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Expression of PTHrp and PTHR (PTH/PTHrP-r) mRNAs and Polypeptides in Bovine Ovary and Stimulation of Bovine Blastocyst Development *in vitro* Following PTHrP Treatment during Oocyte Maturation[*]

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Abstract

Parathyroid hormone related protein (PTHrP) and its receptor have well established roles in the development and regulation of many tissues, including bone and mammary gland. The objectives of this study were: 1) to characterize the distribution of mRNAs encoding parathyroid hormone (PTH)-related protein (PTHrP) and receptor (PTHR) in bovine ovary; 2) to characterize the distribution of PTHrP and PTHR polypeptides in bovine ovary; and 3) to examine the influences of PTHrP (1-141) treatment during bovine oocyte maturation *in vitro* on blastocyst development. mRNAs encoding PTHrP and PTHR were detected by *in situ* hybridization methods in oocytes, and granulosa cells in all follicles from primordial to large antral. PTHrP and PTHR polypeptides displayed distinct distribution patterns with PTHrP polypeptides primarily confined to oocytes from primordial to large antral follicles. PTHrP polypeptides were detectable but at a reduced level in ovarian stroma and in granulosa and thecal layers. PTHR polypeptides were detected in oocytes of all follicular stages but were predominantly found in ovarian stroma, granulosa and theca follicular layers. Supplementation of serum-free cSOFMaa oocyte maturation medium with PTHrP (1-141) resulted in a concentration-dependent increase in development to the blastocyst stage *in vitro*. The results suggest that granulosa cells may be a primary site of PTHrP production and release. Oocytes from all follicular stages stained strongly for PTHrP polypeptides and PTHrP enhanced development to the blastocyst stage *in vitro*.

Introduction

With the characterization of effective, defined and protein-free media to support the maturation of mammalian oocytes *in vitro* it is now possible to examine the roles played by specific hormone and growth factor modulators during oocyte maturation and upon subsequent early embryonic development (Saeki et al., 1991; Gardner, 1994; Eckert et al., 1995; Keskintepe and Brackett, 1996; Hill et al., 1997; Avery et al., 1998; Krishner and Bavister, 1999; Watson et al., 2000). Parathyroid hormone related protein (PTHrP) was originally isolated from patients suffering from humoral hypercalcemia of malignancy (Spiegel et. al., 1983; Strewler et. al., 1987; Stewart et. al., 1987). As its name suggests, this molecule shares structural homology with parathyroid hormone (PTH) (Stewart et. al., 1987; Horiuchi et. al. 1987), which enables PTHrP and PTH to signal through a common PTH/PTHrP receptor (Rodan, et. al., 1988; Abou-Samra, et. al., 1992). While PTH is predominantly (if not exclusively) produced by the parathyroid glands, PTHrP is synthesized by many tissues including skin, brain, pancreas, adrenal glands, smooth muscle, heart, lung, lactating breast, uterus, ovary and placenta (Urena et. al., 1993; Tian et. al., 1993; Lee et al., 1995; Weaver et. al., 1995; Downey et. al., 1997; Li et. al., 1995; Vasavada, et. al., 1998; Curtis et. al., 1998; Ferguson et. al., 1998; Moseley and Gillespie, 1995; Gutmann et. al., 1993). PTHrP is predominantly a paracrine/autocrine regulator of cellular growth and differentiation (Moseley and Gillespie, 1995). The expression and distribution of gene products encoding PTHrP and the PTHR have not been investigated within the bovine ovary. The objectives of the present study were: 1) to characterize the distribution of mRNAs encoding parathyroid hormone (PTH)-related protein (PTHrP) and receptor (PTHR) in the bovine ovary; 2) to characterize the distribution of PTHrP and PTHR polypeptides in the bovine ovary; and 3) to examine the influences of PTHrP (1-141) treatment during bovine oocyte maturation *in vitro* on blastocyst development.

