FINAL FOLLICULAR MATURATION IN THE COW AND ITS EFFECTS ON THE DEVELOPMENTAL POTENTIAL OF THE OOCYTE

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Finale Rijping van Follikels in het Rund en de Effecten daarvan
op de Ontwikkelingspotentie van de Eicel

(met een samenvatting in het Nederlands)

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ABBREVIATIONS

A	androstenedione
AI	artificial insemination
anti-eCG	monoclonal antibody against eCG
BRL	Buffalo rat liver
BSA	bovine serum albumin
cAMP	adenosine 3', 5'-cyclic monophosphate
cDNA	complementary deoxyribonucleic acid
CL	corpus luteum
COC	cumulus oocyte complex
d	days
DAPI	4, 6, diamino-2-phenyl indol
E	estradiol-17ß
EBBS	Earle's balanced salt solution
eCG	equine chorionic gonadotropin
EGF	epidermal growth factor
EM	electronmicroscopy
ET	embryo transfer
FCS	fetal calf serum
FF	follicular fluid
FGF	fibroblast growth factor
FSH	follicle stimulating hormone
GnRH	gonadotropin-releasing hormone
GV(BD)	germinal vesicle (breakdown)
h	hours
IGFBP	insulin-like growth factor binding protein
LMW IGFBPs	low molecular weight IGFBPs
IGF-I	insulin-like growth factor I
im	intramuscular
IU	international units
iv	intravenously
IVC	in vitro culture
IVF	in vitro fertilization
IVM	in vitro maturation
IVP	in vitro embryo production
LH	luteinizing hormone
LM	lightmicroscopy
MI	metaphase I
MII	metaphase II
mRNA	messenger ribonucleic acid

n	number
O-OVDX	ovario-oviductectomy
OPU	ultrasound-guided ovum pick up
OVX	ovariectomy
P	progesterone
PAS	periodic acid-Schiff
PBS	phosphate buffered saline
PG	prostaglandin $F_2\alpha$
RIA	radio-immuno assay
RT-PCR	reverse transcriptase-polymerase chain reaction
SD	standard deviation
SEM	standard error of the mean
SO	superovulation
TBS	tris buffered saline
TGF	transforming growth factor

CONTENTS

GENERAL INTRODUCTION

The availability and increased accessibility of assisted reproduction techniques render promising tools to advance cattle breeding. Using these techniques the standard of 'one calve per cow per year' is no longer limiting in genetic improvement of livestock. Following artificial insemination, superovulation (SO, in vivo embryo production) in combination with embryo transfer (ET) and transvaginal ultrasound-guided ovum pick up (OPU) in combination with in vitro production of embryos (IVP) and ET are now widely applied. Moreover, the production of calves by cloning or nuclear transfer is an upcoming issue. In 1996, worldwide and in Europe, around 450,000 and 118,169 bovine embryos were transferred, respectively; 6-8% of these embryos were produced in vitro (Thibier et al., 1997; Table 1).

Table 1. Number of transfers of in vivo or in vitro produces bovine embryos in the world, Europe and The Netherlands in 1996.

	Number of embryo transfers in 1996	
	In vivo produced	In vitro produced
World	412,996	
Europe	111,321	6,848
The Netherlands	24,272	1,885

Data retrieved from Thibier et al. (1997).

During the normal estrous cycle in the bovine, on average 21 days in length, one ovulation takes place. In The Netherlands, under optimal farm management conditions, cows will be bred or inseminated 40-60 days after parturition and will, depending on the breed, deliver a calf on average 280 days later; one calf per cow per year is produced. Treatment for SO induces on average 15 ovulations per estrous cycle resulting in 4.5 calves per SO treatment; during one OPU session, 7 usable oocytes are collected, which eventually result in 0.5 calves. Theoretically, repetitive use of SO/ET (6 times per year) and OPU/IVP/ET (100 times per year) could increase the number of offspring per cow to 27 and 50 calves per year, respectively. However, when the efficiency per used oocyte is calculated, instead of the efficient use of oocytes during natural mating, using SO/ET only 30% efficiency is reached. OPU/IVP/ET generates only 8 calves per 100 immature oocytes (Table 2).

Table 2. Loss of efficiency during subsequent steps in production of calves after superovulation (SO) or transvaginal ultrasound-guided ovum pick up (OPU) in combination with in vitro production of embryos (IVP).

	SO/ET	OPU/IVP/ET
Initial number of preovulatory oocytes	100	
Initial number of immature oocytes		100
Number of matured oocytes	(72[1)])	(80[2)])
Number of fertilized oocytes	75	70
Number of embryos at day 7	70	25
Number of pregnancies	35	12
Number of calves	30	8

R&D team/IVP laboratory, Holland Genetics B.V., Arnhem, The Netherlands, 1997 (Personal communication). Numbers between brackets are obtained from the IVP laboratory of the Department of Herd Health and Reproduction, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands: 1) de Loos et al., 1991b; 2) de Loos et al., 1994.

Losses in reproductive efficiency occur during each step involved in the production of a healthy calf: during maturation (Leibfried-Rutledge et al., 1987), fertilization (van Soom and de Kruif, 1992), early embryonic development (Greve et al., 1993), embryo transfer (Farin and Farin, 1995; Garry et al., 1996) and, in particular after cloning or nuclear transfer, during parturition (Hasler et al., 1995; Walker et al., 1996) and neonatal development (Garry et al., 1996). In foregoing and current research many (successful) attempts have been made to improve efficiency of laboratory assisted reproduction. In these studies, the process of oocyte maturation is relatively underexposed. Considering that the basis for the increase in the number of offspring is increment of the number of usable oocytes per estrous cycle and that deviations in maturation will affect all further processes involved in creating offspring, a better understanding of oocyte maturation is desirable. Studying maturation, the regulatory mechanisms and events occurring during in vivo maturation in normally cyclic cows should always be considered as the guiding principle.

ANTRAL FOLLICULAR DEVELOPMENT DURING THE NORMAL ESTROUS CYCLE

Recruitment, growth and selection

During each estrous cycle, 2 or 3 and exceptionally 1 or 4 waves of follicular growth occur (Savio et al., 1988; Sirois and Fortune, 1988; Driancourt, 1991; Taylor and Rajamahendran 1991; Lucy et al., 1992; Fortune, 1993; Adams, 1994; Ginther et al., 1996). The onset of the first wave of an estrous cycle is detected as a group of 4-mm

follicles just before the day of ovulation. During the next days of follicular growth, one of the follicles will be selected to become dominant and continue to grow while the others undergo atresia. A second wave emerges at about 10 days after ovulation and, for three wave cycles, is followed by an additional wave at day 16; the preovulatory follicle originates from the final wave of follicular growth (Ginther et al., 1996, Figure 1).

The occurrence of follicular waves is predominantly regulated by surges of FSH in the peripheral blood (Sunderland et al., 1994; Gong et al., 1995; Bodensteiner et al., 1996a). The recruitment of growing follicles of a wave occurs during the incline in the FSH surge. The peak of the FSH surge occurs near the time that the future dominant follicle reaches a mean diameter of 4 mm and the initial decline in FSH concentrations after the peak occurs when the future dominant follicle is approximately 6 mm.

Also locally produced members of growth factor families, such as the fibroblast growth factor- (FGF), epidermal growth factor- (EGF), transforming growth factor- (TGF) and insulin-like growth factor (IGF) family, may be involved in controlling growth of antral follicles. They have been shown to regulate growth and differentiation of granulosa and thecal cells in vitro (review, Monniaux et al., 1997; Table 3). However, knowledge of all elements of these systems is far from complete and their role in folliculogenesis is still hypothetical.

Factors produced by the dominant follicle play an important role in decreasing the peripheral FSH concentration during a surge; the decrease in FSH concentrations coincides with enhanced secretion of inhibin and estradiol-17ß by the dominant follicle (Findley, 1993). The proteinaceous components of follicular fluid, which include inhibin, inhibit FSH concentrations and follicular growth when administered to cattle (Kastelic et al., 1990; Turzillo and Fortune, 1993) and also antiserum against inhibin increases circulating FSH concentrations (Kaneko et al., 1995). In addition, large doses of exogenous estradiol-17ß suppress FSH secretion (Barnes et al., 1981; Bolt et al., 1990) but there is paucity of information on the effect of physiological concentrations of estradiol-17ß on the FSH surge.

Apparently, the final suppression of the FSH surge is a function of the mechanism that causes deviations in growth rates and functionality between the dominant and subordinate follicles. The question remains why the dominant follicle and not the subordinate follicles can continue growth by decreased FSH plasma concentrations. Ginther et al. (1996) suggest a change in dependency of the dominant follicle from FSH to LH based on an increase in the number of LH receptors of the dominant follicle in the bovine (Xu et al., 1995; Bodensteiner et al., 1996b). Stimulation of these receptors by LH may increase cellular cAMP levels, which would prevent atresia of the dominant follicle; the subordinate follicles, lacking LH receptors, would not be able to maintain intracellular levels of cAMP and therefore undergo atresia. Studies in sheep suggest that the dominant follicle has an enhanced sensitivity to FSH when compared to subordinate follicles (Henderson et al., 1985, 1987). Driancourt (1991) proposed that this increased sensitivity is due to amplification of the FSH signal by

IGF-I. Indeed, in the bovine, increased follicular estradiol-17ß concentrations coincide with increased follicular concentrations of IGF-I (Echternkamp et al., 1994) and decreased levels of IGF binding proteins (IGFBPs) (Echternkamp et al., 1994; Funston et al., 1996) suggesting enhanced bioavailability of IGF-I in large follicles with high follicular estradiol-17ß concentrations.

Table 3. The action of growth factors in vitro on proliferation and differentiation of bovine follicular cells. Differentiation is assessed by steroidogenesis.

		Granulosa cells	Thecal cells
Proliferation	Stimulating action	EGF TGF-α bFGF IGF-I	TGF-α
	Inhibiting action	TGF-β	TGF-β
Differentiation	Stimulating action	IGF-I	TGF-β Inhibin
	Inhibiting action	EGF bFGF Activin A Cytokines	TGF-α Activin A

EGF=epidermal growth factor, TGF=transforming growth factor, FGF=fibroblast growth factor, IGF=insulin-like growth factor. Adapted from Monniaux et al. (1997).

Preovulatory follicular development

Increasing plasma levels of estradiol-17ß in combination with decreasing progesterone levels during the follicular phase of the estrous cycle coincide with a high frequency, low amplitude pattern of LH secretion (Rahe et al., 1980; Walters and Schallenberger, 1984; Cupp et al., 1995). This increase in LH pulsatility prevents atresia of the dominant follicle and stimulates its development up to the preovulatory stage (Savio et al., 1993).

Recently, it has been demonstrated that preovulatory follicular development comprises more than just the changes in follicle and oocyte that occur during only final maturation. Final maturation of the oocyte is not the activation of a quiescent gamete, but rather the culmination of a long series of preparatory processes that might be summarized as prematuration of the oocyte (review, Hyttel et al., 1997). It can be speculated that prematuration also exists at the level of the complete follicle in order to allow it to recognize and react to the preovulatory LH surge adequately.

Final maturation proceeds according to a precisely fixed sequence of hormonally regulated changes of biosynthesis, morphology and interaction between follicle and oocyte (Dieleman et al., 1983a, b; Kruip et al, 1983; Dieleman and Blankenstein 1984,

1985; de Loos et al., 1991a; Hyttel et al., 1991; Kastrop et al., 1991; Dieleman and Bevers 1993). Upon the preovulatory LH peak (defined as the maximum of the preovulatory LH surge), theca cell androstenedione synthesis is immediately inhibited (Dieleman et al., 1983a) thereby depleting granulosa cells of its precursor for estradiol-17ß synthesis. Estradiol-17ß levels in the follicular fluid surrounding the maturing oocyte decrease within 6 h after the LH peak. Shortly before ovulation, synthesis of progesterone becomes dominant (Dieleman and Blankenstein, 1985) concomitant with luteinization of the follicular tissue showing then, breakdown of the basal membrane and invasion of eosinophylic granulocytes (Dieleman et al., 1983a; de Loos et al., 1991a). Coinciding with these follicular changes, the oocyte completes nuclear and cytoplasmic maturation. Resumption of meiosis becomes apparent 6 h after the LH peak when germinal vesicle (GV) break down occurs (Kruip et al., 1983; de Loos et al., 1991a). Before ovulation, one set of chromosomes is retained within the oocyte while the other is extruded in the first polar body. At this stage, metaphase II (MII), the oocyte becomes arrested again. Cytoplasmic maturation of the oocyte is observed as re-arrangement of cell organelles like cortical granules, mitochondria, Golgi apparatus and endoplasmatic reticulum and an increase of lipid storage (Kruip et al., 1983; Hyttel et al., 1997). Ovulation, which coincides with inflammatory like changes in the follicle tissue (Dieleman et al., 1983b; Espey, 1994), occurs approximately 24 h after the LH peak (Dieleman et al., 1983b).

There is little doubt about the preovulatory LH surge as the trigger for final maturation. However, as during growth and selection of follicles, also growth factors are assumed to play an important role in the regulation of preovulatory follicular development. Beneficial effects of IGF-I, EGF and TGF-α in vitro, on cumulus cell expansion (IGF-I: Izadyar et al., 1997), nuclear maturation of the oocyte (IGF-I: Izadyar et al., 1997; EGF: Coskun et al., 1991; Harper and Brackett, 1993; Kobayashi et al., 1994; Lorenzo et al., 1994; TGF-α: Kobayashi et al., 1994) and on programming of the oocyte during IVM for further development (IGF-I: Harper and Brackett, 1992) have been described.

DEVIATIONS IN FOLLICULAR DEVELOPMENT DURING SUPEROVULATION

The principle of superovulation is based on bypassing the mechanism of follicular selection by administration of a large dose of FSH-like acting gonadotropins at a time that a new cohort of follicles is being recruited (Figure 1); in follicles > 1.7 mm atresia is prevented, leading to an increase in the number of follicles that have the potential to become preovulatory (Monniaux et al., 1983; Monniaux et al.,1984; Moor et al., 1984). However, not all presumptive preovulatory follicles eventually ovulate and/or result in an embryo. Deviations in preovulatory follicular development are considered

to be responsible for this (Moor et al., 1984; Callesen et al., 1986; Dieleman et al., 1993).

In superovulated cows, the period of preovulatory follicular development is shortened due to a decreased interval between luteal regression and LH peak from 61 to 41 h in comparison to unstimulated cows (Dieleman et al., 1986; Bevers and Dieleman 1987). In addition, during preovulatory development, endogenous basal secretion, pulse frequency and pulse amplitude of FSH and pulse frequency of LH are decreased by more than 50% in comparison to normally cyclic cows (Bevers et al., 1989).

Figure 1. Schematic model of follicular dynamics on the ovary and changes in progesterone and LH concentrations in the peripheral blood during the estrous cycle in the cow. A commonly used scheme for superovulation with eCG, starting at day 10 of the estrous cycle, is depicted in the top of the figure (in the open rectangle). Using OPU, oocytes can be collected throughout the estrous cycle; in general, oocytes are aspirated from 3-6 mm-sized follicles and immediately subjected to IVM.  = Collection of oocytes for IVM. LH= LH surge, ov(‘s)= ovulation(s).

Upon treatment for SO, not all preovulatory follicles mature synchronously (interfollicular asynchrony, Vos et al., 1994), and also not all follicles mature in unison with their oocytes (intrafollicular asynchrony, de Loos et al., 1991b; Hyttel et al., 1991; Dieleman and Bevers 1993). In normally cyclic cows, the preovulatory

follicle is independent of FSH support for further development, whereas, after SO only ± 25% of the preovulatory follicles reached independence of support by exogenous gonadotropins at the time of the LH surge (Vos et al., 1994). Also, at the time of ovulation, the preovulatory oocyte of normally cyclic cows is arrested in MII stage; after superovulation, 20% of the oocytes are still in GV, 7% are in metaphase I (MI) and 73% in MII stage (de Loos et al., 1991b). Occasionally, those oocytes in GV are found in a completely luteinized follicle with expanded cumulus and also, oocytes in MII stage can be found in non-luteinized follicles (de Loos et al., 1991b). In addition, at the time of the LH surge, oocytes can already be found in MII stage (Hyttel et al., 1986) at the time a preovulatory follicle normally still produces predominantly estradiol-17ß.

DEVIATIONS IN FOLLICULAR DEVELOPMENT DURING OPU/IVP

The possibility of increasing the number of offspring using OPU/IVP is based on the fact that the continuously occurring waves of follicular growth on the ovary, even in pregnant and prepubertal animals, can be used as a source of oocytes. These oocytes, usually collected from 3-6 mm follicles, then have to be matured in vitro (IVM) before they can be fertilized. In comparison with oocytes undergoing preovulatory development in normally cyclic cows, the immature oocytes undergoing IVM lack major parts of antral follicular development: both follicular growth and selection and also preovulatory development before the occurrence of the LH surge are bypassed. Despite this, IVM has been ‘successful’ since 1935 (Pincus and Enzmann, 1935); when oocytes are removed from the follicle resumption of meiosis takes place spontaneously and 80% of the oocytes are in MII after 22- 24 of IVM (de Loos et al., 1994).

However, ultrastructural studies of bovine oocytes demonstrate the during IVM deviations in cytoplasmic maturation occur, as for example in the spread in cortical granules along the oolemma (Hyttel et al., 1986). Inadequate cytoplasmic maturation might be responsible for the decreased capacity of in vitro matured oocytes to be fertilized or to further develop into embryos. This hypothesis is supported by studies in which the addition of growth hormone during IVM, that improves the spread of the cortical granules around the oolemma during IVM (Izadyar et al., 1998), promotes subsequent in vitro embryo development (Izadyar et al., 1996). Also, larger follicles provide oocytes, which are more competent to develop into transferable embryos after fertilization than oocytes derived from smaller follicles (Lonergan et al., 1994; Blondin and Sirard, 1996). This better development of oocytes from larger follicles is not due to a better nuclear maturation (Ectors et al., 1995), but presumably to better cytoplasmic maturation.

AIM OF THE THESIS

The increase in the number of offspring per cow after SO and OPU/IVP is based on the utilization of more than one oocyte during one estrous cycle (Figure 1). However, at present, only a minor part of the used oocytes will eventually result in an alive born calf (SO: 30%; IVP: 8%). It has been shown that both during SO and IVP deviations in oocyte maturation occur. Preovulatory follicular development in normally cyclic cows proceeds according to a precisely fixed sequence of hormonally regulated changes of biosynthesis, morphology and interaction between follicle and oocyte. In this thesis concepts of final follicular maturation in unstimulated, normally cyclic cows are applied to maturation during SO and IVP in order to gain better understanding of deviations in follicle and oocyte development using these techniques. Also, preovulatory follicular development after SO is used as a model to study maturation in vivo.

Upon superovulation not all follicles mature synchronously. Thus, at the time of the preovulatory LH surge, which is induced relatively early by a group of advanced follicles, possibly not all follicles are prepared to start final maturation yet. Prolongation of the period of preovulatory follicular development to a time that is similar to that in normally cyclic cows might reduce this heterogeneity in functionality of follicles and give more follicles enough time to acquire all features necessary to respond to the LH signal. Prolongation of preovulatory development can be obtained by temporary postponement of the LH surge. In **chapter 2**, the effects of temporary postponement of the LH surge on ovulation rate, fertilization, early embryonic development and finally embryo yield after SO are described and discussed in relation to the possibly reduced heterogeneity in functionality of the stimulated follicles. Furthermore, the possibility of the use of this protocol in studies towards final maturation in vivo and also its practical implications in cattle breeding is discussed.

During IVP, immature oocytes from 3-6 mm follicles are directly subjected to in vitro maturation (IVM). Thus, compared to the situation in vivo, these immature ooyctes lack the part of preovulatory follicular development that occurs before the preovulatory LH surge. Furthermore, conditions during IVM are poor and static compared to the wide array of hormones and factors that in changing concentrations are involved in in vivo maturation. In **chapter 3**, we investigate to what extend IVM contributes to limiting the yield of viable embryos in currently used IVP. The developmental potential during IVF and IVC of oocytes collected from 2-8 mm sized follicles and matured in vitro is compared to that of in vivo matured oocytes. In order to have the disposal of a large pool of in vivo matured oocytes at a predictable, similar stage of development at a fixed time, these oocytes are collected from eCG-stimulated heifers with controlled LH surge (chapter 2).

The decreased developmental potential during IVF and IVC of in vitro matured oocytes that was found in chapter 3 might be due to IVM conditions per se or to a difference in startcompetence of the used oocytes. Therefore, in **chapter 4**, the effect

of in vivo versus in vitro maturation on developmental potential of oocytes is compared, which presumably have an equivalent startcompetence. For this purpose, again oocytes are collected from eCG-treated animals with a controlled LH surge at the time of the expected LH surge (oocytes to be matured in vitro) and shortly before ovulation (matured in vivo). Also, the effect of follicular dysfunction, on the basis of deviating concentrations of estradiol-17ß and progesterone in the follicular fluid, on developmental competence of oocytes is discussed in this chapter.

In **chapter 5**, preovulatory follicular development after eCG-treatment for SO is used as a model to study the possible role of the insulin-like growth factor system during final maturation in vivo. eCG treatment induces deviations in preovulatory follicular development in part of the stimulated follicles. These changes, which are, among others, reflected in deviant concentrations of steroid hormones in the follicular fluid, coincide with impaired function of the follicles. It is investigated whether eventual differences in IGF-I and IGF binding protein (IGFBP)-levels in fluids of follicles with normal or deviant follicular function may elucidate the role of the IGF system during final maturation. Finally, the regulation of the presence of IGFBPs in preovulatory follicles shortly before the onset of final maturation (**Chapter 6)** is investigated; mRNA levels for IGFBP2, -3, -4 and -5 are compared in normal and deviant eCG-stimulated preovulatory follicles.

