of normal process of IVM indicated that the presence of pen-strep had no adverse effect on this process.

In conclusion, the analysis of data presented in this study suggest that the process of nuclear maturation of bovine oocytes is positively influenced by the presence of antibiotic in maturation media, supplemented with rhFSH and FCS. Although, pen-strep had no notable effect on the pattern of CGs distribution but embryo development was negatively affected by the presence of antibiotics during IVM, probably due to its adverse effects on the pattern of distribution and/or structures of other organells such as mitochondria, vesicles and microvilli which in turn led to interference with the timing of cleavage events, either by delaying or blocking embryo development.

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