Materials and methods

Detection of PTHrP and PTHR mRNAs in Bovine Ovary by *in situ* Hybridization

Bovine ovaries were collected at a local slaughterhouse and were quartered and placed into 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.2 fixative for overnight fixation. Ovarian pieces were then processed for routine paraffin embedding. *In situ* hybridization (ISH) was carried out using biotin-labeled sense and antisense riboprobes and the Genpoint CSA kit (Dako Diagnostics, Mississauga, ON, Canada) as described (Watson, et. al., 2000a; Watson, et. al., 2000b). Labeled riboprobes were prepared from plasmids containing either a 2Kb cDNA corresponding to rat PTHR mRNA (Pausova, et.al., 1994; Amizuka, et.al., 1997) or a 0.7 Kb cDNA corresponding to rat PTHrP mRNA (Yasuda et.al., 1989) using T3 and T7 RNA polymerases (Gibco, Burlington, ON, Canada) and a nucleotide labeling mix containing biotin-16-UTP (Boerhinger-Mannheim, Burlington, ON, Canada). Tissue sections were deparaffinized and rehydrated in a standard xylene and alcohol series and ISH performed according to the manufacturer's directions. Briefly, slides were heated in Target Retrieval Solution and proteinase K to undo protein cross-links caused by fixation. Endogenous peroxidase activity was quenched in 0.3% hydrogen peroxide in methanol for 50 min at room temperature. Sections were hybridized for 2 hours at 42^{o}C with 5 ng/ml biotin-labeled riboprobe in the supplied RNA hybridization buffer. After stringent washing at 55^{o}C, sections were treated with successive incubations in primary streptavidin-horse radish peroxidase, biotinyl-tyramide solution and secondary streptavidin-horse radish peroxidase (Dako) for 15 minutes each at room temperature. Color (golden-brown) was developed with the supplied diaminobenzidine diluted as directed (Dako) and sections counterstained with Carazzi's haematoxylin (blue) prior to dehydration and mounting. We have employed this procedure using the same cDNAs to generate riboprobes to localize PTHrP and PTHR mRNAs in rat tissues (including ovary) in previous studies (Watson, et. al., 2000a; Watson, et. al., 2000b).

Immunocytochemical (ICC) Detection of PTHrP and PTHR polypeptides in Bovine Ovary

ICC was performed as described (Watson et. al., 1995; Watson et. al., 2000a; Watson et. al., 2000b). Briefly, tissue sections (as described above) were deparaffinized and rehydrated in a standard xylene-ethanol series, post-fixed in 4% paraformaldehyde for 15 minutes and incubated for 10 minutes with 10 μg/ml proteinase K (Gibco, Burlington, ON, Canada) at room temperature to retrieve fixation-concealed antigens. Sections were stabilized in 4% paraformaldehyde for a further 15 minutes before proceeding with the ICC procedure using the Vectastain ABC kit (Vector Labs, Burlington, ON, Canada) following the manufacturer's directions. A polyclonal rabbit anti-rat PTHR antibody was used: antibody PTH-IV raised against a portion of the first extracellular loop (TLDEARLTEEELH; aa 249-262) (Babco, Berkeley, CA, USA) (Largo, Gomez-Garre, et. al., 1999). The PTHrP antibody used was a mouse monoclonal raised against amino acids 34-53 of human PTHrP (Oncogene Research Products, Cambridge, MA, USA). Both antisera are fully characterized and their specificity for PTHrP and PTHR polypeptides is well-established (Watson et. al, 1999a; Watson et. al., 1999b; Ferguson et. al., 1998, Largo et. al., 1999). Primary antibodies were diluted to 100 μg/ml in 1% BSA-PBS. As a control, some sections were treated with PTHR primary antibody that had been pre-absorbed overnight at 4^{o}C with the appropriate peptide. For the PTHrP antiserum, control sections were treated with normal mouse serum instead of primary antibody. All sections were counterstained with Carazzi's hematoxylin prior to dehydration and mounting.