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EFFECT OF BRIEF POSTPONEMENT OF THE PREOVULATORY LH SURGE ON OVULATION RATES AND EMBRYO FORMATION IN ECG/PG-TREATED HEIFERS

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The aim of this study was to investigate whether extension of the period of preovulatory follicular development after superovulation to one that resembles that in normally cyclic cows, reduces heterogeneity of oocytes of stimulated follicles with respect to the potential to mature, to ovulate, to be fertilized and to develop into embryos. Heifers were treated with eCG on day 10 and prostaglandin (PG) 48 h later. At the time of eCG administration part of the heifers received a norgestomet implant (N) to suppress the LH surge. After 96-104 h, N was removed and an LH surge was induced with GnRH (G) (N/G); the other animals served as controls (Co). Matured oocytes (experiment (exp) A: n=9, 139 (N/G) and 11, 125 (Co) heifers, oocytes), zygotes and oviducts (exp B: n=8, 44 (N/G) and 9, 72 (Co) heifers, zygotes) and embryos (exp C: n=11, 205 (N/G) and 11, 165 (Co) heifers, embryos) were collected at 22-26 h, 38-52 h and 7 days after the LH surge, respectively. Hatched blastocyst formation of matured oocytes (exp A) was analyzed after 11 days of IVC after IVF. In vivo fertilization rate of zygotes, the presence of PAS positive granules in the oviduct (exp B) and stage of development of embryos (exp C) were analyzed microscopically. The mean interval between PG and the LH surge was 53.8±3 (SD) (N/G) vs. 42.4±4 h (Co). The response to superovulation was higher in the N/G than in the Co heifers (25.4±2 (N/G) vs. 18.7±2 (Co)). Hatched blastocyst formation rate (37.4 (N/G) vs. 33.6% (Co)), in vivo fertilization rate (69.0±14 (N/G) vs. 73.0±10% (Co)) and the yield of embryos (3.8±1 (N/G) vs. 5.6±2 (Co)) did not differ between groups. The % of heifers with abundant presence of PAS positive granules in the distal ampulla (0 (N/G) vs. 31% (Co)) was reduced after N/G treatment. Extension of the period of preovulatory development after superovulation clearly increased the number of ovulations but not the yield of embryos, possibly due to an impaired oviductal environment as a result of the treatment. It is unclear whether heterogeneity of oocytes from stimulated follicles was reduced since final maturation- and fertilization rates were not affected by the treatment.

INTRODUCTION

Superovulation is the most widely used method to increase the number of offspring of genetically valuable cows. However, the yield and quality of embryos raised after superovulation are still unpredictable. Besides cow factors (genetic background, age, breed, reproductive and lactation status (Hasler et al., 1983; Donaldson 1984a, b; Lerner et al., 1986), the status of the follicle population at the time of stimulation with exogenous gonadotropins may influence the response to superovulation. For example,

superovulation in the absence of a dominant follicle results in higher ovulation rates than superovulation in the presence of a dominant follicle (Bungartz and Niemann, 1994). Also, deviations in preovulatory follicular development induced by the use of exogenous gonadotropins may influence ovulation rates and embryo yields. Upon superovulation, not all follicles mature synchronously; when gonadotropic support is stopped at the time of occurrence of the LH surge only 25% of the present preovulatory follicles is able to continue development up to ovulation, whereas the remaining follicles are still dependent on exogenous gonadotropic support to do so (interfollicular asynchrony, Vos et al., 1994a). Thus, maybe at the time of induction of the LH surge by the group of advanced follicles not all follicles are prepared to start final maturation yet, resulting in an asynchrony in development between the follicle and its oocyte (intrafollicular asynchrony, de Loos et al., 1991; Hyttel et al., 1991; Dieleman and Bevers, 1993). Reduction of the period of preovulatory follicular development, due to a decreased interval between luteal regression and the occurrence of the LH surge from 61 h in unstimulated cows (Dieleman et al., 1986) to 44 h in superovulated cows (Bevers and Dieleman, 1987) might be responsible for this.

Prolongation of the interval of preovulatory development after superovulation could reduce follicular heterogeneity and give more follicles enough time to acquire all features necessary to respond to the LH signal. Vos et al. (1994b) demonstrated that prolongation of the interval between luteal regression and the start of maturation could be achieved by the use of a progesterone block to suppress the endogenous preovulatory LH surge followed by administration of GnRH to subsequently induce an LH surge. It is not known whether this procedure improves superovulatory responses. Recently, d'Occhio et al. (1997) used a GnRH agonist to desensitize the pituitary to GnRH and then at any time after FSH stimulation an LH surge can be generated with exogenous LH; in this study extension of the period between luteal regression and start of final maturation did not increase ovulation rates.

The treatment to postpone the preovulatory LH surge should warrant optimal conditions for oocyte maturation, fertilization and early embryonic development. The objective of the present study is to investigate the effect of brief postponement of the preovulatory LH surge after superovulation using a protocol similar to that described by Vos et al. (1994b) on ovulation rate, oocyte maturation, fertilization and early embryonic development.

MATERIALS AND METHODS

Experimental design

Superovulation responses of eCG/PG/anti-eCG treated animals with spontaneous preovulatory LH surges (control group) were compared to those of eCG/PG/anti-eCG treated heifers with temporarily postponed, GnRH-induced LH surges (norgestomet/

GnRH group) in three consecutive years, during different seasons. To test the effects of this treatment on early embryonic development, oocytes, zygotes and embryos were collected at the end of the period of maturation (experiment A), fertilization (experiment B) and early embryonic development (experiment C), respectively (see Figure 1). In experiment B, also oviducts were collected for morphological examination.

Figure 1. Schedule of treatments for superovulation and ex vivo collection of matured oocytes, zygotes and embryos from eCG/PG/anti-eCG treated heifers with a spontaneous LH surge (control) and of heifers with a temporarily postponed, GnRH-induced LH surge (norgestomet/GnRH treated). Day 0 is estrus of a synchronized cycle. Exp = experiment, PG= administration of prostaglandin; max of the LH surges = the period in which the endogenous LH surges occur. Max of the LH surge = the time at which the induced LH surge occurs. Stippled area represents presence of norgestomet ear implant in the norgestomet/ GnRH treated animals.

Superovulation procedure

Holstein-Friesian heifers (n=68) were selected from our herd on the basis of general clinical examination and normal ovarian cyclicity during at least 3 weeks as established by the progesterone concentration in peripheral blood samples taken 3 times a week and at random assigned to the control or norgestomet/GnRH treated group. The heifers were housed in groups of 6 animals, were fed silage and concentrate (to a maximum of 1 kg per heifer per day) and supplied water ad libitum.

Before the experiments estrus was synchronized using an ear implant for 9 d (3 mg norgestomet, Crestar; Intervet International BV, Boxmeer, The Netherlands) accompanied by treatment with 3 mg norgestomet with 5 mg estradiol-valerate (im). Two days before removal of the implant prostaglandin (PG, 15 mg Prosolvin im; Intervet International BV) was administered to ensure complete regression of the corpus luteum (Vos et al., 1996).

Stimulation for superovulation was carried out on day 10 (estrus is day 0) with eCG (2500 IU Folligonan im; Intervet International BV) followed by PG (15 mg Prosolvin im) 48 h later. To prevent eventual suppressive action of dominant follicles on the follicle population to be stimulated, superovulation was performed in absence of follicular dominance. In experiment A and B the dominant follicle was disabled by transvaginal ultrasound-guided puncturing at day 8 of the synchronized cycle. In experiment C, stimulation was performed in the absence of a dominant follicle as established by daily ultrasound scanning; a growing follicle was considered to become dominant when its size exceeded 8 mm and to remain dominant for 3 d after it stopped growing (Huhtinen et al., 1992).

To postpone the release of the endogenous preovulatory LH surge a norgestomet ear implant (Crestar) was inserted concomitantly with administration of eCG; the accompanying im norgestomet and estradiol-valerate were not administered. After 96-104 h these ear implants were removed and 1.0 mg GnRH (Fertagyl, Intervet International BV, im) was administered to induce an LH surge. LH surges were expected 2 h after GnRH treatment (Vos et al., 1994b).

In control heifers, the LH surge was considered to occur when the LH concentration increased in three successive blood samples as determined by a rapid radio-immunoassay (RIA) which was performed every h from 30 until 50 h after injection of PG according to Dieleman and Bevers (1987).

Both the control and the norgestomet/GnRH treated animals, received a dose of a monoclonal antibody against eCG (5 ml Neutra-PMSG iv, Intervet International BV) at 8 h after the determined (spontaneous LH surge, control animals) or expected (induced LH surge, norgestomet/GnRH treated animals) LH surge which was sufficient to neutralize 2500 IU within 1 h (Dieleman and Bevers, 1987). Finally, heifers of experiments B and C were inseminated 8 h after the LH peak with two straws of semen from a bull of known high fertility depositing the contents of one straw per uterine horn.

Superovulation response

To evaluate the response to superovulation, in experiment A, the number of preovulatory follicles (i.e. follicles > 8 mm) present at the time of ovariectomy were counted. In experiment B and C, the number of fresh ovulation sites and corpora lutea

on the ovaries were counted at the time of collection of zygotes and embryos, respectively.

The overall response to superovulation was calculated as the mean of the individual responses of each cow (n=28 and n=31 heifers for norgestomet/GnRH and control group, respectively).

Maturation (Experiment A)

Four experiments were carried out in a period of two months; two groups of control heifers (n=6 and n=5) and two groups of norgestomet/GnRH treated heifers (n=4 and n=5) were ovariectomized between 22 and 26 h after the LH surge by laparotomy through flank incision under local infiltration anesthesia (Dieleman et al., 1983). To obtain the ovaries between 5 a.m. and 4 p.m., control animals were selected from a group of 17 heifers on the basis of the time of occurrence of the LH surge, i.e. 35 – 46 h after PG administration. Ovaries were collected in saline (0.9% (w/v) NaCl) (25°C) and transported immediately to the laboratory. Cumulus oocyte complexes (COCs) were obtained by aspiration of follicles > 8 mm, which were considered to be preovulatory. Only COCs with an expanded cumulus mass were selected for IVF. Until insemination, selected oocytes were kept in fertilization medium (Fert-TALP) as described by Parrish et al. (1988) with slight modifications (Izadyar et al., 1996). Insemination was performed within 2.5 h after ovariectomy for each heifer. COCs were inseminated and cultured in groups of 15-33 oocytes (average 20.8 ± 4 (SD) and 27.8 ± 5 for control and norgestomet/GnRH treated animals, respectively) as described previously (Izadyar et al., 1996). Briefly, insemination was carried out during 19-22 h (39°C, 5% CO_2, in humidified air) with $0.5x10^6$ spermatozoa/ml of a bull with proven in vitro fertility. COCs were freed from cumulus cells and transferred to a well of a 4-well culture plate (Nunc A/S, Roskilde, Denmark) containing 500 µl M199 (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (FCS, Gibco BRL) and a monolayer of Buffalo rat liver cells (39°C, 5% CO_2, in humidified air). At day 4 of IVC, the cleavage rate was evaluated and non-cleaved structures were removed, at day 4 and 8 of culture embryos were transferred to fresh co-culture wells, and at day 7, 9 and 11 numbers of blastocysts and hatched blastocysts were scored. Day 1 of culture was defined to start immediately after IVF was finished. IVP conditions were checked routinely every week (n=4) with 100-150 oocytes (35 oocytes per well) recovered from 2-8 mm sized follicles from ovaries obtained from the slaughterhouse. These cultures were performed by the same investigators under similar conditions. Blastocyst formation and hatching rates after 11 days of IVC were 29.5 ± 6.4% (SD) and 23.1 ± 4.3%), respectively, indicating that the conditions during IVP were constant throughout the experimental period.

Fertilization (Experiment B)

Sedated heifers (3 mg detomidine-hydrochloride, Domosedan iv; SmithKline Beecham, Zoetermeer, The Netherlands) were ovario-oviductectomized (O-OVDX) by laparotomy through left flank incision in a standing position under local infiltration anesthesia. Before excision of the oviduct, the cranial tip of the uterine horn, including the A. and V. ovarica and, if necessary, also the important branches of the V. ovarica and V. uterina in the mesoviduct were ligated (Vicryl 2, Ethicon, Hamburg, Germany). An umbilical cord clamp (Instruvet, Amerongen, The Netherlands) was placed around the mesovarium, caudal of the ovary. O-OVDX were performed between 38-52 h (control group, n=9) or at 44 h (norgestomet/GnRH treated group, n=8) after the preovulatory LH surge. To obtain oviducts between 8 a.m. and 3 p.m., control heifers were selected from a group of heifers (n=12) on the basis of the time of occurrence of the preovulatory LH surge, i.e. 34-50 h after PG administration. Ovaria and oviducts were placed in saline (37°C) and transported immediately to the laboratory. Oviducts were flushed through a blunt needle (inner diameter 2 mm) with 10 ml 1X Earle's Balanced Salt Solution, 37°C (EBSS, Gibco BRL) from the infundibulum towards the isthmus region under gentle massage. In a preliminary experiment the effect of different pressures during massage on oviduct morphology was evaluated (n=5 cows) and no damage of the epithelial cells was observed. The EBSS was collected into an embryo recovery filter (Embryo Concentrator, Immuno Systems Inc., Spring Valley, WI, USA). The embryonic structures were collected by rinsing the filter with 10 ml 1X EBBS.

Fertilization rate. Fertilization rates in norgestomet/GnRH treated animals and control animals were compared using light microscopical (LM) evaluation of the zygotes. To be able to further analyze eventual differences in fertilization rates between groups, half of the collected zygotes were fixed for light microscopical examination of 1 µm thick semi-thin Epon sections. All remaining zygotes were processed for LM (DAPI or aceto-orcein staining).

For semi-thin sectioning zygotes were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH=7.3) for 1 h at 4°C and then stored in 0.1M cacodylate buffer (pH=7.3) at 4°C. Subsequently they were post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer, dehydrated and Epon embedded. Semi-thin sections were cut and finally stained with 1% basic toluidine blue for LM evaluation.

For DAPI staining, zygotes were fixed in 2.5% glutaraldehyde and processed according to the protocol described by Mori et al. (1988). For aceto-orcein staining zygotes were fixed in 25% v/v glacial acetic acid, stained with 1% w/v orcein (Sigma, St. Louis, MO, USA) in 40% v/v acetic acid and rinsed with 20% v/v glacial acetic acid, 20% v/v glycerol.

All zygotes with two or more pronuclei or zygotes that already underwent cleavage were considered as being fertilized.

Oviduct morphology. The oviduct was trimmed from the excess peritoneal fat and serosa. To assure identical sampling sites between animals, all oviducts were divided into 12 segments of the same length per cow; segment 1 was the most abdominal end with fimbriae, segment 6 the transition from ampulla to isthmus and segment 12 the tip of the uterine horn. Tissue samples of the oviduct were prepared from segment 1: infundibulum, segment 3 and 5: proximal and distal ampulla and segment 7: isthmus.

Tissue samples of the left oviduct were fixed for 24 h in Bouins fluid and those of the right oviduct in 0.1 M phosphate buffered (pH 7.4) formaldehyde (4%). After fixation, the tissues of both oviducts were dehydrated in a graded series of ethanol and embedded in paraffin (Paraclean, Klinipath B.V., Zevenaar, The Netherlands). Five µm thick tissue sections were mounted on a slide, deparaffinized and then stained with periodic acid-Schiff (PAS) and Harris's hematoxylin (Bancroft and Stevens, 1996). To evaluate the secretory potential of the oviductal epithelium, tissue samples from the left and right oviducts were scored to be PAS-negative, weakly PAS-positive, moderately PAS-positive or strongly PAS-positive when no secretory cells, few stained cells, abundantly and barely stained cell clusters, or overall abundance of stained cells were found in the epithelium, respectively. Per tissue sample 5-10 sections were analyzed; variations in classification for PAS staining between different sections of one tissue sample were negligible.

For immunohistochemical studies of the tissue samples from the right oviduct endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 10 min. After washing in distilled water, slides were brought in 10 mM citrate buffer (pH 6) and placed in a microwave oven for 10 min (95°C). After rapid cooling and washing in 0.01 M Tris buffered saline (TBS, pH 7.6; 3 times for 5 min), slides were preincubated with 10% normal goat serum in TBS for 30 min at room temperature. Slides were then incubated overnight at 4°C with either i) monoclonal anti-human estrogen receptor (Clone ER-7G5; 1:1) or ii) monoclonal anti-human progesterone receptor (Clone RR-2C5; 1:1) (both from Zymed Laboratories Inc., San Francisco, CA, USA). Subsequently, slides were rinsed in TBS (3 times for 5 min) and incubated for 60 min in a 1:100 dilution of biotinylated goat-anti-mouse serum (Vector Laboratories Inc., Burlingame, USA) in TBS containing 0.5% w/v BSA (TBS-BSA). After incubation, slides were again rinsed in 0.01 M phosphate buffered saline (PBS, pH 7.5, 3 times for 5 min) and placed in Vectorstain ABC kit-elite (Vector laboratories Inc.) in a 1:500 dilution in TBS-BSA for 60 min. Subsequently, slides were rinsed in TBS (2 times for 5 min) and 0.05 M Tris-HCl buffer (pH 7.6, 10 min), whereafter bound antibody was visualized by incubating slides with a 0.6% solution of 3,3'-diamino benzidine tetrahydrochloride (Sigma) in 0.05 M Tris-HCl (pH 7.6) with 0.03% hydrogen peroxide for 10 min. Counterstaining was done with hematoxylin. Controls include replacing the antibody by either TBS or normal mouse serum. The following criteria were used to score the presence and distribution of the immunohistochemical reactions with the light microscope: - no reaction, + weak reaction, i.e. less than one third of the number of epithelial nuclei show brown

immunostaining, ++ moderate reaction, i.e. one to two thirds of the number of epithelial nuclei show brown immunostaining, and +++ strong reaction, i.e. more than two thirds of the number of epithelial nuclei show brown immunostaining.

Embryonic development (Experiment C)

Cows (control: n=11, norgestomet/GnRH: n=11) were slaughtered at the local slaughterhouse at day 7 after AI and reproductive tracts were recovered immediately and kept at body temperature until flushing ex vivo within 2 h. After removal of the ovaries the uterine horns were separated and the oviducts were dissected from the ligaments. For first flushing the caudal side of a uterine horn was blocked with a large hemostatic forceps and 30 ml phosphate buffered saline (PBS) at 37°C was introduced through a blunt needle which was inserted into the oviduct infundibulum. After massaging the uterine horn and oviduct, the PBS was collected into an embryo recovery filter (Embryo Concentrator, Immuno Systems Inc.). The oviduct and uterine horn were flushed a second time with 20 ml PBS directly into the filter. All collected developmental stages were counted and classified as one cell-, 2-32 cell stage or embryo, using a stereomicroscope. Embryonic structures that were qualified as 'multicellular' were also DAPI stained to count the number of nuclei. From two heifers all recovered embryonic structures were DAPI stained to evaluate the accuracy of the stereomicroscopical observations. The embryos were graded as excellent, good, fair and poor according to the method described by Lindner and Wright (1983). In this study, embryos graded as excellent, good or fair are referred to as viable embryos and those graded as poor are considered to be non-viable embryos.

Bloodsampling and RIA of progesterone, estradiol-17ß and LH

Blood samples were taken from the jugular vein into heparinized vacuum tubes and stored in melting ice before centrifugation at 4°C. Plasma was either stored at -25°C or used immediately for rapid RIA of LH and stored afterwards.

During experiment C, bloodsamples were taken frequently to monitor plasma estradiol-17ß and LH concentrations. Plasma estradiol-17ß concentrations were measured every 6 h from administration of eCG until 96 h after PG. In addition, plasma LH concentrations were measured hourly from 30 h until 50 h after PG administration (control animals) or until 12 h after GnRH injection (norgestomet/GnRH treated animals).

During experiment A and B, hourly bloodsamples to detect the preovulatory LH surge were taken from 30 h after PG administration until after the occurrence of the surge in the control group and from removal of the ear implant until 10 h after GnRH administration in the norgestomet/GnRH group. At relevant times bloodsamples were

taken to analyze the pattern of the concentration of estradiol-17ß in the peripheral blood.

Concentrations of progesterone and estradiol-17ß in the peripheral blood were estimated by validated solid-phase ^{125}I RIA methods (Coat-A-Count TKPG and TKE respectively: Diagnostic Products Corporation, Los Angeles, CA, USA) as described by Dieleman and Bevers (1987). The sensitivities were 0.15 nmol l^{-1} and 7.5 pmol l^{-1}, and the interassay coefficients of variation were 11% and 8.9%, respectively.

Concentrations of LH were estimated by a validated RIA method as described by Dieleman et al. (1983, 1986). The intra- and interassay coefficients of variation were < 9%. The sensitivity was 0.4 µg l^{-1} NIH-LH-B4. Cross-reactivity of eCG (highly purified eCG, PM23-2P; Intervet International BV) was 0.2%.

Data handling and statistical analysis

Unless marked differently, values are mean ± standard error of the mean (SEM). Differences are considered significant if $P < 0.05$.

The duration of the LH surge was defined as the period that the LH concentration was higher than the preceding mean basal level plus 3xSD. The area under the curve of the preovulatory LH surge was taken as a measure for the total amount of LH released. Two-sample t-test was used to analyze differences in mean hormone concentrations between groups which in case of heterogeneity of variance was performed on log transformed data.

The difference in response to the superovulation treatment between groups during the three experiments (control: n=31 heifers, norgestomet/GnRH: n=28 heifers) was also analyzed using two sample t-test on log transformed data.