Oocyte Collection, Insemination and Embryo Culture:

Cumulus oocyte complexes (COCs), were collected by razor blade slashing of slaughterhouse ovaries within 4 h of removal from the animal (Wiemer et al., 1991;Watson et al., 1994). The COCs were collected in oocyte collection medium (HEPES-buffered TCM-199 + 2% (v/v) NCS; Gibco BRL, Burlington, ON, Canada) and then were washed 4 times in serum-free medium prior to placement in oocyte maturation medium. Only denuded oocytes were discarded and a COC selection strategy was not employed in this study. Following oocyte maturation (see below for specific experimental conditions), oocytes were inseminated *in vitro* with frozen thawed bovine semen (Semex Canada Inc., Guelph, ON, Canada) prepared using a "swim-up" method in Sperm TL medium (HEPES-buffered modified Tyrodes solution as described in (Parrish et al., 1986)**.** Matured COCs were washed in sperm TL and placed in equilibrated fertilization drops (50 COCs/ 300 μl drop) composed of bicarbonate-buffered modified Tyrodes solution under light paraffin oil (Parrish et al., 1986) BDH Inc., Toronto, ON, Canada). COCs and sperm ($2.25x10^5$ motile spermatozoa/drop) were incubated for 18h at 39^{o}C under 5% CO_2 in air atmosphere before removal with a fine bore glass pipette of the cumulus investment including all corona cells. Inseminated oocytes (40-50) were placed into embryo culture consisting initially of 20 μl microdrops of citrate (0.5mM) and polyvinylalcohol (PVA; 3 mg/ml) supplemented synthetic oviduct fluid medium (cSOFMaa) (Keskintepe et al., 1995; Kestinkepe and Brackett, 1996; Watson et al., 2000) + 1X non-essential amino acids (NEA, Sigma-Aldrich Canada Ltd, Oakville, Ontario) and 1X essential amino acids (MEM, Gibco, BRL) under paraffin oil in a humidified 5% CO_2 / 7% O_2 / 88% N_2 culture atmosphere. Two days following initiation of culture, the microdrops were increased in volume by addition of 20 μl of cSOFMaa medium. On days 5 and 7 of culture 20 μl of medium was removed from each and replaced with 20 μl of fresh medium, thus keeping the microdrop volume constant for the remainder of the eight day culture interval. Cleavage and blastocyst frequencies were assessed on day 3 and 8 post insemination respectively.

Experimental Design:

A PTHrP concentration experiment (0, 10^{-10} M, 1.59 ng/ml; 10^{-8} M, 15.9 ng/ml; and 10^{-6} M, 159 ng/ml PTHrP) was conducted employing a randomized design that allocated equivalent numbers of non-selected COCs (total number of 442 COCs representing 4 experimental replicates with 25-30 oocytes allocated to each treatment group per replicate) to gonadotrophin and estradiol 17-β -free cSOFMaa oocyte maturation medium employing a 5% CO_2 in air atmosphere at 39^{o}C for 22 h. Following maturation, oocyte pools were inseminated and zygotes were placed into culture for assessment of frequency of development to the blastocyst stage as described.

Statistical Analysis:

Culture data were analyzed using the SigmaStat (Jandel Scientific) software package. One-way analysis of variance (ANOVA), followed by pairwise multiple comparisons (Bonferronis Method), were used for analysis of differences in the means for two or more populations. Differences of P# 0.05 were significant differences.

Results

Detection of PTHrP and PTHR mRNAs in Bovine Ovary by in situ Hybridization

PTHrP and PTHR mRNAs were localized in bovine ovarian tissue with biotinylated antisense riboprobes. Experiments were applied to tissue sections obtained from three bovine ovaries collected at slaughter and all *in situ* experiments were conducted a minimum of three times. In total over 100 follicles including stages from primordial to large antral follicles were examined. mRNAs encoding PTHrP were detected by *in situ* hybridization methods in oocytes, and granulosa cells in all follicles from primordial to large antral (Fig. 1 B, D, F, E). The granulosa cells of antral follicles displayed more intense signals for PTHrP mRNA (Fig 1E). A positive signal for PTHrP mRNAs was observed in thecal layers of larger follicles and in ovarian stroma in general (Figure 1 B,D,F, E). The signal observed for PTHrP sense controls (Fig. 1 A, C) was very low and indicated the specificity of the antisense PTHrP riboprobe. mRNAs encoding PTHR were detected in oocytes and granulosa cells in all follicles from primordial to large antral (Fig2 B,D,F, E). A positive signal for PTHR mRNA was observed in thecal layers of larger follicles and in ovarian stroma (Fig. 2 B,D, E,F). Sense PTHR mRNA controls displayed a very low background signal in granulosa and thecal layers (Fig. 2 A,C).

Immunocytochemical Detection of PTHrP and PTHR polypeptides in Bovine Ovary

PTHrP and PTHR immunoreactivity was studied in intact bovine ovarian tissue sections to assess the distribution of these polypeptides within the bovine ovary. PTHrP and PTHR polypeptides displayed distinct distribution patterns with PTHrP polypeptides primarily confined to oocytes from primordial to large antral follicles (Fig. 3 B,D,F). PTHrP polypeptides were also localized to a lesser extent in ovarian stroma and reduced signals were obtained in granulosa cell and thecal layers (Fig. 3 B,D, E, F). PTHrP immuno-specificity was determined by demonstrating a very low background signal in normal mouse serum-treated tissue sections (Fig. 3 A,C). PTHR polypeptides were detected in oocytes of all follicular stages (Fig 4, B, D, F). However, the most intense signals for PTHR polypeptides were localized to ovarian stroma, granulosa cell and thecal follicular layers (Fig 4 B,D,E, F). Pre-absorbed antibody controls indicated a high level of specificity for the PTHR immunolocalization signal (Fig 4A, C).

Influence of PTHrP during Oocyte Maturation on Development of Bovine Zygotes *In vitro*

cSOFMaa culture medium employed for bovine oocyte maturation was supplemented with PTHrP and influences on development to the blastocyst stage were examined. No significant differences in cleavage were observed among the four oocyte maturation treatment groups ($p< 0.05$; Fig. 5 and 6). Likewise, development to the 6-8 cell stage was not significantly influenced by PTHrP treatment during oocyte maturation (Fig. 5 and 6). However, development to the blastocyst stage as assessed by the proportion of total oocytes inseminated (Figure 5) or as a proportion of cleaved zygotes progressing to the blastocyst stage was significantly enhanced ($p<0.05$) by the addition of PTHrP to the oocyte maturation medium. The beneficial influence of PTHrP during oocyte maturation on blastocyst formation arose following the 6-8 cell stage since PTHrP treatment during oocyte maturation lead to a significant increase ($p<0.05$) in the proportion of 6-8 cell zygotes progressing to the blastocyst stage (Figure 6).

DISCUSSION

This study characterized the distribution of mRNAs and polypeptides encoding PTHrP and the PTHR within bovine ovary and investigated the influence of PTHrP treatment during *in vitro* maturation of bovine oocytes on development to the blastocyst stage. The results indicate that the bovine ovary expresses both the mRNAs encoding PTHrP and PTHR and their polypeptides. The granulosa layer may be a primary site of PTHrP production (high mRNA signals) but the majority of this PTHrP does not appear to be stored within the granulosa layer (low polypeptide signal). Why the granulosa highly expresses PTHrP mRNA but contains little peptide is unclear. It may be that granulosa PTHrP mRNA is not translated well (or at all) or even that the PTHrP peptide is secreted to the antral cavity. The answer to these possibilities awaits further experimentation. Oocytes from all follicular stages stained intensely for PTHrP polypeptides. The cognate receptor for PTHrP, the PTHR, was expressed in both oocytes and granulosa cells of the bovine ovary suggesting that PTHrP may act in both an autocrine and paracrine fashion to regulate bovine follicle development. PTHrP may also be an important regulator of oocyte maturation since addition of PTHrP to bovine oocyte maturation medium enhanced the development of fertilized bovine eggs to the blastocyst stage *in vitro.*