Recovery rates of zygotes (experiment B) and embryonic structures (experiment C) were calculated relative to the number of fresh ovulation sites or corpora lutea at the time of collection, respectively, and analyzed by logistic regression with overdispersion (McCullagh and Nelder, 1989).

Cleavage-, blastocyst formation- and hatching rates (experiment A) were calculated relative to the total number of oocytes from all heifers in one treatment group at day 1 of IVC and were analyzed using Chi-square analysis. Differences between groups in the percentage of cows with abundant PAS reaction in specific regions of the oviduct were also analyzed using Chi-square analysis.

Percentages of ‘indefinable’ structures, 2-32 cell stages, viable embryos and total number of embryos (experiment C) were calculated as the mean from individual percentages of each of the eleven cows in both treatment groups. Differences in percentages of ‘indefinable’ structures, 2-32 cell stages and total number of embryos between groups were analyzed using generalized estimating equations, using a Poisson distribution and an unspecified correlation structure (Diggle et al., 1994). This model uses the individual data per cow. Differences between groups in the fraction of

viable embryos of the total number of embryos were analyzed using logistic regression with overdispersion (McCullagh and Nelder, 1989).

RESULTS

Hormone concentrations in peripheral blood

In all heifers (n=59) that were selected for the experiments, the progesterone concentration decreased to values < 3.5 nmol l^{-1} within 24 h after PG administration indicating that luteal regression was completed. During norgestomet treatment LH surges were not detected, and one single LH surge was always observed with a maximum at 2 h after subsequent GnRH administration. The average interval between the administration of PG and the maximum of the LH surge was 42.4 ± 4.2 (SD) and 53.8 ± 3.2 h for control (n=31) and norgestomet/GnRH treated heifers (n=28), respectively. The mean maximum estradiol-17ß concentration at the occurrence of the preovulatory LH surge was significantly higher in norgestomet/GnRH treated than in the control heifers (Table 1) and in all animals a decrease of the estradiol-17ß concentration was observed between 6 and 8 h after the maximum of the LH surge.

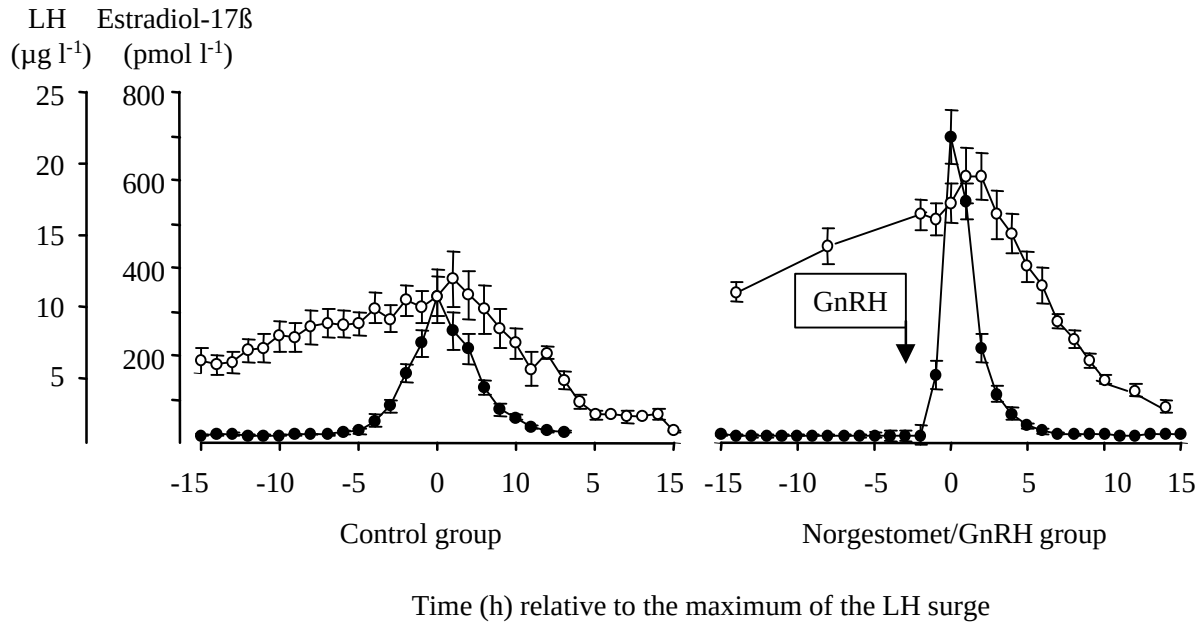

Figure 2. Concentrations (mean ± SEM) of estradiol-17ß (o) and LH (•) in peripheral blood of eCG/PG/anti-eCG treated heifers with a spontaneous LH surge (control, n=11) and of heifers with a temporarily postponed, GnRH-induced LH surge (norgestomet/GnRH treated, n=11). Arrow indicates administration of GnRH (1.0 mg, im).

Figure 2 is representative for the pattern of LH and estradiol-17ß concentrations in control and in norgestomet/GnRH treated heifers. The amplitude of the induced LH surge was significantly higher, but its duration was significantly less than that of control heifers (6.0 ± 0.9, n=11, and 9.8 ± 0.9 h, n=11, respectively). The total amount of LH released during the preovulatory signal calculated as the area under the curve (53.4 ± 9.3 and 65.8 ± 4.9 µgh l^{-1}) was similar for control and norgestomet/GnRH treated heifers.

Superovulation response

During the three experiments superovulation responses were always higher in the norgestomet/GnRH treated than in the control heifers and the average overall response to superovulation was significantly higher in the norgestomet/GnRH group than in the control group (Table 1).

Table 1. Maximum plasma estradiol-17ß concentrations in peripheral blood and follicular responses after stimulation with eCG/PG of heifers with a spontaneous preovulatory LH surge (control) and of heifers with a temporarily postponed, GnRH-induced LH surge (norgestomet/GnRH treated).

	Control			Norgestomet/GnRH treated		
Experiment	n	Maximum E (pmol l^{-1})	Response[1)]	n	Maximum E (pmol l^{-1})	Response
Maturation (A)	11	463 ± 51	20.8 ± 2.9	9	656 ± 131	26.0 ± 3.0
Fertilization (B)	9	361 ± 54	14.0 ± 1.8	8	238 ± 30	18.9 ± 2.4
Embryo Develop. (C)	11	379 ± 43	20.5 ± 2.3	11	638 ± 102	29.6 ± 3.8
Overall	31	403 ± 17*	18.7 ± 1.5[a]	28	529 ± 36**	25.4 ± 2.0[b]

Values are mean ± SEM. Max mean estradiol-17ß concentrations (*,**) and superovulation responses ([a,b]) with different superscripts are significantly different ($P<0.05$). [1)] Response to superovulation on the basis of the number of (A) preovulatory sized follicles (> 8 mm) shortly before ovulation, (B) fresh ovulation sites 44 h after the LH surge and (C) corpora lutea 7 days after AI; n=number of heifers; E=estradiol-17ß.

Maturation

Ovariectomies were performed in norgestomet/GnRH treated heifers and in control heifers at similar times after the maximum of the LH surge, i.e. 24 h ± 42 min (SD) and 24 h ± 94 min after the LH surge, respectively. Also, the average intervals between ovariectomy and in vitro insemination were similar for the two groups (norgestomet/GnRH treated vs. control heifers, 2 h ± 49 min (SD) and 2 h 9 min ± 33 min, respectively). The recovery rates of COCs were 60.8 ± 18.3% (SD) for norgestomet/GnRH treated heifers and 62.0 ± 25.9% for control heifers. COCs not showing expanded cumulus mass were excluded from further IVF and IVC (n=2 of

145 and n=7 of 132 for norgestomet/GnRH treated and control animals, respectively). At removal of the cumulus cells of the COCs after IVF, 4 oocytes of norgestomet/GnRH treated heifers were lost.

Cleavage rates at day 4 of IVC after IVF were not significantly different between control and norgestomet/GnRH treated heifers. Also no differences were found between the rates of blastocyst formation (42.4 vs. 46.8%) and hatching (33.6 vs. 37.4%) for control and norgestomet/GnRH treated heifers at day 11 of IVC (Figure 3).

Figure 3. Rates of cleavage, blastocyst formation and hatching during IVC after IVF of in vivo matured oocytes obtained from eCG/PG/anti-eCG treated heifers with a spontaneous LH surge (control, open bars) and of heifers with a temporarily postponed, GnRH-induced LH surge (norgestomet/GnRH treated, stippled bars). Day 1 is start of IVC. Total number of COCs at day 1 was 125 for control animals and 139 for norgestomet /GnRH treated heifers. Numbers above bars represent the number of zygotes or (hatched) blastocysts present at the day of observation.

Fertilization

Ovario-oviductectomy was performed at 46 h ± 5 h (SD) after the spontaneous LH surge in the control heifers and at 44 h after the induced LH surge in the norgestomet/GnRH treated heifers. From one norgestomet/GnRH treated heifer, the

right oviduct could not be collected intact and therefore no tissue or zygotes were collected from this oviduct and in some cases oviductal tissues from segment 1 and 7 were lost during surgery. In total 53 of 64 (norgestomet/GnRH) and 67 of 72 (control) of the expected tissue samples were collected.

Fertilization rates. The recovery rate of the zygotes from the oviducts tended to be lower in norgestomet/GnRH treated animals (43.8% ± 21.5 (SD)) than in control animals (64.9% ± 21.3), (P=0.056). Overall, 72 embryonic structures recovered from control heifers and 44 from norgestomet/GnRH treated heifers were analyzed. In control animals, on average, 73.0 ± 10.3% (n=9, heifers) of the analyzed zygotes were fertilized from which 14.0% (7 zygotes) showed polyspermy. In norgestomet/GnRH treated heifers, on average, 69.0 ± 14.0% (n=8, heifers) of the analyzed zygotes were fertilized and 2 cases of polyspermy were found (6%). The LM examination did not reveal differences in fertilization rates between the two groups and therefore no further microscopical analysis of the semi-thin sections was performed.

Oviduct morphology. Two tissue samples (1: norgestomet/GnRH, 1: control) appeared to be collected from the isthmus instead of the distal ampulla, and were therefore excluded from further calculations. The left and the right oviduct of each heifer showed similar distribution of PAS positive granules and therefore the data were combined in the following calculation.

Examples of unstained, weakly, moderately, and strongly PAS stained tissue samples are shown in Figure 4. For analysis of the results, tissue samples showing absence of staining or a weak PAS reaction were grouped (no/weakly PAS positive). The same was done for tissue samples that stained moderately or strongly with PAS (abundant PAS positive). The incidence of negative samples in the no/weakly PAS positive group was 60%; that of the moderately PAS stained samples in the abundantly PAS positive group was 50%. In general, PAS reactions were most abundant in the infundibulum and obviously less present in the isthmus where no or only a few cells stained. However, in the ampulla of control heifers abundant PAS positive staining tended to be (proximal ampulla, P=0.28) or was significantly more often present (distal ampulla, P=0.045) than in that of norgestomet/GnRH treated heifers (Figure 5).

Throughout the whole oviduct progesterone and estradiol-17ß receptors were detected in the nuclei of the epithelial cells by immunohistochemistry. The infundibulum and the isthmus showed a strong and the ampulla a moderate immuno-reaction (Figure 4). No differences were found between groups, indicating a similar expression of the steroid receptors in both groups.

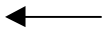

Figure 4. Morphological evaluation of tissue samples of bovine oviducts collected at 38-52 h after the LH surge from eCG/PG/anti-eCG treated heifers with a spontaneous (control) or temporarily postponed GnRH-induced LH surge (norgestomet/GnRH treated). 1-4: Representative examples of the four classes of abundance of PAS positive cells in the epithelium of the ampulla after periodic acid-Schiff (PAS) and Harris's hematoxylin staining, x185. 1. Absence of PAS reaction. e= epithelium, c= connective tissue of the lamina propria, m= muscular layer; 2. Weak PAS reaction: only few epithelial cells are stained (arrows); 3. Moderate PAS reaction in the epithelium; 4. Strong PAS reaction. 5-7: immunohistochemical staining for progesterone receptor in epithelial cell nuclei, x185. 5. Strong immunoreaction in the infundibulum; 6. Moderate immunostaining in the ampulla; 7. Strong immunoreaction in the isthmus. 8. Negative control of progesterone receptor immunoreactivity in the infundibulum after replacement of antibody by normal mouse serum, x115.

Figure 5. Proportion of heifers with abundant (: moderate or strong) PAS reaction in epithelial cells in infundibulum, proximal and distal ampulla and isthmus region of oviducts collected at 38-52 h after the LH surge of eCG/PG/anti-eCG treated heifers with a spontaneous LH surge (control, open bars) and of heifers with a temporarily postponed, GnRH-induced LH surge (norgestomet/GnRH treated, stippled bars). Data obtained from the left and the right oviduct are combined. Bars with different superscripts (*) representing samples of one oviduct segment are significantly different ($P<0.05$).

Embryonic development

As in experiment B, recovery rates of embryonic structures tended to be lower in norgestomet/GnRH treated animals (60.0 ± 19.1 % (SD)) than in control animals (74.0 ± 10.8 %) (P=0.19). Overall, 205 embryonic structures were recovered from the norgestomet/GnRH treated animals and 165 from the control animals. A summary of the recovered embryonic structures is presented in Table 2. The number of nuclei as scored by DAPI staining were similar to the number of cells observed by LM for embryonic structures with > 32 cells. DAPI staining of all recovered structures of 2 at random selected heifers demonstrated that only 23% of the LM classified one cell stages of these heifers had indeed one nucleus. Therefore, the group of stereomicroscopically qualified one cell stages was classified 'indefinable' (Table 2).

A large variation in the respective percentages of collected embryonic structures was found in both the norgestomet/GnRH treated and control heifers (range 0-100%). The proportion of in total collected embryos (Table 2) was not significantly different for norgestomet/GnRH treated and control group, and also the fraction of viable embryos relative to the total number of embryos (60% and 61%, norgestomet/GnRH and control group, respectively) was not different. The percentage of 'indefinable' structures recovered in the norgestomet/GnRH treated group (n=11) was significantly higher than in the control group.

Table 2. Embryonic structures flushed at day 7 after AI from eCG/PG/anti-eCG treated heifers with a spontaneous LH surge (control) and of heifers with a temporarily postponed, GnRH-induced LH surge (norgestomet/GnRH treated).

	Number (percentage) of embryonic structures				
	'indefinable'	2-32 cell	total embryos	viable embryos	total structures
Control (n=11)	4.4 ± 1.6 (26.9 ± 7.4[a])	4.9 ± 0.9 (34.8 ± 6.3)	5.6 ± 1.9 (37.6 ± 10.1)	3.4 ± 1.5 (23.0 ± 8.1)	15.0 ± 1.7
Norgestomet/ GnRH (n=11)	10.7 ± 3.0 (57.5 ± 9.8[b])	3.9 ± 1.3 (19.8 ± 4.8)	3.8 ± 1.4 (21.6 ± 7.7)	2.3 ± 1.0 (12.5 ± 5.1)	18.6 ± 3.9

Values are mean ± SEM; values within one column with different superscripts are significantly different (P<0.05). Values between brackets are the means of the percentages of recovered embryonic structures relative to the total number of structures per heifer. 'Indefinable' structures were all stereomicroscopically classified as one cell structures. Viable embryos comprise embryos graded as excellent, good and fair.

DISCUSSION

The period of preovulatory follicular development between regression of the corpus luteum and onset of final maturation, i.e. the LH surge, is markedly reduced when cows are treated for superovulation. In normally cyclic cows development takes place in about 60 h whereas in eCG-stimulated animals only about 40 h are available. Therefore, it was investigated whether prolongation of the preovulatory period reduces heterogeneity of the stimulated follicle population with respect to the potential to ovulate, and to maturation, fertilization and development into embryos of the oocytes.

The procedure used to postpone the occurrence of the preovulatory LH surge obviously was very effective. Norgestomet always prevented spontaneous release of the LH surge as can be inferred from the LH plasma concentrations remaining low during treatment. To release such an LH surge in these heifers GnRH in all likelihood had to be administered at the time of removal of the norgestomet implant similar to eCG/PG-treated heifers in which the LH surge was suppressed by a progesterone releasing intravaginal device (Vos et al., 1994b). Although the induced LH surges were somewhat but significantly different from those occurring spontaneously with regard to amplitude and duration, similar total amounts of LH were released in both norgestomet/GnRH treated and control heifers. It is assumed, therefore, that subsequent final follicular maturation was initiated similarly in both groups. This is supported by the observed decrease in all animals of the estradiol-17ß plasma concentration 6-8 h after induced or spontaneous LH surge. It cannot be excluded that formation of corpora lutea after ovulation was affected in the norgestomet/GnRH group. Administration of hCG at day 5 of the estrous cycle generates corpora lutea producing more progesterone than those induced by a GnRH agonist (Schmitt et al., 1996). In our study, however, progesterone plasma concentrations at day 7 after superovulation were not different between groups. It is concluded that norgestomet/GnRH treatment during eCG-stimulation results in extension of the period of preovulatory follicular development preceding the LH surge to 54 h, which resembles that in normally cyclic cows. On the basis of the variable time of occurrence of the spontaneous LH surge, this period was 6-14 h shorter in control heifers.

Extension of the period of preovulatory follicular development clearly increased the number of mature preovulatory follicles capable of responding properly to the LH surge. The maximum estradiol-17ß plasma concentration which is correlated with the number of preovulatory follicles (Bevers and Dieleman, 1987) was on average 1.3 times higher in norgestomet/GnRH treated than in control heifers. The increased number of preovulatory follicles in the norgestomet/GnRH treated heifers corresponded with the observed on average 1.4 times higher ovulation rate. It is likely that the increase in superovulatory response was primarily due to more follicles being

stimulated as observed by the increased number of preovulatory-sized follicles at the time of ovulation. Oocytes out of these follicles showed similar in vitro developmental potential when collected from norgestomet/GnRH or control heifers indicating that heterogeneity of the follicular population was still present with regard to final maturation in vivo. It cannot be excluded, however, that reduction of heterogeneity of the preovulatory-sized follicular population present at the time at which the spontaneous LH surge would have occurred was a major factor contributing to the improved superovulatory response, and that the increased number of preovulatory-sized follicles observed at ovulation was merely a consequence of more follicles getting stimulated additionally due to extension of the stimulation period. These additional follicles would then contribute the part of the follicular population that is not mature.

When spontaneous release of the LH surge is prevented by prolonged administration of a GnRH agonist instead of by norgestomet no extra increase of the ovulation rate is observed after treatment for superovulation (d'Occio et al., 1997). The difference in effect of postponement of the LH surge on superovulatory response may be explained by absence of pulsatile release of LH during GnRH agonist treatment as reported by Gong et al. (1996) for non-stimulated heifers which were continuously infused with a GnRH agonist. On the contrary, during norgestomet treatment pulsatile release of LH is maintained (Sanchez et al., 1995) and LH pulse frequency is higher than in the presence of a functional corpus luteum (Kojima et al., 1992). It is supposed that pulsatile release of LH is essential for oocytes to acquire developmental competence (Greve et al., 1995).

The observed increase of the ovulation rate in heifers with postponed LH surges was not reflected by a corresponding increase of the number of viable embryos at day 7 after AI. In both groups the yield of embryos was noticeably reduced compared to previously reported results for the eCG/PG/anti-eCG procedure (Dieleman et al., 1989; Bevers et al., 1993). A marked difference, however, concerns the two fold higher proportion of ‘indefinable’ structures that were recovered at day 7 after AI from the norgestomet/GnRH treated heifers in comparison to control superovulated heifers. These structures appeared to be non-fertilized oocytes upon lightmicroscopic examination, but after staining for nuclei in an at random selection of samples, the majority was exposed as fertilized zygotes that were arrested during very early stages of embryonic development. This is supported by the high rate of fertilization observed at about 20 h after the period of multiple ovulations in both groups of heifers. It is, therefore, unlikely that postponement of the LH surge caused intrafollicular aging of oocytes as has been reported in rats (Mattheij et al., 1994) that were treated with low doses of LH before onset of final maturation. Although prolonged exposure to LH-like activity of eCG in the norgestomet/GnRH treated group may have occasionally initiated premature resumption of meiosis, the process of final maturation was not adversely affected as shown by the high in vitro developmental potential of oocytes after in vivo maturation which was similar to that in control heifers.

After fertilization a substantial and crucial part of further embryonic development takes place in the oviduct at the junction of the ampulla and isthmus. Amongst the multitude of factors influencing embryo development, products of the oviduct epithelium play a role of which secretion is controlled by steroid hormones (Gandolfi, 1994). Oviductal glycoproteins have been demonstrated to associate with the zona pellucida of in vivo developing embryos (Gandolfi et al., 1989) and in vitro embryonic development is promoted by co-culture of early embryos with oviduct cells (Rexroad and Powell, 1988) or their products (Eyestone and First, 1989). In the present study, the abundance of PAS positive secretory cells in the epithelium of the ampulla was considerably decreased in norgestomet/GnRH treated heifers compared to control heifers. This may reflect a decreased production or an advanced secretion of the granules to the lumen of the oviduct. In the normally cyclic cow, maximal secretory activity of the oviduct with regard to amount and composition of the fluid in the oviducts occurs in response to estradiol-17ß when concentration of progesterone is basal (Killian et al., 1989). Also in sheep, progesterone appeared to act antagonistically to estradiol with regard to the stimulatory effect of estradiol on the secretory cells of the epithelium when both steroids were administered simultaneously (Willemse, 1975). In the norgestomet/GnRH treated heifers the progestagen activity of norgestomet was almost continuously present when high concentrations of estradiol-17ß were produced by the growing follicles whereas in the control heifers the progesterone concentrations started to decrease when prostaglandin was administered to reach basal levels ample time before estradiol-17ß concentrations became maximal. However, these differences of the patterns of steroid concentrations were not accompanied by changes in the distribution of progesterone and estradiol-17ß receptors in the epithelium of the oviduct. Nevertheless, the observed reduction in secretory activity of this epithelium probably is a consequence of the prolonged norgestomet treatment and may have caused a premature arrest in embryo development during the first cell cleavages.

In conclusion, prolongation of the period of preovulatory follicular development until the occurrence of the LH surge to one that resembles that in normally cyclic cows improves development of more mature, estradiol-17ß producing follicles after eCG stimulation. This also results in increased ovulation rates. It is suggested that the heterogeneity of the follicular population is reduced as is present in normally stimulated heifers at the time of the spontaneous LH surge. The treatment with norgestomet to postpone the LH surge did not adversely affect final maturation and fertilization but early embryonic development appeared to be impaired which may be due to reduced secretory activity of the cells of the epithelium of the oviduct. Further experiments are required to examine whether the norgestomet/GnRH treatment can be applied in practice to improve superovulation with regard to AI and administration of anti-eCG at a fixed time after removal of the implant without detection of estrus being necessary. It is clear that the treatment procedure can be used to collect follicles and oocytes at fixed stages in vivo to study the impact and mechanisms of specific steps of

development on potential to grow into viable embryos (Hendriksen et al., 1998; van de Leemput et al., 1998).

Acknowledgments

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IMPROVEMENT OF IN VITRO EMBRYO DEVELOPMENT USING IN VIVO MATURED OOCYTES FROM HEIFERS TREATED FOR SUPEROVULATION WITH A CONTROLLED PREOVULATORY LH SURGE

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In bovine IVP, the IVM step appears to be rather successful with 80% of the oocytes reaching the MII stage. However, to what extent IVM contributes to limiting the yield of viable embryos in IVP is still hardly known. Therefore, we compared the developmental capacity during IVC after IVF of oocytes which had been matured in vitro with that of oocytes which were collected after in vivo maturation. IVM was carried out for 22 h with oocytes (n=417) obtained from 2-8 mm follicles of ovaries from a slaughterhouse in M199 with 10% fetal calf serum (FCS), 0.01 IU LH per ml and 0.01 IU FSH per ml. In vivo matured oocytes (n=219) were aspirated from preovulatory follicles at 22 h after the LH surge which was induced by GnRH at a fixed time in eCG/PG/anti-eCG-superovulated heifers; endogenous release of the LH surge was suppressed by a norgestomet ear implant. This allowed synchronization of the in vitro and in vivo maturation processes and, as a consequence, simultaneous IVF of both groups of oocytes. Overall the in vitro developmental potential of in vivo matured oocytes was two times higher ($P<0.01$) than that of in vitro matured oocytes with blastocyst formation and hatching rates after 11 days IVC of 49.3 ± 6.1 (SEM) (n=10 heifers) versus 26.4 ± 1.0% (n=2 replicates) and 39.1 ± 5.1% versus 20.6 ± 1.4%, respectively. It is concluded that IVM is one of the major factors limiting the yield of viable embryos in IVP, although it cannot be excluded that other factors contributed to the observed differences such as the lack of normal preovulatory development for the in vitro matured oocytes which precedes final maturation.

INTRODUCTION

In vitro embryo production (IVP) combined with the possibility of collecting oocytes by transvaginal ultrasound-guided ovum pick up (OPU) renders a promising tool to increase the number of offspring of cows of specific interest. This technique provides several advantages over the conventional method of superovulation (SO).

OPU offers the possibility of collecting oocytes throughout the estrous cycle and the same animal can be used repeatedly as oocyte donor (Hanenberg et al., 1997). It can be calculated that continuous use of OPU/IVP may double the number of calves per year from one donor cow compared to repeated SO. Also, OPU/IVP appears to be a more reliable and constant embryo production system since this technique is without the unpredictable variability in response to superovulation treatment with either FSH or eCG.

However, the efficiency of OPU/IVP is far from optimum when calculated on the basis of the number of oocytes necessary to produce one calf. In general, 100 oocytes result in 55 and 30 live born calves after artificial insemination of normally cyclic and SO-treated cows, respectively, whereas OPU/IVP generates only 8 calves from 100 collected oocytes.

Many efforts have been undertaken to improve in vitro embryo production systems, almost exclusively concentrating on in vitro fertilization (IVF) and in vitro culture (IVC) (Gordon and Lu, 1990). Only limited research has been focused on the contribution of in vitro maturation (IVM) to the success rate of IVP. This may be due to the apparently high yield of IVM. In vitro matured oocytes do show expanded cumulus mass and about 80% of the oocytes is in MII stage at the end of the maturation period (de Loos et al., 1994). In 1987, Leibfried-Rutledge et al. (1987) reported that oocytes clearly acquired enduring features when matured in vivo but not in vitro that benefited their developmental potential up to the morula stage after IVF and subsequent transfer to sheep oviducts. It could very well be that this observed difference in developmental potential for in vitro vs. in vivo matured oocytes can no longer be distinguished when using current day IVM, IVF and IVC procedures.

Therefore, we investigated to what extent IVM contributes to limiting the yield of viable embryos in currently used IVP by comparing the developmental potential of in vitro and in vivo matured oocytes. Results of a preliminary study (van de Leemput et al., 1996) indicated that the developmental potential during IVC after IVF was increased when pooled in vivo matured oocytes from superovulated heifers were used. However, an extensive variation in developmental potential was observed between replicates which was probably due to animal variations with regard to oocyte quality (Hyttel et al., 1991) and response to superovulation. Substantial differences between the periods of maturation occurred as a consequence of the variation in time of occurrence of the preovulatory LH surge initiating maturation. In general, the interval between treatment with prostaglandin and the LH surge shows a wide range in superovulated cows (Dieleman et al., 1993). To overcome the irregularity in maturation period superovulation may be carried out with a controlled preovulatory LH surge by suppressing endogenous release and subsequent induction with GnRH at a fixed time (Vos et al., 1994b). It has been demonstrated that such a procedure produces in vivo matured oocytes with an in vitro developmental potential after IVF which is equivalent to that of oocytes obtained from normally superovulated heifers (Vos et al., 1996a). Moreover, superovulation with a controlled LH surge allows precise synchronization of in vivo and in vitro maturation to be succeeded by simultaneous IVF, thereby preventing experimentally induced artifacts during IVP (Sirard and Blondin, 1996).

In this study, development into hatched blastocysts during IVC was compared for in vitro and in vivo matured oocytes after IVF. In vivo matured oocytes obtained from heifers treated for superovulation with a LH surge at a fixed time were cultured in

separate wells per heifer to account for differences in oocyte quality due to variation between heifers.

MATERIALS AND METHODS

Experimental design

A preovulatory LH surge was induced at a fixed time initiating maturation in two groups of 5 heifers treated for superovulation to obtain in vivo matured oocytes at a fixed time (Table 1). Concomitantly, in vitro maturation of immature oocytes from ovaries obtained from a slaughterhouse was started 5 h later to account for the time interval associated with the induction of the LH surge and the ovariectomies to collect the in vivo matured oocytes. Thus, IVF could be accomplished simultaneously for in vivo and in vitro matured oocytes with the same batch of sperm, and subsequent culture of the embryos took place under the same conditions.

Table 1. Superovulation treatment to obtain in vivo matured oocytes.

Time day	h	Treatment / event
0		estrus
8		removal of eventual dominant follicle
10	0	onset of superovulation with eCG
		implantation of norgestomet
12	48	induction of luteal regression with prostaglandin
15	102	removal of norgestomet implant
		induction of LH surge with GnRH
	104	maximum of the preovulatory LH surge
		start of in vivo maturation
	112	neutralization of eCG
16	126	ovariectomy
		completion of in vivo maturation

Oocyte collection and preparation

In vivo matured oocytes. For recovery of in vivo matured oocytes Holstein-Friesian heifers (n=10) were selected from our herd on the basis of general clinical examination and normal ovarian cyclicity indoors during at least 3 weeks as established by the progesterone concentration in peripheral blood samples taken 3

times a week. The heifers were housed in two groups of 5 animals put together at random, were fed silage and concentrate (to a maximum of 1 kg per heifer per day) and supplied water ad libitum. The experiments were carried out during November and December. The two groups were prepared for superovulation by synchronization of the estrous cycle, using an ear implant (3 mg norgestomet, Crestar; Intervet International BV, Boxmeer, The Netherlands) accompanied by treatment with 3 mg norgestomet with 5 mg estradiol-valerate (im). The heifers received prostaglandin (PG) 2 d before removal of the implants after 9 d (15 mg Prosolvin im; Intervet International BV) to ensure complete regression of the corpus luteum (Vos et al., 1996b). At day 8 (estrus is day 0) of the subsequent cycle all follicles > 8 mm were disabled by transvaginal ultrasound-guided puncturing to prevent eventual suppressive action of dominant follicles on the follicle population to be stimulated. Superovulation was carried out at day 10 with eCG (2500 IU Folligonan im; Intervet International BV) and PG (15 mg Prosolvin, im) 48 h later.

To facilitate simultaneous fertilization of in vivo and in vitro matured oocytes endogenous release of the preovulatory LH surge was suppressed during eCG stimulation by a temporary ear implant (Crestar) without accompanying treatment. Upon removal of the ear implant, the LH surge was induced with GnRH (1.0 mg Fertagyl in 10 ml saline im; Intervet International BV) (Table 1). Previous similar experiments (Vos et al., 1994b) showed that LH surges were to be expected at 56 h after treatment with PG, an interval corresponding to the average period of preovulatory follicular development in superovulated heifers. Finally, the heifers received a dose of monoclonal antibody against eCG (5 ml Neutra-PMSG iv; Intervet International BV) at 10 h after GnRH which was sufficient to neutralize 2500 IU of eCG within 1 h (Dieleman and Bevers, 1987).

Heparinized blood samples were collected from the jugular vein every other day from day 0 (estrus) until day 10, every 4 h from administration of eCG until PG administration, then every 2 h until 52 h after PG, hourly from 2 h before until 12 h after administration of GnRH, and finally, every 4 hours until ovariectomy. Blood samples were centrifuged at 4°C and stored at –25°C until use.

Heifers were ovariectomized under local anesthesia (Dieleman et al., 1983) at 22-24 h after GnRH administration. Ovaries were collected in saline (25°C) and transported immediately to the laboratory. Cumulus oocyte complexes (COCs) were obtained by aspiration of presumably preovulatory follicles > 8 mm and selected on the presence of an expanded cumulus mass. COCs with compact cumulus investment and degenerated oocytes were discarded. Until fertilization, selected COCs were kept in maturation medium, i.e. M199 (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (FCS, Gibco), 0.01 IU porcine FSH (Sigma, St. Louis, MO, USA) per ml and 0.01 IU equine LH (Sigma) per ml.

In vitro matured oocytes. Bovine ovaries were collected at a slaughterhouse from cows of unknown reproductive state in a thermos flask and transported to the

laboratory. COCs were obtained by aspiration of 2-8 mm follicles and selected on the presence of a multilayered compact cumulus investment. On average, 10 usable COCs were collected per ovary. Selected COCs were once rinsed with HEPES buffered M199 supplemented with 10% FCS and once with maturation medium. Groups of 35 oocytes were randomly allocated to each well of an 4-well culture plate (Nunc A/S, Roskilde, Denmark) containing 500 µl maturation medium per well and then cultured for 22 h (39°C, 5% CO_2 in humidified air).

IVF and IVC

IVF and IVC were performed as described by Izadyar et al. (1996). Briefly, in vitro matured oocytes were fertilized and cultured in groups of 35 oocytes per well of a 4-well culture plate whereas in vivo matured oocytes obtained from one animal were fertilized and cultured in an individual well. Both in vivo and in vitro matured oocytes were fertilized at the same time with the same batch of spermatozoa from a bull with proven in vitro fertility. Before fertilization oocytes in each well were rinsed twice with 2 ml HEPES buffered M199 medium per well.

Frozen/thawed semen used for IVF was centrifuged over a percoll gradient for 30 min at 700 g at 25°C. The sperm sample was collected by removing the gradient except for the last 150 µl containing the sperm pellet. COCs were transferred to 0.43 ml of fertilization medium (Fert-TALP) as described by Parrish et al. (1988) but without glucose and with 10 µl/ml penicillin/ streptomycin instead of gentamycin. Twenty microlitres of sperm suspension (final concentration $0.5x10^6$ spermatozoa/ml), 20 µl heparin (final concentration 10 µl/ml) and 20 µl PHE (consisting of 20 µM D-penicillamine, 10 µM hypotaurine, 1 µM epinephrine) were added. After 18 to 20 h of incubation (39°C, 5% CO_2 in humidified air), the zygotes were freed from cumulus cells by vortexing for 3 min and all zygotes were placed in a co-culture system of 0.5 ml of M199 containing 10% FCS on a monolayer of Buffalo rat liver (BRL) cells in each well (39°C, 5% CO_2 in humidified air). BRL cells, separated from the BRL cell line from the American Type Culture collection (ATCC) (Coon, 1968) were cultured routinely in an 1:1 mixture of Ham's F12 medium and Dulbecco's Modified Eagle's medium supplemented with 7.5% FCS and antibiotics. These cells differ from those currently available from ATCC in that they exhibit contact inhibition of growth.

On the fourth and eighth day of culture embryos were transferred to fresh co-culture wells. At day 4, the cleavage rate was evaluated and non-cleaved structures were removed. From cinematographic studies (Grisart et al., 1994) of in vitro developing bovine zygotes it is known that first cleavage activity occurs within a few hours after the end of the IVF period and the peak of cleavage activity emerges shortly thereafter, so it is very unlikely that oocytes would have cleaved after day 4 of IVC.

At day 7, 9 and 11 of culture numbers of blastocysts and hatched blastocysts were scored. Day 1 of culture was defined to start immediately after IVF was finished.

Radioimmunoassay of progesterone, estradiol-17ß and LH

Concentrations of progesterone and estradiol-17ß in peripheral bloodsamples were estimated by solid-phase ^{125}I RIA methods (Coat-A-Count TKPG and TKE, respectively; Diagnostic Products Corporation, Los Angeles, CA, U.S.A.) with modifications as described by Dieleman and Bevers (1987). The sensitivity was 0.15 nmol l^{-1} and 7.5 pmol l^{-1}, and the interassay coefficient of variation 11% and 8.9%, respectively.

Concentrations of LH were estimated by a validated RIA method as described by Dieleman et al. (Dieleman et al., 1983, 1986). The intra- and interassay coefficients of variation were < 9%. The sensitivity was 0.4 μg l^{-1} NIH-LH-B4. Cross-reactivity of eCG (highly purified eCG, PM23-2P; Intervet International BV) was 0.2%.

Data handling and statistical analysis

Unless marked otherwise, all values are expressed as mean ± SEM. When $P<0.05$, differences were considered significant.

Plasma estradiol-17ß concentrations (n=10 heifers) around the occurrence of the preovulatory LH surge, were analyzed with multivariate repeated measurements. In order to find the first significant decrease in time, Helmert contrasts were used with Bonferroni intervals (Johnson and Wichern, 1992). The correlation between the number of preovulatory-sized follicles, present at the time of ovariectomy, and the maximum plasma estradiol-17ß concentration within one animal was estimated by Pearson's correlation.

Cleavage, blastocyst formation and hatching rates are calculated as percentages of the numbers of (non)-fertilized oocytes at day 1; average percentages at each timepoint as depicted in Figure 2 are calculated as the mean from individual percentages of each of the ten cows for in vivo matured oocytes or of both replicates for in vitro matured oocytes. Differences in these percentages of cleaved zygotes and (hatched) blastocysts at each day of culture after in vivo or in vitro maturation were tested for significance using generalized estimation equations for dependent data with a binomial distribution and an exchangeable correlation structure, as described by Diggle et al. (1994). This model uses the individual data per cow (Table 2).

The progress of embryonic development at each day of IVC (Figure 3) is expressed as the number of blastocysts observed at that particular day, relative to the ultimate number of blastocysts at day 11 of IVC. To examine differences in the progress of

embryonic development between in vivo in vitro matured oocytes, these percentages were analyzed with the Chi-square test.

RESULTS

Hormone concentrations in peripheral blood

To evaluate the progress of the superovulation procedure progesterone, LH and estradiol-17ß concentrations in peripheral plasma were measured. In general, all cows (n=10) showed the same pattern of hormonal concentrations (Figure 1).

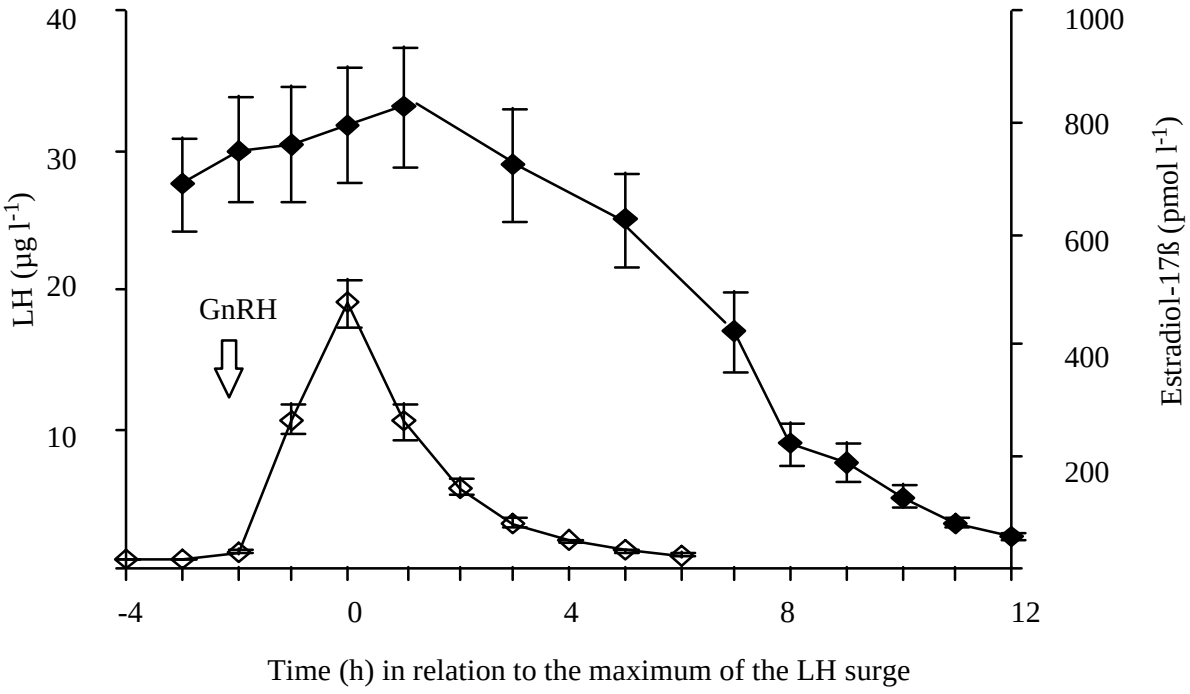

Figure 1. Concentrations (mean ± SEM) of estradiol-17ß (◆) and LH (♢) in peripheral plasma of heifers (n=10) treated for superovulation with induced LH surge to obtain in vivo matured oocytes. Arrow indicates injection of GnRH (1.0 mg, im).

Luteal regression was completed within 24 h after PG administration (i.e., peripheral progesterone concentration < 3.5 nmol l^{-1}). GnRH injection always resulted in a preovulatory LH surge with a duration of 6 h and a maximum of 19.7±1.7 µg l^{-1} occurring 2 to 3 h after GnRH injection. Maximum estradiol-17ß concentrations (825±105 pmol l^{-1}) were observed 1 h after the maximum of the LH surge and started to decrease significantly at 3h after the LH surge to basal values reaching 192±35 pmol l^{-1} at 9 h. Maximum estradiol-17ß concentrations (range 338-1402 pmol l^{-1}) were positively correlated (r=0.927; P<0.0001) with the number of preovulatory-sized follicles (>8 mm) present on the ovaries at time of ovariectomy (range 15-56 preovulatory-sized follicles).

Table 2. The effect of in vivo maturation of bovine oocytes on cleavage and blastocyst formation in vitro after IVF.

	Number of oocytes after IVF	Number of Cleaved embryos	Number of blastocysts				
			Total			Hatched	
	day 1	day 4	day 7	day 9	day 11	day 9	day 11
After IN VITRO maturation							
replicate 1	209	129	32	52	53	26	46
replicate 2	208	149	32	62	57	21	40
SUM	417	278	64	114	110	47	86
After IN VIVO maturation							
replicate 1							
H1	26	23	1	2	3	1	2
H2	10	8	1	3	3	1	3
H3	20	17	2	10	11	1	10
H4	21	18	2	9	12	0	6
H5	15	15	2	11	11	0	9
replicate 2							
H6	23	21	10	11	11	6	8
H7	37	33	9	23	22	10	17
H8	32	29	7	16	16	5	14
H9	20	13	2	7	7	1	6
H10	15	14	5	10	11	4	9
SUM	219	191	41	102	107	29	84

H1-10 = heifer 1-10. Day 1 is start of IVC.

In vivo matured oocyte collection

On average, ovariectomies were performed at 22 h 15 min ± 29 min (SD) after the maximum of the LH surge. The number of preovulatory-sized follicles that were aspirated was 33 ± 13 (SD) per cow (n=10). Aspiration was completed at 23 h 30 ± 52 min (SD) and COCs were stored in maturation medium until fertilization at 25 h 20 min ± 15 min (SD) after the LH surge. The maximum time necessary for processing all the COCs of a group of 5 heifers for fertilization was 2 h 54 min.

The average recovery rate after processing of the COCs until fertilization was 72.0 ± 7.7% (SD) (i.e. a total of 231 COCs out of 326 follicles) producing 23 ± 8 (SD) COCs per cow (range 11-38). The 8 COCs (3.5%) not showing an expanded cumulus mass and the 4 oocytes (1.7%) that were clearly degenerated were excluded from further IVF and IVC.