PTHrP and its receptor, the PTHR, are widely expressed in both skeletal and extraskeletal tissues (Lee et al., 1995; Urena et. al., 1993; Tian et. al., 1993; Watson et. al, 2000a). Reports suggest that the distribution of PTHrP and its receptor are nearly ubiquitous since this receptor ligand duo are described in tissues as diverse as skin (Kaiser et. al., 1992), smooth muscle (Moseley and Gillespie, 1995; Williams et. al., 1994; Yamamoto et. al., 1992; Watson, et. al., 2000a), bone (Lee et. al., 1993; Kronenberg et. al., 1998), kidney (Lee et. al., 1996; Amizuka et.

al., 1997; Yang et. al., 1997; Watson, et. al., 2000a), uterus-placenta (Williams et. al., 1994; Tucci and Beck, 1998; Watson, et. al., 2000b), brain (Weaver et. al., 1995), breast (Downey et. al., 1997), intestine (Li et. al., 1995; Watson, et. al, 2000a), pancreas and cardiovascular system (Vasavada, et. al., 1998). Few studies to date have investigated the expression or role of PTHrP and the PTHR in the mammalian ovary. Ovarian expression of PTHrP was first demonstrated by the humoral hypercalcemia of malignancy associated with ovarian small cell carcinomas and PTHrP is now widely used as an ovarian tumour expression marker (Matias-Guiu et. al., 1994; Inoue et. al., 1995). Northern blots were used to identify PTHR transcripts in rat ovary (Urena et. al., 1993; Joun et. al., 1997) and PTHrP expression has been demonstrated in the ovary of the developing frog (Danks et. al., 1997) and most recently in the porcine ovary (Garmey et al., 2000). Both the PTHR and its ligand, PTHrP, have been identified in rat ovary (Watson, et. al, 2000a). Only one study has identified PTHrP as a component of human ovarian follicular fluid and demonstrated that the granulosa-luteal cells were the source of the PTHrP (Gutmann et. al., 1993). None of these studies have attempted to localize mRNAs encoding PTHrP and the PTHR or their polypeptides as we have in the present study. In a recent study reporting nuclear localization of the PTHR, we observed that transcripts encoding both the PTHR and PTHrP and their polypeptides were present in rat ovary (Watson et. al., 2000a).

PTHrP is implicated as a regulator of embryo development (Behrendtsen et al.,1995; Lee et al., 1995; Nowak et al., 1999). PTHrP and the PTHR are expressed during differentiation of embryonal carcinoma or stem cells to primitive and parietal endoderm (van de Stolpe et. al., 1993) and PTHrP is essential to the outgrowth of parietal endoderm from isolated mouse embryo inner cell mass (ICM) regardless of the substratum used for outgrowth (Behrendtsen et al., 1995).

In the developing mouse embryo, expression of PTHR transcripts was detected in parietal endoderm from day 5.5 p.c. onwards. In the embryo proper, PTHR transcripts were highly expressed at sites of epithelial-mesenchyme interaction in developing intestine, lung, kidney and dermis starting at day 9.5 p.c. (Karperien, et. al., 1994). A comprehensive survey of PTHrP and PTHR expression during rat fetal development found that, in extraskeletal tissues, PTHrP mRNA was largely expressed in surface epithelia while PTHR mRNA was mainly localized to the adjacent mesenchyme (Lee et al., 1995). This was true for tissues as diverse as lung, choroid plexus, teeth, heart and skin. These results are strongly suggestive of a paracrine role for PTHrP during development. It has been proposed that PTHrP is a local, autocrine/paracrine regulator of cell growth and differentiation (Tucci and Beck, 1998; Moseley and Gillespie, 1995). A physiological role for PTHrP during mammalian endochondral bone formation and modeling is well documented (Lee, et.al., 1996; Lanske et. al., 1996; Lanske and Kronenberg, 1998). In other tissues PTHrP regulates smooth muscle relaxation, placental calcium transfer, breast development and lactation, vascular smooth muscle tension and pancreatic β-cell growth (Mosely and Gillespie, 1995; Vasavada et. al., 1998; Porter et. al., 1998). Very recently Nowak, et al., (1999) reported that PTHrP and transforming growth factorβ (TGF-β) both interact to promote murine blastocyst outgrowth demonstrating that PTHrP may be an important regulator of early developmental events.