Table 3. Effect of individual heifer on rates of blastocyst formation and hatching of in vivo matured oocytes as observed at day 11 of IVC after IVF.

Heifer	1	2	3	4	5	6	7	8	9	10
% of Blastocysts										
Total	11.5	30.0	55.0	57.1	73.3	47.8	59.5	50.0	35.0	73.3
Hatched	7.7	30.0	50.0	28.6	60.0	34.8	45.9	43.8	30.0	60.0

Rates are calculated as percentage of the number of oocytes at day 1 of IVC.

IVM

In vitro matured oocytes were collected from slaughterhouse ovaries and matured in maturation medium for 21 h 54 min ± 20 min (SD) until start of IVF.

IVF and IVC

Total fertilization time was 19 h 21 ± 18 min (SD) for both in vivo and in vitro matured oocytes. On average 21.9 ± 8.1 (SD) in vivo matured oocytes were fertilized and cultured per well per heifer. Embryonic development during IVC of in vivo and in vitro matured oocytes is depicted in Table 2.

Embryo development in the two replicates with the in vitro matured oocytes was similar during the 11 days of IVC ($P > 0.12$). Therefore, data obtained from the two replicates were combined.

Overall, in vivo matured oocytes performed better during IVC after IVF than in vitro matured oocytes showing significantly higher ($P < 0.01$) rates of cleavage (86.9 ± 3.0 (n=10, heifers) versus 66.7 ± 5.0% (n=2, replicates)) and, at day 11, of blastocyst formation (49.3 ± 6.1 versus 26.4 ± 1.0%) and hatching (39.1 ± 5.1 and 20.6 ± 1.4%) (Figure 2).
However, the progress of blastocyst formation during IVC after IVF was slower ($P < 0.05$) for in vivo matured oocytes than for in vitro matured oocytes (Figure 3). A similar difference was observed when hatching stage was taken as end point.

In vivo matured oocytes obtained from individual animals were cultured separately. A large variation between animals was found in blastocyst formation rate (range 11.5 - 73.3%) and in hatching rate (range 7.7 - 60%) (see Table 3).

Figure 2. Rates of cleavage, blastocyst formation and hatching during IVC after IVF of in vitro matured bovine oocytes (open bars; n = 417 at start of IVC, n=2 replicates) and in vivo matured oocytes (stippled bars; n = 219 at start of IVC) collected from superovulated heifers (n = 10) at 22 h after the induced preovulatory LH surge. Rates are calculated as percentages of oocytes after IVF (mean ± SEM). Bars with different superscripts at one stage of development are significantly different ($P<0.01$). Day 1 is start of IVC.

Figure 3. Progress of blastocyst formation during IVC after IVF of bovine oocytes matured in vitro (open bars) or in vivo (stippled bars). Blastocyst formation is expressed as percentage (mean ± SEM) of the ultimate number of blastocysts present at day 11. Day 1 is start of IVC.

DISCUSSION

Our study was conducted to investigate the extent of the contribution of IVM to the limited yield of viable embryos in IVP despite of the apparent success of IVM of 80% of the oocytes reaching the MII stage. Therefore, the in vitro developmental potential

after IVF was compared for oocytes which were matured in vitro or in vivo. To obtain sufficient numbers of in vivo matured oocytes at a fixed time heifers were treated for superovulation using a procedure with GnRH-induction of the preovulatory LH surge. In vivo maturation took place for 22 h, as calculated from the interval between the maximum of the LH surge and the time of ovariectomy which corresponded with the 22 h of culture of oocytes for IVM. Although in vivo maturation was considered to be terminated upon ovariectomy, preparation of the in vivo matured oocytes for IVF took a further 3 h during which period the oocytes were kept in their follicles and, after aspiration, stored in maturation medium. It is not clear whether and how this prolongation affected the in vitro developmental potential of the oocytes after IVF. Normally, in eCG/PG/anti-eCG superovulated cattle, ovulation takes place between 24 and 30 h after the LH surge (Dieleman and Bevers, 1987) and most oocytes have completed nuclear maturation at the time (de Loos et al., 1994). Thus, despite of the 3 h postponement of IVF of the in vivo matured oocytes, the period between LH surge and fertilization still resembles the physiological maturation time. In addition, it has recently been shown (Dominko and First, 1997) that the success rate of IVF was higher when oocytes were maintained in culture for several hours after reaching the MII stage compared to oocytes that were fertilized immediately. This suggests that shortening of the IVM interval might be more harmful then postponement of IVF.

On the basis of peripheral patterns of hormones and superovulation responses it can be concluded that the used experimental design resulted in all heifers in synchronized preovulatory LH surges with normal follicular function (Figure 1). Plasma estradiol-17ß concentrations decreased significantly starting within 3 h after the maximum of the LH surge as has been reported for superovulated animals using normal procedures Bevers and Dieleman, 1987; Callesen et al., 1990). These findings are in agreement with previous experiments (Vos et al., 1994b) in which the preovulatory LH surge was suppressed using a progesterone releasing intravaginal device. The number of preovulatory-sized follicles present at the time of ovariectomy was high in all heifers and appeared to be increased compared to the numbers observed in heifers after normal superovulation. Improved yields after superovulation in the absence of a dominant follicle at the time of stimulation has been described before (Huthinen et al., 1992; Bungartz and Niemann, 1994). However, this increase might also be due to the delay of the preovulatory LH surge which may have decreased inter- and intrafollicular asynchrony coherent with superovulation (Hyttel et al., 1986; Dieleman and Bevers, 1993; Dieleman et al., 1993; Assey et al., 1994) by enabling a larger cross section of the stimulated follicle population to respond properly to the LH signal (Vos et al., 1994a). It was concluded that sufficiently high numbers of in vivo matured oocytes had been collected per heifer at a precisely defined time for IVF and subsequent IVC.

The overall developmental potential during IVC after IVF expressed as blastocyst formation rate or hatching rate was two times higher for in vivo matured oocytes

compared to that for in vitro matured oocytes (Figure 2). This difference is only partly a result of the observed differences in cleavage rate between the two groups of oocytes. The progress of embryo development shows that in vitro matured oocytes develop faster compared to in vivo matured oocytes. After in vitro maturation the majority of ultimately produced blastocysts was formed before day 7 of IVC whereas after in vivo maturation most embryos blastocysed between day 7 and 9 (Figure 3). It has been reported that progress of embryo development is correlated with embryo quality when this quality is expressed as post-thawing survival rate (Mahmoudzadeh et al., 1995) or as the total cell number of the blastocysts (Jiang et al., 1992). However, it is unclear whether this difference in embryo quality also applies to our observations since the referred studies only relate to embryos from in vitro matured oocytes. Therefore, our results indicate that in vivo maturation preceding IVF and IVC improves the production of viable embryos. It should be noticed, however, that the sources of in vivo and in vitro matured oocytes were different being from preovulatory follicles and 2-8 mm follicles, respectively. It is obvious that the oocytes from 2-8 mm follicles lack the processes of normal preovulatory development such as selection and growth which are accompanied by a change in pulsatile release of LH and FSH leading to prematuration as described by Hyttel et al. (Hyttel et al., 1997). Although the administration of eCG used to obtain in vivo matured oocytes also depresses the pulsatile release of gonadotropins (Bevers et al., 1989) it is not known what would be the effect of a complete bypass of preovulatory development on developmental potential of oocytes as with IVM. Thus, both the conditions during maturation as the source of the used oocytes might be responsible for the low blastocyst yield after IVM.

Only a very low number of clearly degenerated and immature oocytes was excluded after in vivo maturation. Yet it can be expected that various stages and qualities of preovulatory oocytes were entered into further IVF and IVC despite of the decrease of inter- and intrafollicular asynchrony achieved by the superovulation procedure used. In general, a maximum of 50% of the oocytes results in viable embryos upon flushing of donor cows (Dieleman et al., 1989) and extensive variation in oocyte quality has been observed between cows after superovulation (Hyttel et al., 1991). This may explain the substantial variation between heifers in rates of blastocyst formation and hatching found in our study. Oocytes collected from heifer 1 performed poorly compared to those of other heifers throughout the IVP process. When the results of this heifer are excluded the hatching rates still varied from 28.6 to 60%. Another factor that may have reduced the outcome of the in vivo matured oocytes is the bypassing of he normal selection mechanism of the dominant follicle from a large pool of recruited follicles (Driancourt 1991). It can be speculated that the observed difference in developmental potential during IVC after IVF would even have been larger when oocytes of preovulatory follicles from normally cyclic heifers had been used.

The mechanisms underlying the decreased developmental potential after in vitro maturation are not clear. Although the majority of in vitro matured oocytes shows an expanded cumulus and proper nuclear maturation (de Loos et al., 1991) differences with in vivo matured oocytes have been reported with regard to protein synthesis (Kastrop et al., 1990, 1991) and progress of rearrangement of cortical granules along the oolemma (Hyttel et al., 1986).

In conclusion, the two-fold increase in developmental potential during IVC after IVF of in vivo matured oocytes strongly indicates that IVM is one of the major factors limiting the yield of viable embryos in IVP but the importance of follicular development preceding the LH surge needs to be elucidated.

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DIFFERENCES IN BLASTOCYST FORMATION FOLLOWING IN VIVO OR IN VITRO MATURATION OF BOVINE OOCYTES AFTER PREMATURATION IN FOLLICLES STIMULATED WITH ECG

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In bovine IVP, IVM appears to be an important factor limiting embryo yield. It is not known whether the origin of the oocytes (2-8 mm sized follicles) or the maturation conditions per se are responsible for this. Therefore, blastocyst formation after 11 days of IVC after IVF was compared of cumulus oocyte complexes (COCs) which had matured in vivo or in vitro after a similar period of prematuration in vivo. All COCs were collected from preovulatory-sized follicles of heifers (n = 33) treated with eCG/PG and a simultaneous norgestomet implant to suppress endogenous release of the preovulatory LH surge. After removal of the implant, at 54 h after prostaglandin administration, in 12 heifers maturation was initiated in vivo by induction of a preovulatory LH surge with GnRH and from the other 21 heifers, COCs were recovered upon OVX for maturation in vitro. In vivo matured COCs were recovered after OVX at 19.5 h after the maximum of the LH surge and maturation was continued to 22-24 h in M 199 with 10% fetal calf serum, 0.01 IU equine LH per ml and 0.01 IU porcine FSH per ml. IVM was carried out in the same medium for 22-24 h. After IVF, IVC was started with 175 and 161 COCs which had been matured in vivo or in vitro, respectively. Overall, the in vitro developmental potential of in vivo matured COCs was markedly higher than that of in vitro matured COCs with blastocyst formation and hatching rates after 11 days of IVC of 52.0 and 40.6% vs. 32.3 and 26.1% relative to the number of COCs, respectively. Estradiol-17ß (E) and progesterone (P) concentrations were determined in all follicular fluids to detect eventual follicular dysfunction due to the eCG treatment. The average E/P ratios per heifer showed no exceptional deviations in follicular development. It is concluded that the conditions during IVM are one of the major factors responsible for limited blastocyst yield after IVP and not the lack of prematuration of oocytes.

INTRODUCTION

In vitro embryo production (IVP) following ultrasound guided ovum pick up, generates numerous possibilities to improve livestock production (Gordon and Lu, 1990). However, implementation of this technique in veterinary practice is retarded by amongst others the limited efficiency of only 8 calves per 100 oocytes.

Previous experiments indicated that the IVM procedure is one of the important factors limiting IVP embryo yield in the cow. When in vivo matured oocytes, collected from

preovulatory follicles, instead of in vitro matured oocytes, collected from 2-8 mm follicles, are subjected to IVF and subsequent IVC, the number of hatched blastocysts after 11 days of culture is doubled (van de Leemput et al., 1996, 1997). The mechanisms underlying the decreased developmental potential after in vitro maturation are not fully understood. In general, nuclear maturation during IVM appears to be rather successful with 80% of the oocytes reaching the MII stage (de Loos et al., 1994), but cytoplasmic maturation appears to be disturbed frequently compared to in vivo maturation (Hyttel et al., 1986). One of the features of this process is rearrangement of the cortical granules to a position along the oolemma at completion of maturation preventing occurrence of polyspermia during subsequent fertilization (Szollosi, 1967). It has been observed that enhanced rates of fertilization and blastocyst formation in vitro were paralleled by improvement of the pattern of cortical granules spread (Izadyar et al., 1998).

It is unclear whether the previously reported (van de Leemput et al., 1996, 1997) decreased embryonic development was due to IVM conditions per se or to a difference in startcompetence of the used oocytes. The in vivo matured oocytes originated from preovulatory follicles shortly before ovulation in eCG/PG-treated (PG: prostaglandin) heifers and the oocytes for IVM had been collected from a heterogeneous population of 2-8 mm follicles of ovaries obtained from the slaughterhouse. Contrary to preovulatory follicles, 2-8 mm follicles lack the processes of normal preovulatory development comprising that of selection and growth which is accompanied by a change in pulsatile release of LH and FSH. This process in the preovulatory follicle leads to prematuration of the oocyte (Hyttel et al., 1997).

Therefore, this study was conducted to compare the effect of in vivo maturation on developmental potential of oocytes with that of in vitro maturation using oocytes which presumably have an equivalent startcompetence after prematuration. Treatment with eCG/PG stimulates a population of follicles to undergo preovulatory follicular development and the majority of their oocytes acquires startcompetence shortly before onset of final maturation. When this is accompanied by suppression of the endogenous release of the LH surge (Vos et al., 1994a), large numbers of oocytes may be obtained at a fixed time after prematuration. It has been demonstrated (Vos et al., 1996a) that such oocytes still possess normal developmental potential after in vivo maturation, similar to that after regular superovulation treatment. Using the reported (Vos et al., 1996a) eCG/PG treatment we initiated maturation in vivo of the oocytes of part of the heifers by induction of an LH surge at a fixed time and used the oocytes of the other part of the heifers for maturation in vitro. Subsequently, IVF and IVC were carried out in one particular well per heifer to identify eventual deviating responses to the superovulation treatment. Follicular (dys)function as a consequence of eCG treatment was established on the basis of estradiol-17ß and progesterone concentrations in the follicular fluid.

MATERIALS AND METHODS

Experimental design

Heifers were treated with eCG/PG to stimulate a large population of follicles to undergo the normal events of prematuration, such as selection and growth. During stimulation release of the preovulatory LH surge was prevented using a norgestomet ear implant. After prematuration was continued for a period corresponding to that in normal estrous cycles ear implants were removed. At the time, in part of the heifers final maturation was initiated by induction of a preovulatory LH surge with GnRH, and from the other part of the heifers oocytes of preovulatory follicles were collected for in vitro maturation. Subsequently, IVF and IVC were performed simultaneously for in vivo and in vitro matured oocytes.

Figure 1. Schedule to obtain oocytes from eCG stimulated follicles undergoing prematuration before in vivo or in vitro maturation. Stippled area represents presence of norgestomet ear implant. PG= administration of prostaglandin; OVX = ovariectomy; LH peak = the maximum of the preovulatory LH surge.

Superovulation procedure

Holstein-Friesian heifers (n=36) were selected from our herd on the basis of general clinical examination and normal ovarian cyclicity during at least 3 weeks as established by the progesterone concentration in peripheral blood samples taken 3 times a week. The heifers were housed in groups of 6 animals and were fed silage and concentrate (to a maximum of 1 kg per heifer per day) and supplied water at libitum. The experiments were carried out from February until April 1997.

Before the experiments estrus was synchronized using an ear implant for 9 d (3 mg norgestomet, Crestar; Intervet International BV, Boxmeer, The Netherlands) accompanied by treatment with 3 mg norgestomet with 5 mg estradiol-valerate (im). Two days before removal of the implants prostaglandin (PG, 15 mg Prosolvin im; Intervet International BV) was administered to ensure complete regression of the corpus luteum (Vos et al., 1996b). At day 8 (estrus is day 0) of the subsequent cycle all follicles > 8 mm were disabled by transvaginal ultrasound-guided puncturing (Vos et al., 1994b) to prevent eventual suppressive action of dominant follicles on the follicle population to be stimulated, and stimulation for superovulation was carried out on day 10 with eCG (2500 IU Folligonan im; Intervet International BV) followed by PG (15 mg Prosolvin im) 48 h later. An ear implant (Crestar) was inserted concomitantly with the administration of eCG (see Figure 1) to suppress endogenous release of the preovulatory LH surge; the accompanying im norgestomet and estradiol-valerate were not administered. The ear implant was removed 54 h after PG treatment at the time prematuration was assumed to be completed, and heifers were at random assigned to a group donating in vivo matured oocytes and a group donating oocytes for IVM.

Then GnRH (1.0 mg Fertagyl in 10 ml saline im; Intervet International BV) was administered to 12 heifers to start in vivo maturation; this dose of GnRH induces an LH surge 2 h later (Vos et al., 1994a). Finally, these heifers received a dose of monoclonal antibody against eCG (5 ml Neutra-PMSG iv; Intervet International BV) at 10 h after GnRH which was sufficient to neutralize 2500 IU of eCG within 1 h (Dieleman and Bevers, 1987). The heifers were ovariectomized 21.5 - 22.0 h after GnRH (i.e. 19.5 - 20.0 h after the maximum of the LH surge) to recover in vivo matured oocytes. Oocytes for IVM were obtained after OVX (n=21, heifers) which was carried out within 1.5 - 2 h after removal of the ear implant.

OVX was performed by laparotomy through flank incision under local infiltration anesthesia (Dieleman et al., 1983b). Ovaries were collected in saline (0.9% (w/v) NaCl) (25°C) and transported to the laboratory for immediate aspiration of cumulus oocyte complexes (COCs) from presumably preovulatory follicles (diameter > 8 mm). Every week, 4 heifers had been scheduled to be ovariectomized. However, 2 heifers were excluded due to the presence of a cystic follicle at the time of removal of the dominant follicle (day 8) and a further one from which no COCs were recovered. During each week OVX of heifers donating oocytes for IVM and OVX of heifers donating in vivo

matured oocytes was carried out on consecutive days which allowed simultaneous execution of IVF and IVC.

Heparinized blood samples were collected from the jugular vein daily from day 0 (estrus) until day 10 and twice a day from administration of eCG until removal of the ear implant. From the group of heifers donating in vivo matured oocytes further blood samples were collected every hour between administration of GnRH and of anti-eCG, and then every 4 h until ovariectomy. In addition, a blood sample was taken during ovariectomy of each animal.

Oocyte collection

In vivo matured oocytes. COCs were obtained by aspiration using a winged infusion set (18 G needle, tubing length 30 cm, volume 0.44 ml, Terumo Europe N.V., Leuven, Belgium) connected to a pump (Air compressor, MagneTek type SP, Thomas Industries Inc., Monroe, LA, U.S.A.) keeping the pressure between 0.2-0.4 bar. After aspiration the tubing of the equipment was flushed with 0.9% (w/v) NaCl and follicular fluid (FF) and flush fraction were collected in separate tubes for each follicle. One hour after aspiration of the first follicle of a heifer recovery of the COCs from FF (kept at 21-25°C) was started using a stereomicroscope. When a COC could not be recovered from the FF the flush fraction was examined as well. After recovery of the COCs, 50 IU heparin (10 µl) was added to the FFs before storage of the FFs at –25°C until RIA.

COCs showing expanded cumulus mass were selected and placed in collection medium (HEPES buffered M199 (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (FCS, Gibco BRL) in a 4-well culture plate (Nunc A/S, Roskilde, Denmark). All COCs from one heifer were allocated to one particular well, which was continued throughout subsequent IVF and IVC. When collection was completed for one heifer COCs were transferred to another well containing maturation medium (M199 supplemented with 10% FCS, 0.01 units/ml porcine FSH (Sigma, St Louis, MO, USA) and 0.01 units/ml equine LH (Sigma)) and stored at 39°C in 5% CO_2, humidified air for 1.5 h until IVF was started at 22-24 h after the LH peak.

Maturation time of the in vivo matured oocytes was defined as the interval between the maximum of the preovulatory LH surge and time of OVX plus the period between OVX and end of temporary culture preceding IVF.

Oocytes for in vitro maturation. Aspiration and collection of COCs and FFs was performed following the same procedure as used for the in vivo matured oocytes. Only COCs with a multilayered compact cumulus investment were used for IVM. Selected COCs from one heifer were allocated to one particular well of a 4 well culture plate containing collection medium. Maximally two hours after recovery of the first COC, COCs were transferred to maturation medium. COCs were cultured for 22 h at 39°C, in 5% CO_2 in humidified air.

Maturation time of the in vitro matured oocytes was defined as the interval between the time of recovery of the oocyte from its FF and the end of culture at onset of IVF.

IVF and IVC

IVF and IVC were started and carried out simultaneously for both groups of COCs as described previously (Izadyar et al., 1996; van de Leemput et al., 1997). Briefly, COCs were rinsed twice with 2 ml HEPES buffered M199 medium per well before transfer to fertilization medium as described by Parrish et al. (1988) with slight modifications (Izadyar et al., 1996). Fertilization was then carried out during 18-20 hours with $0.5x10^6$ spermatozoa/ml of a bull with proven in vitro fertility after which COCs were freed from cumulus cells and transferred to a well containing 500 µl M199 supplemented with 10% FCS and a mono layer of Buffalo rat liver cells. At day 4 of IVC, the cleavage rate was evaluated and non-cleaved structures were removed, on the 4^{th} and the 8^{th} day of culture embryos were transferred to fresh co-culture wells, and at day 7, 9 and 11 numbers of blastocysts and hatched blastocysts were scored. Day 1 of culture was defined to start immediately after IVF was finished.

IVP conditions were checked routinely throughout the experimental period each week (n=9) with 70 oocytes (2 wells with 35 oocytes) recovered from 2-8 mm sized follicles from ovaries obtained from the slaughterhouse. These cultures were performed by the same investigators under similar conditions. Blastocyst formation and hatching rates after 11 days of IVC were 27.1 ± 5.2% and 21.9 ± 5.0% relative to the number of COCs, respectively, indicating that the conditions during IVP were constant throughout the experimental period.

Immunoassays for LH, progesterone and estradiol-17ß

Preceding the experiment normal ovarian cyclicity was established on the basis of peripheral progesterone concentrations in the blood as estimated by enzyme immunoassay (van de Wiel and Koops, 1986).

During the superovulatory treatment concentrations of progesterone and estradiol-17ß in peripheral blood samples were estimated by solid-phase ^{125}I RIA methods (Coat-A-Count TKPG and TKE, respectively; Diagnostic Products Corporation, Los Angeles, CA, U.S.A.) with modifications as described by Dieleman and Bevers (1987). The sensitivity was 0.15 nmol l^{-1} and 7.5 pmol l^{-1}, and the interassay coefficient of variation 11% and 8.9%, respectively. Concentrations of LH were estimated by a validated RIA method as described by Dieleman et al. (1983b). The intra- and interassay coefficients of variation were < 9%. The sensitivity was 0.4 µg l^{-1} NIH-LH-B4.

In FF, progesterone and estradiol-17ß concentrations were estimated by the solid-phase ^{125}I RIA methods (Coat-A-Count TKPG and -TKE) according to the manufacturer. For RIA of progesterone 4 µl of FF were diluted to 100 µl with the zero calibrator plasma. The sensitivity and the interassay coefficient of variation of the assay were 0.32 nmol l^{-1} and 13.4%, respectively. For RIA of estradiol-17ß aliquots of FF were diluted with 0.02 M borate buffer (pH 8.5) in 0.9% (w/v) NaCl, that is 707 times for FF from follicles collected before the preovulatory LH surge and 61 times for those collected shortly before ovulation. The sensitivity and the interassay coefficient of variation of the assay were 0.07 nmol l^{-1} and 14.1%, respectively.

Data handling and statistical analysis

Unless marked otherwise, all values are expressed as mean ± SD. Although COCs obtained from one heifer were cultured in one well during IVM, IVF and IVC to allow identification of blastocysts from a particular heifer, rates of cleavage, blastocyst formation and hatching are calculated after gathering of the results per experimental group as percentages of the number of (non)-fertilized oocytes at day 1 of IVC. This was done to avoid improper effects due to variation in embryonic development as observed previously to oocytes of different animals (van de Leemput et al., 1997), and to variation in initial numbers of oocytes per heifer (Table 2). Numbers of cleaved oocytes, blastocysts and hatched blastocysts and the progress of embryonic development at each time point were analyzed with the Chi-square test and $P \leq 0.05$ was considered significant.

Deviant follicular function of heifers ovariectomized before the LH surge was defined as an average estradiol-17ß / progesterone ratio (E/P ratio) in the FF below or above the mean E/P ratio of all heifers ± 1 SD.

RESULTS

Superovulation procedure

All heifers showed luteal regression (i.e., peripheral progesterone concentration<3.2 nmol l^{-1}) within 28 h after PG administration. The LH concentration in peripheral blood of heifers providing oocytes for IVM (in vitro group) did not exceed 0.7 µg l^{-1} in the samples collected 3 h before and at OVX. In the heifers providing the in vivo matured oocytes (in vivo group), GnRH injection always resulted in a preovulatory LH surge, with a duration of 6 h and a maximum of 20.0 ± 1.4 (SEM, n=12) µg l^{-1} occurring 2 h after GnRH. The estradiol-17ß concentration in the peripheral blood at removal of the

ear implant shortly before onset of maturation was similar for both groups of heifers (354 ± 71 (SEM, n=12) pmol l^{-1} and 315 ± 35 (SEM, n=21) pmol l^{-1} for the in vivo and in vitro group, respectively), and it decreased significantly at 6 h after the maximum of the LH surge in the in vivo group.

Superovulation responses, defined as the number of follicles > 8 mm present at the time of OVX were similar for the in vivo and in vitro group (19.8 ± 9.7 (n=12) and 18.3 ± 7.3 (n=21), respectively).

Oocyte recovery

For the in vivo group 191 COCs were recovered from 237 preovulatory-sized follicles (recovery rate: 84.0 ± 12.4%; n=12 heifers). Denuded oocytes (n=2) and COCs not showing cumulus expansion (n=2) were discarded.

For the in vitro group 212 COCs were recovered from 385 preovulatory sized follicles (recovery rate: 56.9 ± 19.8%; n=21 heifers). COCs that did not have an intact multilayered compact cumulus investment (n=41, 19.0%) and those already showing cumulus expansion (n=5, 2.3%) were discarded.

Overall, 34.8% of the selected in vivo matured oocytes (65/187) and 6.0% of the oocytes selected for IVM (10/166) were recovered from the saline flush fraction after aspiration instead of the FF.

Maturation

In vivo maturation. The average number of in vivo matured COCs per heifer was 14.6 ± 6.4 (range 4-25; n=12 heifers). On average per heifer, in vivo maturation time was 23 h 45 min ± 9 min and OVX was performed at 19 h 38 min ± 7 min after the maximum of the LH surge. COCs per heifer spent 21 min ± 7 min in the ovary at 25°C and 1 h 9 min ± 7 min in their own FF at 21-25°C. Recovery of COCs from FF per cow took 56 min ± 30 min. Before fertilization, COCs spent an additional 1 h 38 min ± 31 min in maturation medium.

In vitro maturation. During IVM, the average number of COCs per heifer cultured in one well was 7.3 ± 4.2 (range 2-16; n= 21 heifers). Total in vitro maturation time was 23 h 23 min ± 25 min of which 1 h 12 min ± 29 min was performed in collection medium. Each COC matured for at least 21 h 55 min with a maximum of 23 h 30 min.

IVF and IVC

At removal of the cumulus cells of the COCs after IVF, 12 (6.4%) in vivo matured oocytes and 5 in vitro matured oocytes (3.0 %) were lost. Average duration of IVF per heifer was 19 h 47 min ± 28 min.

Table 1. Blastocyst and hatched blastocyst development per replicate after 11 days of IVC following IVF of oocytes matured in vivo or in vitro obtained from follicles that had undergone preovulatory development preceding final maturation in heifers treated for superovulation.

	In VIVO maturation				In VITRO maturation			
Replicate	Number of		Number of blastocysts		Number of		Number of blastocysts	
Number	Heifers	Oocytes	Total	Hatched	Heifers	Oocytes	Total	Hatched
1	2	31	21	15	2	8	1	0
2	2	42	26	21	2	16	5	4
3	2	30	21	16	2	8	1	1
4	1	4	1	0	2	12	7	6
5	1	24	8	7	1	7	5	4
6	1	6	3	2	3	18	4	3
7	1	16	5	5	3	37	15	13
8	1	11	2	2	3	25	8	7
9	1	11	4	3	3	30	6	4
Total	12	175	91	71	21	161	52	42

Embryonic development during IVC after IVF per replicate of oocytes matured in vivo or in vitro is presented in Table 1. In 5 of the 9 replicates in vitro developmental potential was higher for oocytes that were matured in vivo than for oocytes that were subjected to IVM, in 3 replicates it was lower for in vivo matured oocytes and in one replicate no difference was found. When results of replicates are gathered per in vivo or in vitro group (Table 2), cleavage rates at day 4 of IVC after IVF were not significantly different between the two groups (78.3% vs. 68.9%, P = 0.10) but blastocyst formation and hatching rates after 11 days of IVC after IVF were significantly higher for oocytes that had undergone in vivo maturation than for oocytes that were subjected to IVM (52.0 and 40.6% vs. 32.3 and 26.1%, respectively).

To investigate effects of eventual bias, developmental potential of both groups was compared excluding heifers with a recovery rate < 30% (n=2, in in vitro group) and with cultures of < 5 oocytes per well (one heifer of the in vivo group and 6 of the in vitro group). This again resulted in significantly higher blastocyst formation and hatching

rates for the in vivo group than the in vitro group (52.6 and 41.5% vs. 35.3 and 28.8 %, respectively).

The progress of embryonic development in the in vivo group was not significantly different from that in the in vitro group on the basis of the numbers of (hatched) blastocysts observed at each day of IVC relative to the ultimate number of (hatched) blastocysts present at day 11 of IVC.

Table 2. Rates of blastocyst formation and hatching after 11 days of IVC following IVF of in vivo or in vitro matured oocytes obtained from follicles that had undergone preovulatory development preceding final maturation in heifers treated for superovulation.

	Numbers of oocytes after IVF day 1	Number of cleaved embryos day 4 (%)	Number of blastocysts Total day 7 (%)	Total day 9 (%)	Total day 11 (%)	Hatched day 9 (%)	Hatched day 11 (%)
After in vivo maturation	175	137 (78.3)	35 (20.0)	88 (50.3)[a]	91 (52.0)[a]	19 (10.9)	71 (40.6)[a]
After in vitro Maturation	161	111 (68.9)	22 (13.7)	48 (29.8)[b]	52 (32.3)[b]	17 (10.6)	42 (26.1)[b]

Day 1 is start of IVC. Percentages of cleavage, blastocyst formation and hatching are calculated relative to the number of (non)-fertilized oocytes at day 1 of IVC. Values with different superscripts within one column are significantly different ($P \leq 0.05$).

Hormone concentrations in follicular fluid

Follicles donating in vivo matured oocytes. In the in vivo group 230 FFs were collected from 237 preovulatory-sized follicles shortly before ovulation; seven FFs were contaminated with fluid from the adjacent follicle and therefore not analyzed. The average estradiol-17ß (E) and progesterone (P) concentrations per heifer in FF were 0.44 ± 0.19 μmol l^{-1} and 0.40 ± 0.15 μmol l^{-1} (n= 230, follicles), respectively.

Follicles donating oocytes for IVM. In the in vitro group, 374 FFs were collected from 385 aspirated preovulatory-sized follicles shortly before the expected LH surge; 11 FFs from 8 heifers had coagulated before the addition of heparin. Three follicles with an inverse E/P ratio, i.e. an exceptionally high progesterone concentration (average 8.2 μmol l^{-1}) concurrent with a low estradiol-17ß concentration (average 0.06 μmol l^{-1}) were excluded from further calculations. The average estradiol-17ß and progesterone concentrations per heifer in FF were 4.40 ± 1.14 μmol l^{-1} and 0.17 ± 0.05 μmol l^{-1},

n=371, follicles) respectively. The average E/P ratio per heifer was 28.6 ± 10.6 (range: 12.9 - 46.7; Figure 2). Heifers with an average E/P ratio in FFs below or above the mean follicular E/P ratio of all cows ± 1 SD were regarded to have an exceptional follicular function. When these heifers (n=3 below and n=3 above the mean E/P ratio ± 1 SD) were excluded from the calculations, blastocyst formation- and hatching rates after 11 days of IVC following IVF of in vitro matured oocytes, increased to 40.8% (51/125) and 33.6% (42/125), respectively.

Figure 2. Average ratio (•) per heifer of the estradiol-17ß / progesterone concentrations in the fluid of follicles collected shortly before the occurrence of the expected LH surge, after treatment for superovulation. Numbers of follicles per heifer are noted above dots. The stippled area represents the average E/P ratio of all heifers plus or minus one SD (28.6 ± 10.6; n=21, heifers). FF: follicular fluid; E: estradiol-17ß; P: progesterone.

DISCUSSION

In general, IVP in the bovine comprising IVM, IVF and IVC is carried out with oocytes from 2-8 mm follicles. Such oocytes possess a potential to develop into hatched blastocysts which is about half the potential of oocytes from preovulatory follicles that had matured in vivo (van de Leemput et al., 1997). The present study was conducted to investigate the extent to which IVM conditions per se contribute to reduced embryonic development ruling out eventual effects of differences in origin of the oocytes related to

differences in developmental stage of follicles. Therefore, only oocytes were used from preovulatory follicles on the verge of onset of final maturation. At the time oocytes were aspirated after OVX for in vitro maturation (: in vitro group), and for in vivo maturation the oocytes were maintained in situ until shortly before ovulation (: in vivo group). To obtain sufficient numbers of oocytes at precisely defined times heifers were treated for superovulation using a procedure with a controlled LH surge as previously tested (Vos et al., 1994a, 1996a; van de Leemput et al., 1997). Both groups of heifers showed patterns of hormone concentrations in peripheral plasma and responses to superovulation treatment, which were in agreement with patterns reported for superovulated animals (Vos et al., 1994a). In all heifers of the in vivo group administration of GnRH resulted in synchronized LH surges followed by a decrease of the peripheral plasma concentration of estradiol-17ß similar to previously observed (Vos et al., 1994a).

The recovery rate of COCs upon aspiration from preovulatory follicles was strikingly reduced for the in vitro group in comparison to that for the in vivo group. Low recovery rates have been reported (Vos et al., 1994b) for the collection of oocytes from preovulatory follicles using transvaginal ultrasound guided ovum pick-up around the time of occurrence of the LH surge. At onset of final maturation the cumulus of the COCs is still compact and firmly attached to the follicular wall to start expanding and loosening from the wall after the LH surge until ovulation. It can be speculated that this process facilitates collection of the COCs. Although the recovery rate was reduced for the in vitro group it was assumed that the collected COCs were of a representative sample having an average quality similar to that of the COCs of the in vivo group at that stage.

The duration of maturation was similar for the oocytes of both groups lasting 22-24 h. For the vitro group maturation was defined to start when the first COC of a heifer was recovered from the follicular fluid. Around the time of the LH surge preovulatory follicles contain a factor inhibiting maturation (Romero-Arredondo and Seidel, 1996) and COCs spontaneously resume meiosis when they are removed from their follicular fluid. Therefore, the few COCs that were recovered from the saline fraction instead of the follicular fluid probably started maturation about 1 h earlier than the majority of the collected COCs. It was assumed that this did not affect the general outcome of embryonic development of the in vitro group because of the small number of COCs recovered from the saline fraction. For the in vivo group about one-third of the COCs was recovered from the saline fraction in which they remained for 1-3 h before being transferred for the short stay in maturation medium preceding IVF. Whether this brief period in saline impaired their in vitro developmental potential is unclear but the rates of blastocyst formation and hatching were conform to those as previously observed for similar oocytes in the same culture system (van de Leemput et al., 1997). It was concluded that maturation of COCs of the in vivo group sufficiently resembled the physiological maturation until ovulation which starts at 24 h after the maximum of the LH surge in eCG/PG/anti-eCG treated heifers (Dieleman and Bevers, 1987).

The rates of blastocyst formation and hatching at 11 days of IVC after IVF showed a large variation between animals in agreement with previous observations (van de Leemput et al., 1997) which probably is due to differences in response to superovulatory treatment. Superovulation treatment induces anomalies in follicular function (Dieleman and Bevers, 1993; Dieleman et al., 1993) which are considered to affect oocyte quality (Hyttel et al., 1986; Hyttel et al., 1991; Assay et al., 1994). In the in vitro group 6 heifers were found having a population of preovulatory follicles with on average exceptional estradiol-17ß and/or progesterone concentrations in the follicular fluid. When the IVP results of these heifers were excluded from the final observations, the rates of blastocyst formation and hatching increased indicating that the steroid producing capacity of the follicular wall influences oocyte quality. For the in vivo group deviant follicular function in essence can not be distinguished. At the time of collection of the follicles, being 19.5 h after the maximum of the LH surge, large variations in progesterone concentrations in the follicular fluid can be expected since in the preovulatory follicle of normally cyclic heifers progesterone production starts to increase (Dieleman et al., 1993). Longitudinal studies have as yet not been performed of further development of follicles considered to be deviant before the occurrence of the LH surge. Therefore, heifers with exceptional follicular function could not be identified for the in vivo group as was done for the in vitro group, and comparison of the developmental potential of both groups was carried out with the results of the oocytes of all heifers.

In the in vitro group, the rates of blastocyst formation and hatching at day 11 of IVC after IVF were markedly depressed in comparison to those of the in vivo group. Clearly, both groups of oocytes will have had similar developmental potential at onset of final maturation in vivo or in vitro, since both groups originated from follicles which experienced the same prematurational development after stimulation with eCG followed by PG. Therefore, the decrease in developmental potential of the in vitro group can only be attributed to conditions during final maturation being not optimum, and this effect of IVM becomes apparent after cleavage. A further argument is provided by the observation that rates of blastocyst formation and hatching of the in vitro group were similar to those of oocytes from 2-8 mm slaughterhouse follicles cultured parallel to check IVP conditions in general.

Numerous studies have been performed to improve in vitro maturation conditions. Amongst others the effects during IVM have been investigated of gonadotropins, steroids and growth factors on subsequent embryonic development after IVF (Bevers et al., 1997). So far, however, literature is not quite conclusive and difficult to interpret since various culture systems have been used. In our maturation medium LH and FSH were present throughout IVM but in vivo the microenvironment of the maturing oocyte in the preovulatory follicle undergoes a strict sequence of hormonal changes (Dieleman et al., 1983a, b). For example, 4 h after the LH peak in the peripheral blood LH reaches a maximum concentration in the fluid of the preovulatory follicle decreasing thereafter at a slower rate than in peripheral blood to attain basal

levels shortly before ovulation. The concentration of FSH in the follicular fluid also shows a peak value concomitant with that of LH in the fluid followed by a gradual decrease and an increase shortly before ovulation. Extrapolating these in vivo findings, COCs in vitro may demand a changing hormone regime that mimics the sequence of changes in hormonal concentrations in vivo during final maturation in particular during the first hours of IVM.

The progress of embryonic development during IVC was similar for the in vitro and in vivo groups and was conform to that as previously observed for similar oocytes in the same culture system (van de Leemput et al., 1997). Since in that study (van de Leemput et al., 1997) in vitro matured oocytes from 2-8 mm slaughterhouse follicles developed at a faster rate than in vivo matured oocytes from preovulatory follicles it can be suggested now that oocytes undergo certain changes during follicular development from 2-8 mm to preovulatory stage at onset of final maturation which may be involved in programming embryonic development.

In conclusion, oocytes that have experienced follicular prematuration lose a substantial part of their potential to develop into hatched blastocysts when they are matured in vitro. Obviously, in vivo maturation provides a sequence of changes in the microenvironment of the maturing oocyte which is more appropriate than the static conditions during in vitro maturation.

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INSULIN-LIKE GROWTH FACTOR-I AND ITS BINDING PROTEINS IN THE FLUID OF PREOVULATORY FOLLICLES COLLECTED FROM eCG/PG-TREATED COWS AT DIFFERENT STAGES BEFORE AND AFTER THE PREOVULATORY LH SURGE IN RELATION TO FOLLICULAR STEROID CONCENTRATIONS

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In this study, preovulatory follicular development induced by treatment with eCG/PG/anti-eCG in cows was used as a model to study the possible role of the insulin-like growth factor (IGF) system during final maturation. IGF-I and IGF binding protein (IGFBP) levels were compared in the fluids (FFs) of eCG-stimulated follicles with 'normal' or 'deviant' follicular function. Follicular function was determined on the basis of progesterone, estradiol-17ß and androstenedione concentrations in FF. Concentrations of these steroids similar to those in FFs collected from untreated normally cyclic cows at corresponding times were considered to indicate 'normal' follicular function. Cows were stimulated with eCG (3000 IU im, day 10 (estrus=day 0)) followed by administration of prostaglandin (22.5 mg im) 48 h later. FFs of follicles > 8 mm were collected at 12 h before (P0, n=86) and 3 h (P1, n=63), 12 h (P2, n=78) and 22 h (P3, n=108) after the LH surge. Concentrations of IGF-I were measured by RIA and the amount of IGFBPs by Western-ligand blotting, in ± 20 'normal' and ± 20 'deviant' FFs per puncture time. Of the FFs collected at P0, P1, P2 and P3, 39.5, 23.8, 24.4 and 57.4% were classified 'deviant', respectively. Only minor differences were found between IGF-I and IGFBP3 levels in 'normal' FF collected at the consecutive puncture times and between 'normal' and 'deviant' FF collected at the same puncture time. LMW IGFBPs were found in a higher number of 'deviant' than 'normal' FFs ('deviant': 35, 13, 6 and 0% and 'normal' 0, 0, 0 and 15% at P0, P1, P2 and P3, respectively). It was concluded that 'deviant' follicular development at the onset of final maturation coincides with an increased presence of LMW IGFBPs. Since during final maturation the presence of the IGF system in 'normal' and 'deviant' follicles was rather similar only a permissive role of the IGF system in this process can be suggested.

INTRODUCTION

In the bovine, the presence of insulin-like growth factor I (IGF-I) and IGF binding proteins (IGFBPs) in the fluid of antral follicles varies during folliculogenesis, suggesting a regulatory role of the IGF system in antral follicular development. In general, growth and atresia of follicles coincide with more or less static levels of IGF-I and IGFBP3 in the follicular fluid and a respective decrease and increase in follicular levels of low molecular weight (LMW) IGFBPs, i.e. IGFBP2, -4 and -5 (cow: Echternkamp et al., 1994; sheep: Monget et al., 1993; pig: Grimes et al., 1994); these LMW IGFBPs are assumed to decrease bioavailability and functionality of IGF-I (rat:

Bicsak et al., 1990; Adashi et al., 1992; sheep: Monget et al., 1993). During normal preovulatory follicular development, IGF-I and IGFBP3 are always present at rather static levels in the fluid of bovine follicles, but LMW IGFBPs are not detectable in the fluids of these follicles (Funston et al., 1996), suggesting maximum bioavailability of IGF-I during preovulatory follicular development.