The characterization of optimized culture conditions for mammalian oocytes and early embryos is an important priority. Progress in the development of effective defined media for embryo culture has occurred rapidly in the last few years. Media supplements such as serum have, however, only recently been removed from standard culture protocols and little research has investigated the role of ovarian factors on oocyte maturation and early development *in vitro* under defined serum-free culture conditions (Saeki et al., 1991; Gardner, 1994; Eckert et al., 1995; Keskintepe and Brackett, 1996; Hill et al., 1997; Avery et al., 1998; Krishner and Bavister, 1999; Watson et al., 2000). Research from our laboratories has characterized cSOFMaa medium for bovine oocyte maturation (Watson et al., 2000). Our results indicate that supplementation of cSOFMaa oocyte maturation medium with PTHrP may be of benefit for supporting enhanced numbers of bovine zygotes through to the blastocyst stage. Future studies will explore the mechanism underlying this positive influence and will determine whether PTHrP should become a routine supplement to mammalian oocyte maturation and embryo culture media.

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Figure 1: Detection of PTHrP mRNA in bovine ovary. Representative photomicrographs display A) sense control, B) detection of PTHrP mRNAs in primordial follicles included oocytes and pre-granulosa cells (arrowheads), C) sense control, D) PTHrP mRNAs in oocyte (o) and granulosa(g) layer of a secondary follicle and stromal (s) tissue, E) PTHrP mRNAs in granulosa and theca (t) cell layers of antral follicles (a), F) PTHrP mRNAs in oocyte, granulosa cells, and theca of antral follicles. Bar = 50 μM.

Figure 2: Detection of PTHR mRNAs in bovine ovary. Representative photomicrographs display A) sense control, B) detection of PTHR mRNAs in primordial follicles included oocytes and pre-granulosa cells (arrowheads), C) sense control, D) PTHR mRNAs in oocyte (o) and granulosa (g) layer of primordial and secondary follicles and stromal (s) tissue, E) PTHR mRNAs in granulosa and theca (t) layers of antral (a) follicles, F) PTHR mRNAs in oocyte, granulosa cells, and theca of early antral follicle. Bar = 50 μM

Figure 3: Detection of PTHrP polypeptides in bovine ovary The photomicrographs represent A) mouse normal serum control, B) detection of PTHrP polypeptides in primordial follicles included oocytes and pre-granulosa cells (arrowheads), C) control, D) PTHrP polypeptides in oocyte (o) of secondary follicle, E) PTHrP polypeptides in theca (t) layers of antral follicles, F) PTHrP polypeptides in oocyte of early antral (a) follicle and in ovarian stroma (s). g, granulosa Bar = 50 μM.

Figure 4: Detection of PTHR polypeptide in bovine ovary The photomicrographs represent A) pre-absorbed control, B) detection of PTHR polypeptides in primordial follicles included oocytes and pre-granulosa cells (arrowheads) and ovarian stroma (s), C) control, D) PTHR polypeptides in oocyte (o) and granulosa (g) cells of secondary follicle and stromal tissue, E) PTHR polypeptides in granulosa and theca (t) layers of antral (a) follicles and stroma, F) PTHR polypeptides in oocyte, granulose cells and theca of antral follicle. Bar = 50 μM

Figure 5: Influence of PTHrP during oocyte maturation on blastocyst development. Proportion of inseminated oocytes cleaving (cleavage), or reaching the 6-8 cell stage, 6-8/insem, or morula and blastocyst stage of development, mor/bl/insem and blastocysts blast/insem displayed by oocytes matured in 0, 1.59, 15.9, and 159 ng/ml (0, 0.1, 1 and 10 nM, respectively) of human PTHrP (1-141)–supplemented cSOFMaa. Cleavage, development to the 6-8 cell and morulae /blastocyst stages did not vary significantly among the treatment groups. The proportion of zygotes that progressed to the blastocyst stage varied significantly among the treatments. Bars represent the mean ± SEM of n=4 replicates. Values with different superscript letters are significantly different ($p<0.05$).