Results from in vitro studies suggest a stimulatory role of IGF-I on final maturation of follicle and oocyte. The presence of IGF-I during in vitro maturation stimulates cumulus cell expansion and nuclear maturation of the oocyte (Izadyar et al., 1997). Also when IGF-I, in combination with FSH, is present during in vitro maturation, blastocyst formation after IVF and IVC is increased (Harper and Brackett, 1992). The presence of insulin during IVM, that is structurally related to IGF-I and at higher concentrations occupies the IGF-I receptor, induces cumulus expansion and has a positive effect on the blastocyst cell number (Zhang et al., 1991).

A better understanding of the role of the IGF system during preovulatory follicular development in vivo can be obtained by comparing the presence of IGF-I and IGFBPs in follicles undergoing normal preovulatory development to that in follicles undergoing deviant preovulatory development. The use of exogenous gonadotropins to induce multiple ovulations in cows generates both preovulatory follicles with deviant characteristics and preovulatory follicles with characteristics that resemble those of follicles at corresponding stages of development in normally cyclic cows. For example a substantial proportion of the stimulated preovulatory follicles remains dependent for growth and development on the FSH-like activity of eCG until the occurrence of the LH surge (Vos et al., 1994b). Also, an asynchrony in development between the follicle and its oocyte may occur (Dieleman et al., 1988; de Loos et al., 1991; Hyttel et al., 1991). These deviations in follicular development are reflected in, among others, changes in the concentrations of progesterone, estradiol-17ß and androstenedione in the fluid of preovulatory follicles. Alterations in steroid concentrations in the follicular fluid have been shown to be related to impaired oocyte development; the proportion of oocytes that developed into blastocyst after IVM, IVF and IVC, is lower in oocytes collected from eCG-stimulated follicles with low follicular estradiol-17ß concentrations at the time of the occurrence of the preovulatory LH compared to oocytes derived from concurrently developing follicles with high follicular estradiol-17ß concentrations (van de Leemput et al., 1998).

In the present study, preovulatory follicular development induced by treatment with eCG is used as a model to study the possible role of the IGF system during final maturation in vivo.

IGF-I and IGFBP levels are compared in the fluids of eCG-stimulated preovulatory follicles with ‘normal’ follicular function to those in follicles with ‘deviant’ follicular function, collected just before and at three times after the occurrence of the preovulatory LH surge. Follicular function is determined on the basis of concentrations of progesterone, estradiol-17ß and androstenedione in follicular fluid;

concentrations of these hormones similar to those in the fluid of preovulatory follicles of untreated normally cyclic cows at corresponding stages of development are considered to indicate 'normal' follicular function.

MATERIALS AND METHODS

Animals

Holstein-Friesian non-lactating cows were selected from our herd on the basis of general clinical examination and normal ovarian cyclicity during at least 3 weeks as established by the progesterone concentration in peripheral blood samples taken 3 times a week. The cows were housed in groups of 6 animals and were fed silage and concentrate (to a maximum of 1 kg per cow per day) and supplied water ad libitum.

Before the experiments estrus was synchronized using an ear implant for 9 d (3 mg norgestomet, Crestar; Intervet International BV, Boxmeer, The Netherlands) accompanied by treatment with 3 mg norgestomet with 5 mg estradiol-valerate (im). Following synchronization, only cows showing a normal luteal phase progesterone pattern, i.e. peripheral progesterone concentration >6.4 nmol l^{-1} at day 10 of the synchronized cycle (day 0: estrus) (Dieleman et al., 1986), were stimulated for superovulation on this day with eCG (3000 IU Folligonan im; Intervet International BV) followed by prostaglandin (PG; 22.5 mg Prosolvin im, Intervet International BV) 48 h later. Finally, the cows received a dose of a monoclonal antibody against eCG (anti-eCG; 5 ml Neutra-PMSG iv, Intervet International BV) 6 h after the maximum of the LH surge which was sufficient to neutralize 3000 IU eCG within 1 h (Dieleman and Bevers, 1987).

Only cows were selected for the experiment having more than 8 follicles with a diameter > 8 mm at 28-30 h after PG injection following eCG stimulation, as assessed by transrectal ultrasonography (Aloka, type SSD-210 DX, Tokyo, Japan, linear array 7.5 MHz), and showing a preovulatory LH surge in the period of 30-52 h after PG treatment.

Collection and classification of follicles

Collection of follicular fluids (FFs) from preovulatory follicles was performed using the transvaginal ultrasound-guided puncture technique as described by Vos et al. (1994a). In each cow (n=23), the FF of 4-5 follicles was collected at 30 h after PG administration (time: P0). The fluid of the remaining follicles was collected at one of the subsequent puncture times P1 (3 h after the maximum of the LH surge, n=6 cows),

P2 (12 h after the maximum of the LH surge, n=8 cows) or P3 (22 h after the maximum of the LH surge, n=9 cows). FFs were centrifuged at 4°C and stored at -25°C until use.

The follicles were qualified as 'normal' or 'deviant' on the basis of steroid hormone concentrations (progesterone, estradiol-17ß and androstenedione) in the collected FF samples. Selection criteria were calculated from the steroid concentrations in FF as reported for preovulatory follicles of untreated normally cyclic cows before and at corresponding times after the preovulatory LH surge (Dieleman et al, 1983a,b, Table 1). FFs with steroid concentrations within the range (Table 1) were classified as 'normal' and those with steroid concentrations exceeding the boundaries as 'deviant'.

Table 1: Range of concentrations of progesterone, estradiol-17ß and androstenedione in the fluid of preovulatory follicles of untreated, normally cyclic cows around the LH surge.

Time relative to LH peak	Steroid	Range of steroid concentrations (µmol l^{-1})[1)]
12 h before	P	0.06-0.60
	E	4.31-8.66
	A	0-3.63
0-6 h after	P	0.13-1.62
	E	0.93-6.55
	A	0-2.62
6-20 h after	P	0.11-0.49
	E	0-3.14
	A	0-0.73
20-24 h after	P	0.51-2.51
	E	0-0.78
	A	0-0.66

P: progesterone; E: estradiol-17ß; A: androstenedione.[1)] Mean concentrations ± 2 or 3 SD as reported by Dieleman et al. (1983a,b).

Bloodsampling and RIA of progesterone, LH, estradiol-17ß and androstenedione in plasma and follicular fluid

Blood samples were taken from the jugular vein into heparinized vacuum tubes and stored in melting ice before centrifugation at 4°C. Plasma was either stored at 25°C or used immediately for rapid RIA for LH or progesterone and stored afterwards.

Plasma progesterone concentrations were measured every other day from day 3 to 14 of the synchronized estrous cycle to observe corpus luteum function before

superovulation induction and luteolysis after PG administration on day 12. Further, plasma LH concentrations were determined by a rapid RIA which was performed every hour from 30 h until 50 h after PG administration to detect the preovulatory LH surge (Dieleman and Bevers, 1987), to establish the time of anti-eCG administration and of puncture times P1, P2 or P3. These blood samples were also used to determine the concentration of estradiol-17ß around puncture time P0 and to investigate eventual effects of the puncturing procedure on the remaining follicles.

Concentrations of progesterone and estradiol-17ß in peripheral blood were estimated by solid-phase ^{125}I RIA methods (Coat-A-Count TKPG and TKE, respectively: Diagnostic Products Corporation, Los Angeles, CA, USA) according to the manufacturer with modifications as described by Dieleman and Bevers (1987). The sensitivities were 0.15 nmol l^{-1} and 7.5 pmol l^{-1}, the interassay coefficients of variation <11% and 8.9%, and the intra-assay coefficients of variation 8 and 9%, respectively. Concentrations of LH were estimated by a validated RIA method as described by Dieleman et al. (1983b). The sensitivity was 0.4 µg l^{-1} NIH-LH-B4. The inter- and intra-assay coefficients of variation were < 9%. Cross-reactivity of eCG (highly purified eCG, PM 23-2P, Intervet International B.V.) was 0.2%.

Concentrations of progesterone and estradiol-17ß in FF were estimated by RIA methods as previously validated for bovine FF after extracting appropriate µl aliquots in duplicate using n-hexane (96%, pro analysis (p.a.); J.T. Baker, Deventer, The Netherlands) and fresh diethyl ether (p.a.; Merck AG, Darmstadt, Germany), respectively (Dieleman et al., 1983a). The sensitivities were 1.3 and 0.4 nmol l^{-1}, the intra-assay coefficients of variation were 11 and 8%, and the interassay coefficients of variation were <12% and 9.6%, respectively. FF concentrations of androstenedione were estimated by a solid phase ^{125}I RIA method (Coat-A-count TKA; Diagnostic Products Corporation) according to the manufacturer after extracting appropriate µl aliquots in duplicate, using fresh diethyl ether (p.a.; Merck AG). The sensitivity was 3.5 nmol l^{-1}. The inter- and intra-assay coefficients of variation were < 10%. Main cross-reactivities were 6.3, 4.3, 0.87, 0.75, 0.2 and 0.19% for androsterone, dehydroepiandrosterone, adrenosterone, estrone, testosterone and progesterone, respectively, and <0.05% for other steroids tested according to the manufacturer. A correlation coefficient of r=0.99 (P<0.01) was obtained for the comparison of the androstenedione concentrations (n=20) as estimated by this RIA method with those estimated by the method described previously (Dieleman et al., 1983a).

Measurement of IGF-I and IGFBPs in follicular fluid

In a similar number of FF per puncture time (P0: 20 ‘normal’ (N) and 20 ‘deviant’ (D), P1: 20 N and 15 D, P2: 22 N, 16 D and P3: 20 N and 20 D) IGF-I concentrations

and IGFBP levels were measured. Each selection of follicular fluids comprised a maximum number of cows (see Figure 2).

RIA for total IGF-I. IGFBPs were removed from FF, after acidification of the samples to dissociate IGF-IGFBP complexes, using Sep-pack Plus C18 cartridges (Waters, Etten-Leur, The Netherlands) according to the procedure described by Lee et al. (1991) for serum samples with slight modifications. Briefly, 10 µl FF and 190 µl extraction buffer (63 mM Na_2HPO_4 (pH 7.4), 10 mM Na_2EDTA and 0.25% (w/v) BSA (RIA grade, Fraction V, > 96% albumin, Sigma St-Louis, MO, USA) were acidified with 1.3 ml of 1% aqueous trifluoracetic acid (TFA, ultra gradient HPLC grade, J.T. Baker) for 10 min at room temperature (RT). The cartridges were preconditioned by sequential washes with 100% acetonitrile (HPLC gradient grade, J.T. Baker), deionized water (MilliQ Plus, Millipore B.V., Etten-Leur, The Netherlands) and 0.1% aqueous TFA. Then, the acidified FF (1.5 ml) was loaded on the cartridge and flushed with three 1 ml volumes of 0.1% aqueous TFA to eluate IGFBPs. Finally, free IGF-I was eluated from the cartridge with 2 ml of acetonitrile containing 0.1% v/v TFA. The acetonitrile eluate was evaporated during centrifugation under vacuum at 37°C (Savant AES1000, New Brunswick Scientific Benelux BV, Nijmegen, The Netherlands) and the residu was solubilized in 2 ml of assay buffer (see below).

Twelve samples were extracted at the same time: 10 samples of FF, one reference sample of FF and one sample of FF with 50,000 cpm (corresponding to maximal 125 pg IGF-I) ^{125}I-IGF-I (see below). The latter one was used to estimate the efficiency of the extraction method. Therefore, preceding extraction, each time 10 µl FF was incubated with 10 µl ^{125}I-IGF-I and 180 µl assay buffer for 2 h at RT. The extraction efficiency was 55% ± 4.1 (SD, n=11). When FFs were not acidified preceding extraction only 19.5% of the iodinated IGF-I was found in the IGF-I fraction, indicating that ^{125}I-IGF-I replaced endogenous IGF-I by binding to the IGFBPs. To compare the extraction efficiency in samples with different concentrations of IGF-I, serial volumes (10-150 µl) of FF were extracted and subsequently measured by RIA; a linear correlation between the volumes and the measured IGF-I concentrations was found ($r=0.990$, $P<0.01$). Therefore, IGF-I measurements were corrected for the average extraction efficiency on the basis of recovered iodinated IGF-I.

RIA for IGF-I was performed as described by Nap et al. (1993) for serum samples with a slightly modified assay buffer (63 mM Na_2HPO_4 (pH 7.4), 10 mM Na_2EDTA, 0.25% (w/v) BSA, 0.1% triton X-100 (Sigma) and 2% (v/v) trasylol (10.000 KIE/ml, RVG 05312, Bayer, Germany). 100 µl resolved FF fraction after extraction (0.5 µl FF equivalent) was added to 100 µl antiserum against human IGF-I (UKB 487, NIDDK, USA, final dilution 1:18,000) and 100 µl of assay buffer containing 10,000 cpm ^{125}I-IGF-I. IGF-I (recombinant human IGF-I; Boehringer Mannheim, Almere, The Netherlands) was iodinated using 1,3,4,6-tetrachloro-3",6"-diphenylglycouril (Nap et al., 1993). After incubation for 48 h at 4°C, antibody-bound IGF-I was precipitated by

the addition of 100 µl of anti-rabbit second-antibody-coated cellulose (Sac-Cel, IDS Ltd, Tyne & Wear, UK).

Serial dilutions of extracted FF (100 (103.7 pg/tube IGF-I), 90, 80, 70, 60, 50, 40, 30, 20 and 10 µl) resulted in a displacement parallel to the standard curve (2.5-250 pg/tube IGF-I; recombinant hIGF-I). Accuracy of the RIA procedure was determined by measuring known quantities of exogenous IGF-I added to a reference sample of extracted bovine FF (reference sample 23.6 pg IGF-I/tube) in a range of 0, 25, 50, 100, 150 pg/tube (n=6 for each concentration). The relation between the added and recovered amounts of IGF-I was described by linear regression ($r=0.997$, $P<0.05$). The minimum detectable concentration of IGF-I was 0.025 ng/ml. The intra- and interassay coefficients of variation were 4.6 and 8.1 %, respectively.

Western ligand blot analysis of IGFBPs in FF. A qualitative and quantitative evaluation of IGFBPs in bovine FF was conducted by ligand blot analysis as described by Hossenlopp et al. (1986) with slight modifications. Proteins were separated by one-dimensional SDS-PAGE using 4% stacking gel and 15% polyacrylamide separating gel in a minigel system (Mighty Small SE 250, Hoefer Scientific Instruments, San Fransisco, CA, USA). One microliter of FF with 4 µl of MilliQ water and 5 µl of samplebuffer (0.125 M Tris-HCl, 0.2% (v/v) glycerol, 0.4% (w/v) sodium docedyl sulphate (SDS)) was loaded per lane. A sample of pooled FF from small and medium sized follicles containing IGFBP2, -3, -4 and -5 was included in each gel to enable comparison among gels within an experiment. ^{125}I-IGF-II (recombinant human IGF-II; Boehringer Mannheim, Almere, The Netherlands) was used as a ligand. IGF-II was iodinated using Chlooramine T (Selman, 1995). Nitrocellulose membranes (Hoefer Scientific Instruments) were exposed to phosphorscreens (Fuji Film Imaging Plate Radio active energy sensor, Fuji, P photo Film Co., Japan) for 4 h. After analysis of the phosphorscreen (Fuji Bas 1000 bio imaging analyzer, Fuji, P photo Film Co), band intensities were analyzed using ImageQuaNT software (Biotechnology and LC Systems, Maarssen, the Netherlands). Therefore, the integrated pixel intensity (IPI) on a line, centered through the middle of the lane on the gel including the sample, was analyzed and depicted as a graph (IPI against distance on the line). IPIs per band (units, mm^{-2}) were then measured in an 8 mm wide area under the curve centered at the maximum of the peak for that band. Per gel, IPIs were corrected for a background IPI. This background was automatically established during analysis using linear regression (ImageQuaNT users guide, Biotechnology and LC systems). Relative IPIs per band were obtained by correction of the band intensities of the samples for the average IPI for that band of the reference samples included in each gel. The 49 and 43 KD bands correspond with the doublet of IGFBP3, the 35 KD band with IGFBP2, the 30 KD band with IGFBP5 and the 28 and 22 KD band with the doublet of IGFBP4 as has been demonstrated for bovine FF using Western-immunoblotting (de la Sota et al., 1996). The quantitative presence of IGFBP3 and IGFBP4 was calculated as the respective sums of band intensities of their doublets.

Data handling and statistical analysis

Values are mean ± SEM unless marked differently. Two-sample t-test was used to compare plasma estradiol-17ß concentrations at different times during eCG treatment. Follicular concentrations of progesterone, estradiol-17ß, androstenedione, IGF-I and IGFBP3 were analyzed using generalized estimating equations, using a normal distribution with an exchangeable correlation structure on log transformed data (Diggle et al., 1994). Data were considered to be significantly different if $P < 0.05$.

RESULTS

Hormone concentrations in the peripheral blood and response to superovulation

In all cows, luteal regression was completed within 24 h after PG administration, i.e. peripheral progesterone concentration < 3.2 nmol l^{-1}. The estradiol-17ß concentration showed a significant increase from 12.4 ± 0.6 pmol l^{-1} (n=23) before injection of eCG on day 10 to 154.0 ± 13.5 pmol l^{-1} at the time of the first puncture session (P0); immediately after puncturing of the 4-5 follicles on P0, a temporary fall to 133.6 ± 11.7 pmol l^{-1} in estradiol-17ß concentration could be observed, which was not significant (P=0.2). Thereafter, estradiol-17ß concentrations continued to increase to 173.8 ± 25.9 pmol l^{-1} until the occurrence of the LH surge.

In all cows, on average, the maximum of the LH surge occurred at 43.2 ± 0.9 h (n=23) after administration of PG with a mean amplitude of 21.2 ± 2.0 µg l^{-1}.

The average response to superovulation, i.e. the total number of follicles per cow that were aspirated at the two puncture times, for cows punctured at P0+P1, P0+P2 and P0+P3 were 14.5 ± 2, 13.3 ± 2 and 15.8 ± 3, respectively.

Collection and classification of follicular fluid

P0 was performed 30.6 h ± 0.4 (SD) h (n=23) after PG injection, i.e. 12.8 h ± 3.7 h before the maximum of the LH surge. The subsequent puncture sessions were carried out at 3.7 ± 2.2 (P1, n=6), 12.2 ± 0.3 (P2, n=8) and 22.0 ± 0.4 (P3, n=9) h after the occurrence of the LH surge respectively.

On the basis of the steroid concentrations in the FF, 61.2% of all collected follicles was classified as ‘normal’ and 38.8% as ‘deviant’. In general, the follicle population

per cow comprised follicles of both ‘normal’ and ‘deviant’ classes. The distribution of the numbers of ‘normal’ and ‘deviant’ follicles on the 4 puncture sessions is shown in Table 2. The mean steroid concentrations of progesterone, estradiol-17ß and androstenedione in the fluid of the follicles qualified ‘normal’ are shown in Figure 1.

Table 2. Numbers of ‘normal’ and ‘deviant’ classified follicular fluids collected by transvaginal ultrasound-guided puncture of preovulatory-sized follicles at successive times relative to the LH surge in eCG/PG/anti eCG-treated cows.

Time of collection relative to the LH surge		Number of cows	Number of FF	Number (%) of ‘normal’ FF	Number (%) of ‘deviant’ FF	Reason for ‘deviant’ classification
P0	12 h before LH	23	86	52 (60.5%)	34 (39.5%)	21 E↓ 8 E↓, P↑ 3 E↓, P↓ 1 P↑ 1 A↑
P1	3 h after LH	6	63	48 (76.2%)	15 (23.8%)	12 E↑ 2 E↓ 1 E↓, P↑
P2	12 h after LH	8	78	59 (75.6%)	19 (24.4%)	13 P↓ 1 P↓, E↓ 1 P↓, E↑ 4 P↑
P3	22 h after LH	9	108	46 (42.6%)	62 (57.4%)	47 P↓ 13 P↓, E↑ 1 P↑ 1 E↑, A↑
Overall number of FF			335	205 (61.2%)	130 (38.8%)	

FF: follicular fluid; P: progesterone, E: estradiol-17ß, A: androstenedione. ↑ = Concentration exceeding the upper limit and ↓ = concentration below the lower limit as determined from concentrations measured at corresponding times relative to the LH surge in fluid of preovulatory follicles of untreated, normally cyclic cows (Table 1).

At puncture time P0, the majority of ‘deviant’ follicles (Table 2) had a low estradiol-17ß concentration (n=32, mean concentration 2.57 ± 0.27 µmol l^{-1}, SEM) in the FF, but the androstenedione concentration in these follicles (0.40 ± 0.06 µmol l^{-1}) was

within limits, although significantly less than in ‘normal’ follicles (P=0.002). Eight of the ‘deviant’ follicles contained a progesterone concentration higher than the upper limit (mean, 2.34 ± 0.46 µmol l^{-1}). One follicle was qualified ‘deviant’ on the basis of a too high androstenedione concentration (4.78 µmol l^{-1}).

Figure 1. Progesterone, estradiol-17ß and androstenedione concentrations in the fluid of eCG/PG/anti-eCG stimulated preovulatory follicles with steroid concentrations within the normal range (‘normal’ follicles) as defined from concentrations measured at corresponding times relative to the LH surge in the fluid of preovulatory follicles of untreated, normally cyclic cows (Table 1). Values are mean ± SEM. Numbers above bars represent the number of follicles.

After the LH surge, at puncture time P1, all ‘deviant’ follicles contained deviating estradiol-17ß concentrations, either higher (n=12 follicles (all collected from cow 14), mean 7.46 ± 0.18 µmol l^{-1}) or lower (n=3 follicles, mean 0.18 ± 0.04 µmol l^{-1}) than the

defined limits. The mean androstenedione concentration of these 'deviant' follicles was significantly higher compared to the 'normal' follicles (P=0.013), but within limits.