Figure 5: Influence of PTHrP During Bovine Oocyte Maturation on Development to the Blastocyst Stage

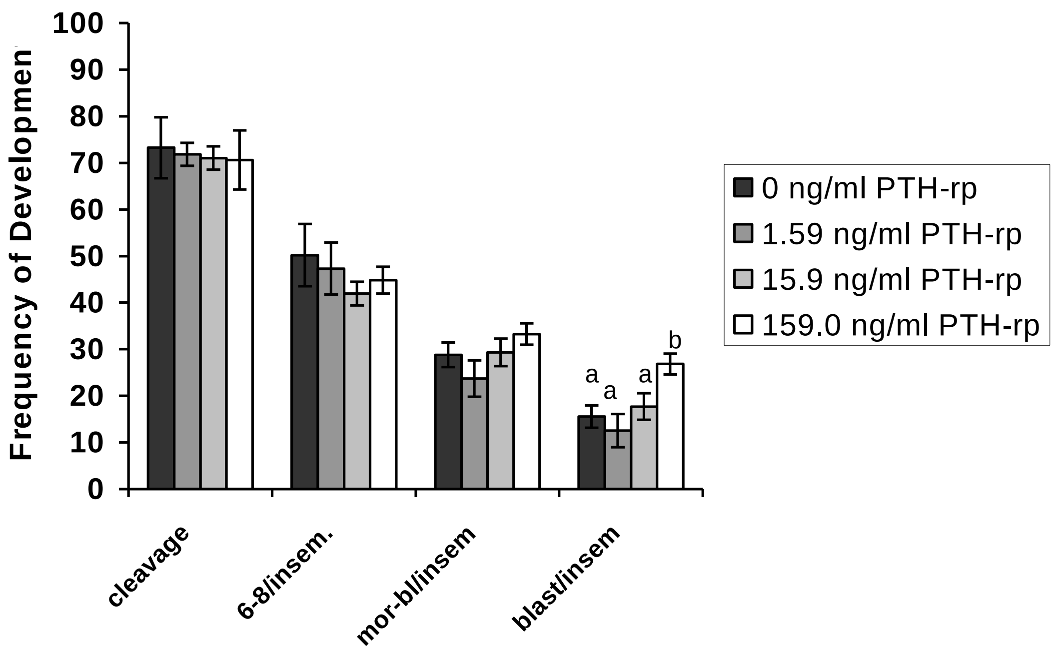

Figure 6: Influence of PTHrP during oocyte maturation on progression of 6-8 cell embryos to the blastocyst stage. Proportion of 6-8 cell stage embryos (over cleaved embryos, 6-8/clvd), morulae –blastocysts (over cleaved embryos, mor-blast/cleaved) blastocysts (over cleaved embryos, blast/clvd) and 6-8 cell embryos progressing to the blastocyst stage, (blast/6-8) displayed by oocytes matured in 0, 1.59, 15.9, and 159 ng/ml (0, 0.1, 1 and 10 nM, respectively) of murine PTHrP (1-141)–supplemented cSOFMaa. Development to the 6-8 cell and morula-blastocyst stages did not vary significantly among the treatment groups. However the proportion of blastocysts and 6-8 cell embryos that progressed to the blastocyst stage varied significantly. Bars represent the mean ± SEM of n=4 replicates. Values with different superscript letters are significantly different ($p<0.05$).

Figure 6: Influence of PTHrP During Oocyte Maturation on Development to the Blastocyst Stage