At puncture time P2, all 'deviant' follicles showed deviating progesterone concentrations; the majority of the progesterone concentrations were lower than the lower limit (n=15 follicles, mean 0.08 ± 0.01 µmol l^{-1}) and in 4 follicles higher than the upper limit (mean 0.56 ± 0.14 µmol l^{-1}).

Shortly before onset of the period of multiple ovulations, at puncture time P3, the majority of the 'deviant' follicles showed a too low progesterone concentration (n=60 follicles, 0.26 ± 0.03 µmol l^{-1}). One follicle contained an excessively high progesterone concentration of 9.54 µmol l^{-1}.

Figure 2. Concentration of total IGF-I in fluids of eCG/PG/anti-eCG stimulated preovulatory follicles with steroid concentrations within ('normal' follicles, stippled bars) or outside ('deviant' follicles, open bars) the normal ranges as defined from concentrations measured at corresponding times in fluid of preovulatory follicles of untreated, normally cyclic cows. Values are means ± SEM. Numbers above bars (f,c) represent the number of follicles (f) analyzed and the number of cows (c) donating those follicles. Stippled bars with different superscripts (a, b) are significantly different (P<0.05).

Concentrations of IGF-I in follicular fluid

Comparing IGF-I concentrations in the fluid of 'normal' follicles, a significant time effect was found between P0 and P3 (P=0.029) and between P1 and P3 (P=0.008). At a particular puncture time, follicular IGF-I concentrations of 'normal' and 'deviant' follicles were not significantly different (Figure 2).

Western-ligand blot analysis of IGFBPs in follicular fluid

In decreasing molecular mass order, six bands could be detected in the analyzed follicles (same series as for IGF-I RIA); 49, 43, 35, 30, 28 and 22 KD, corresponding with IGFBP3 (49 and 43 KD), IGFBP5 (35 KD) IGFBP2 (30 KD) and IGFBP4 (28 and 22 KD). All follicles contained IGFBP3. When LMW IGFBPs were present, always the combination of IGFBP2, -4 and -5 was found. Relative IPIs of the bands for IGFBP2, -4 and -5 in the fluid of preovulatory follicles were in the same order of magnitude as those in the reference sample of pooled small and medium sized follicles analyzed on each gel.

Figure 3. Presence of LMW IGFBPs in fluids of eCG/PG/anti-eCG stimulated preovulatory follicles with steroid concentrations within ('normal' follicles, stippled bars) or outside ('deviant'

follicles, open bars) the normal ranges as defined from concentrations measured at corresponding times in fluid of preovulatory follicles of untreated, normally cyclic cows. Numbers (p/n) above bars represent the number of follicles with LMW IGFBPs in their follicular fluid (p) and the total number (n) of 'normal' or 'deviant' follicles analyzed at that puncture time. Numbers above bars between brackets indicate the number of cows donating the follicles.

Figure 4. Amount of IGFBP3 (Figure 4a) or LMW IGFBPs (Figure 4b) in fluids of eCG/PG/anti-eCG stimulated preovulatory follicles with steroid concentrations within ('normal' follicles) or outside ('deviant' follicles) the normal ranges as defined from concentrations measured at corresponding times in fluid of preovulatory follicles of untreated, normally cyclic cows. Values are means ± SEM. For calculation of the mean amounts of IGFBP2, -4 and -5 only follicles containing these IGFBPs are included. Numbers between brackets above bars represent the number of follicles included in the mean. Different superscripts of bars (Figure 4a) indicate significant differences ($P<0.05$) between 'normal' follicles collected at different puncture times (a, b) or between 'normal' and 'deviant' follicles (*) collected at one puncture time.

In non of the 'normal' follicles, except in 15% of the follicles collected at P3, LMW IGFBPs could be detected. The mean progesterone concentrations of the follicles with LMW IGFBPs in their fluids collected at P3 were higher than of the follicles without

LMW IGFBPs (1.15 ± 0.1 vs. 0.88 ± 0.1 µmol l^{-1}). At P0, the fluid of 35% of the 'deviant' follicles contained LMW IGFBPs, and this percentage decreased during maturation (Figure 3). From the 7 'deviant' follicles with LMW IGFBPs at P0, 6 had too low estradiol-17ß concentrations and also in the fluids of the two 'deviant' follicles with LMW IGFBPs collected at P1 estradiol-17ß concentrations were too low. The progesterone concentration of the one 'deviant' follicle at P3 with LMW IGFBPs exceeded the upper limit.

In general, the amounts of IGFBP3 in the 'normal' follicles did not differ between the four puncture times; only at P2, IGFBP3 levels were higher than at P0 (P=0.04). At P0, P1, and P3 follicular IGFBP3 levels in 'normal' and 'deviant' follicles were similar; at P2 levels were significantly lower in 'deviant' than 'normal' follicles' (Figure 4a). The amounts of LWM IGFBPs are depicted in Figure 4b. Since the number of follicles presenting LMW IGFBPs in their fluid was very low (Figure 3) the depicted means are only based on the amounts of IGFBPs in follicles presenting the LMW IGFBPs and no statistical analysis was performed on these data.

DISCUSSION

In this study, preovulatory follicular development induced by treatment with eCG/PG/anti-eCG to induce multiple ovulations in the cow, was used as a model to study the possible role of the IGF system in bovine final maturation. Levels of IGF-I and IGFBP2, -3, -4 and -5 were compared in the fluid of eCG-stimulated preovulatory follicles with 'normal' or 'deviant' follicular function, as defined on the basis of steroid concentrations in the follicular fluid.

To reduce the influence of the variation in response to eCG treatment of the used group of cows on the number and quality of follicles per puncture time (Bevers and Dieleman, 1987), follicular fluids were collected by transvaginal ultrasound-guided aspiration at two different times during the same preovulatory period per cow; once before the LH surge and once at one of the respective puncture times after the LH surge. Immediately after puncturing of 4 to 5 follicles at 12 h before the LH surge a temporary fall in plasma estradiol-17ß concentration was observed, which is correlated to the number of mature follicles (Bevers and Dieleman, 1987), indicating that functional follicles were collected. The puncture procedure did not affect further follicular development of the follicles remaining on the ovaries, since plasma estradiol-17ß concentrations continued to increase until the occurrence of the preovulatory LH surge, as has been reported before in eCG/PG treated cows (Bevers and Dieleman, 1987). Furthermore, in the selected group of cows the number of preovulatory follicles and the interval between PG administration and the occurrence of the preovulatory LH surge were similar to those reported for eCG/PG treated cows

that did not undergo follicular puncturing before the LH surge (Bevers and Dieleman, 1987).

Final maturation in follicles with ‘normal’ follicular function was characterized by rather constant levels of IGF-I and IGFBP3 in the follicular fluid; some significant time effects were found comparing the concentrations at the different puncture times, but their physiological importance is doubtful. LMW IGFBPs were absent in the fluid of ‘normal’ follicles at the time the oocyte undergoes germinal vesicle breakdown and reached metaphase I stage, but were present in 15% of the fluids collected just before ovulation at relatively high levels. Interestingly, the ‘normal’ follicles containing LMW IGFBPs showed a follicular progesterone concentration, which was higher than that of the follicles without LMW IGFBPs. This suggests that these follicles were relatively more advanced in development and likely to ovulate early during the 6 h period of ovulations that is found in eCG/PG stimulated cows (Dieleman and Bevers, 1987). The presence of IGF and IGFBPs in the fluid of ‘normal’ follicles after eCG-stimulation resembles that of preovulatory follicles of unstimulated normally cyclic cows. These follicles, when collected just before and at 6.5 and 18.5 h after the maximum of the LH surge, contained always constant levels of IGF-I and IGFBP3 and occasionally little but mostly no other IGFBPs (Funston et al., 1996). Extrapolation of the presence of LMW IGFBPs in the fluid of eCG-stimulated preovulatory follicles with ‘normal’ function, collected at 22 h after the LH surge (our study), to the absence of these proteins at 18.5 h after the LH surge in unstimulated cows (Funston et al., 1996) suggests that the reappearance of detectable levels of LMW IGFBPs in follicular fluids during final maturation is precisely timed and occurs only very shortly before ovulation.

In comparison to eCG-stimulated follicles with ‘normal’ function, ‘deviant’ follicles contained similar levels of IGF-I and IGFBP3 but the presence of LMW IGFBPs was different in these fluids. At the onset of final maturation, 35% of the ‘deviant’ follicles contained LMW IGFBPs at relatively high levels compared to a complete absence of these proteins in ‘normal’ follicles. During final maturation, LMW IGFBPs were occasionally found (at 3 and 12 h after the LH surge) or absent (at 22 h after the LH surge) in ‘deviant’ follicles. Before the LH surge, all, except one, of the ‘deviant’ follicles with LMW IGFBPs in their fluid were qualified ‘deviant’ on the basis of too low estradiol-17ß concentrations, which is probably a sign of atresia (Kruip and Dieleman, 1987; McNatty et al., 1994). Also in unstimulated, normally cyclic cows low estradiol-17ß concentrations in large follicles (> 8 mm) coincide with the presence of LMW IGFBPs (Echternkamp et al., 1994). In rat (Adashi et al., 1985) and bovine (Gong et al., 1994) granulosa cell cultures, IGF-I enhances FSH-induced granulosa cell estradiol-17ß synthesis. Thus, assuming that IGF-I has a similar function under in vivo circumstances as in vitro, it can be speculated that the presence of LMW IGFBPs in ‘deviant’ follicles prevented IGF-I from exerting its action on the granulosa cells. Similar actions of LMW IGFBPs have been demonstrated before.

IGFBP2 decreases IGF-I induced proliferation of rat granulosa cell in vitro (Bicsak et al., 1990). In sheep, IGF-I induced granulosa cell proliferation can be inhibited by follicular fluid of small follicles containing LMW IGFBPs, but not when this proliferation is induced by an IGF-I analogue that has only very weak affinity for IGFBPs (Monget et al., 1993).

After the occurrence of the LH surge, at the time that oocytes undergo germinal vesicle breakdown and reach the metaphase I stage, the comparison of the presence of the IGF-I system in fluids of 'normal' and 'deviant' follicles did not reveal an important regulatory role for the IGF system during final maturation. However, during in vitro maturation (IVM), IGF-I has been shown to stimulate cumulus cell expansion and nuclear development of the oocyte (Izadyar et al., 1997). Also, the presence of IGF-I in combination with FSH during IVM increased blastocyst formation after in vitro fertilization and culture of these oocytes (Harper and Brackett, 1992). Maybe, instead of a clear regulatory role, IGF-I has a more permissive function in final maturation.

The appearance of LMW IGFBPs in the fluid of 'normal' but not 'deviant' follicles at the end of maturation indicate a role for these IGFBPs during ovulation and/or corpus luteum formation. In correspondence with our data, an increase in IGFBP2 but not IGFBP3 was found in fluid of porcine preovulatory follicles just before ovulation (Howard and Ford, 1992).

Our data add more evidence to the general assumption that LMW IGFBPs, more than IGFBP3, modulate IGF-I action; IGFBP3 would mainly serve as a carrier protein for IGF-I in the peripheral circulation. However, IGFBP3 can also actively influence IGF-I action as is demonstrated in a study where intrabursal injection of IGFBP3 in eCG-stimulated follicles of immature rats reduces ovulation rates with 55% (Bicsak et al., 1991). Furthermore evidence is growing that each of the individual IGFBPs, including IGFBP3, might affect IGF-I action in a specific way, possibly due to characteristic binding affinities of IGFBPs for IGF-I and/or of IGF-IGFBP complexes for IGF receptors. For example, IGFBP3 is a more potent inhibitor of IGF-I induced estradiol-17ß and progesterone synthesis by cultured rat granulosa cells than IGFBP2 in a similar concentration (Bicsak et al., 1992).

It can be concluded that low estradiol-17ß concentrations in preovulatory follicles at the onset of maturation coincide with the presence of LMW IGFBPs in the follicular fluid. According to the situation in vitro, these LMW IGFBPs might inhibit the stimulatory action of IGF-I on estradiol-17ß production by granulosa cells. Final maturation in the bovine is characterized by rather constant presence of IGF-I and IGFBP3 in combination with in general an absence of LMW IGFBPs in both fluids of 'normal' and 'deviant' follicles after eCG-stimulation. Therefore, only a permissive role of IGF-I in final maturation can be expected. Just before ovulation, LMW IGFBPs reappear in fluids of follicles with 'normal' function, indicating a different role of the IGF system during ovulation and corpus luteum formation.

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mRNA Levels for Insulin-like Growth Factor Binding Proteins in eCG-Stimulated Bovine Follicles, Collected Shortly before the Preovulatory LH Surge in Relation To Follicular Estradiol-17ß Concentrations

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In the bovine, eCG-stimulation induces deviations in preovulatory follicular development that coincide with altered insulin-like growth factor binding proteins (IGFBPs) presence in the follicular fluid. To study the regulation of IGFBPs during preovulatory development, gene expression for IGFBPs in eCG-stimulated follicles with normal and deviant function were compared using RT-PCR. Follicular function was established on estradiol-17ß (E) concentrations in the follicular fluid. Heifers (n=21) were stimulated with eCG (2500 IU) on day 10 of the estrous cycle followed by prostaglandin (15 mg) 48 h later. Concomitantly with eCG administration a norgestomet implant was applied for 102 h to suppress the LH surge. After ovariectomy, at 2 h after removal of the implant, fluid, wall and COC from follicles > 8 mm were collected. mRNA Analysis was performed in follicles from heifers with lowest (deviant, n= 22, follicles) and highest (normal function, n=19, follicles) mean follicular E concentrations. Amplification products after 40 -(IGFBP2, -4) or 30 and 40 cycles (IGFBP3 and -5) were analyzed; cDNA amplification and gel electrophoresis were performed simultaneously for samples that were compared. Gene expression of IGFBPs was present in all analyzed follicles and quantitative differences could never (IGFBP3) or occasionally be found. IGFBP2 and -5 gene expression tended to be higher in normal follicles, IGFBP4 expression was similar or tended to be lower in normal follicles. These differences in gene expression between normal and deviant follicles can not explain the found differences in IGFBP patterns in those follicles and therefore, it is concluded that the presence of IGFBPs in preovulatory follicles is not primarily regulated by gene expression.

INTRODUCTION

In the bovine, the use of exogenous gonadotropins results in an increase in ovulation rate but the yield of transferable embryos lags behind. This discrepancy can be partly explained by deviations in follicular development induced during selection, growth or maturation of follicles. Stimulation with gonadotropins results in heterogeneity in functionality between stimulated follicles (Vos et al., 1994a; van de Leemput et al., 1997) and also in an asynchrony in development between the follicle and its oocyte (Dieleman et al., 1988; de Loos et al., 1991; Hyttel et al., 1991). Recently is has been demonstrated that deviations in function of preovulatory follicles after stimulation with gonadotropins coincide with changes in the insulin-like growth factor binding protein (IGFBP) pattern

in the follicular fluid; around the time of the LH surge 35% of the follicles with deviant steroid synthesis contain IGFBP2, -4 and -5 (low molecular weight (LMW) IGFBPs) that are not found in concurrently developing normal preovulatory follicles (van de Leemput et al., 1997).

The IGF system, comprising 2 factors (IGF-I and –II), 6 different IGFBPs and three different receptors (IGF-I, IGF-II and insulin receptor) (Sara and Hall, 1990; Rotwein, 1991) plays an important role in, among others, growth and differentiation of bovine granulosa cells in vitro (Spicer et al., 1993; Armstrong et al., 1996). So far, four IGFBPs (IGFBP2, -3, -4, and -5) have been identified in the cow ovary (de la Sota et al., 1996). Studies with rat granulosa cell cultures indicate that IGFBPs regulate both bioavailability and action of IGF-I (Bicsak et al., 1990; Adashi et al., 1992).

In normally cyclic animals, follicular growth and atresia is accompanied by a constant presence of IGFBP3 in the follicular fluid and changes in intrafollicular levels of LMW IGFBPs. In the bovine, as in human (Cataldo and Giudice, 1992), pigs (Mondschein et al., 1991) and sheep (Monget et al., 1993) growth of healthy antral follicles is associated with a decrease in LMW IGFBPs, being undetectable by Western-ligand blotting in the fluid of preovulatory follicles around the time of the occurrence of the preovulatory LH surge (Echternkamp et al., 1994; Funston et al., 1996). On the contrary, atresia of medium sized and large follicles is characterized by increasing intrafollicular levels of these LMW IGFBPs (Echternkamp et al., 1994; Funston et al., 1996). The presence of IGFBPs in the follicular fluid during growth and atresia of follicles is probably mainly regulated by specific proteolysis (human: Chandrasekher et al., 1995; sheep: Besnard et al., 1996a; pigs: Besnard et al., 1997) and by alterations in gene expression (sheep: Besnard et al., 1996b; pig: Samaras et al., 1993; rat: Erickson et al., 1992a, b). These changes in gene expression vary for respective IGFBPs during different processes and in different species. In the bovine, in situ hybridization studies (Armstrong et al., 1998) of follicles of normally cyclic animals demonstrate that gene expression for IGFBP2 is always present in healthy follicles < 8 mm but only in 40% of the analyzed healthy follicles > 8 mm. The localization of mRNA for IGFBP2 in healthy follicles is confined to granulosa cells while during atresia mRNA for IGFBP2 can be found in both granulosa and theca tissue. mRNA for IGFBP4 is always detected in theca cells of growing follicles and in both granulosa and theca cells of atretic follicles. Studies concerning IGFBP3 and -5 gene expression in bovine ovarian tissue are not available yet.

eCG stimulated follicles can be used to study the regulation of IGFBPs during preovulatory follicular development, since after stimulation with exogenous gonadotropins in deviant preovulatory follicles another pattern of IGFBPs has been observed than in concurrently developing normal preovulatory follicles,. Therefore, we investigated the presence of mRNA for IGFBP2 -3, -4 and -5 in normal and deviant preovulatory follicles shortly before onset of final maturation in eCG stimulated heifers, using reverse transcriptase polymerase chain reaction (RT-PCR). The functionality of the follicles was determined on the basis of estradiol-17ß concentrations in the follicular fluid.

MATERIALS AND METHODS

Experimental design

Heifers were stimulated with eCG to create a large heterogeneous population of preovulatory follicles. During stimulation release of the preovulatory LH surge was prevented using a norgestomet ear implant (Vos et al., 1994b) which was removed at the time corresponding to the time of occurrence of the preovulatory LH surge in normally cyclic animals. Heifers were ovariectomized 2 h later. Fluid (FF), cumulus oocyte complex (COC) and a part of the wall were collected of all follicles > 8 mm. To establish follicular function, estradiol-17ß concentrations were measured in the FFs from which a COC was recovered, and COCs were matured, fertilized and cultured in vitro.

In preovulatory follicles, collected from heifers with the lowest and highest mean follicular estradiol-17ß concentrations, semi-quantitative analysis of the presence of mRNA for IGFBP2, -3, -4 and -5 was performed using RT-PCR. To minimize variations in the amount of final PCR product due to the procedure, amplification of cDNA and gel electrophoresis were performed simultaneously for follicles that were compared. Only comparisons within one gel were made.

For comparative purposes, the presence of mRNA for IGFBPs in follicles < 4 mm and in eCG stimulated follicles collected just before ovulation from a further group of heifers was analyzed as well.

Treatment of the animals

Holstein-Friesian heifers were selected from our herd on the basis of general clinical examination and normal ovarian cyclicity during at least 3 weeks as established by the progesterone concentration in peripheral blood samples taken 3 times a week. The heifers were housed in groups of 6 animals, were fed silage and concentrate (to a maximum of 1 kg per heifer per day) and supplied water at libitum.

Before the experiments estrus was synchronized using an ear implant for 9 d (3 mg norgestomet, Crestar; Intervet International BV, Boxmeer, The Netherlands) accompanied by treatment with 3 mg norgestomet and 5 mg estradiol-valerate (im). Two days before removal of the implants prostaglandin (PG, 15 mg Prosolvin im; Intervet International BV) was administered to ensure complete regression of the corpus luteum (Vos et al., 1996a). At day 8 (estrus is day 0) of the subsequent cycle all follicles > 8 mm were disabled by transvaginal ultrasound-guided puncturing (Vos et al., 1994c) to prevent eventual suppressive action of dominant follicles on the follicle population to be stimulated. Stimulation was carried out on day 10 with eCG (2,500 IU Folligonan im; Intervet International BV) followed by PG (15 mg Prosolvin im) 48 h later. An ear

implant (Crestar) was inserted concomitantly with the administration of eCG to suppress endogenous release of the preovulatory LH surge after regression of the corpus luteum; the accompanying im norgestomet and estradiol-valerate were not administered. The ear implant was removed 54 h after PG treatment at the time preovulatory follicular development until onset of final maturation was assumed to be completed and ovariectomy (OVX, n=21 heifers) was performed 2 h later.

Six additional heifers were synchronized and stimulated with eCG according to the same protocol. In addition, at the time of removal of the ear implant, GnRH was administered (1.0 mg Fertagyl in 10 ml saline im; Intervet International BV) to induce an LH surge 2 h later (Vos et al., 1994b), and 10 h after GnRH administration a dose of monoclonal antibody against eCG (5 ml Neutra-PMSG iv; Intervet International BV) was administered which was sufficient to neutralize 2,500 IU of eCG within 1 h (Dieleman and Bevers, 1987). The heifers were ovariectomized 21.5 - 22.0 h after GnRH (i.e., 19.5 - 20.0 h after the maximum of the LH surge).

OVX was performed by laparotomy through flank incision under local infiltration anesthesia (Dieleman et al., 1983). Ovaries were collected in 0.9% (w/v) NaCl (25°C) and transported immediately to the laboratory.

Heparinized blood samples were collected from the jugular vein daily from day 0 until day 10 and twice a day from administration of eCG until removal of the ear implant. From the six heifers ovariectomized at 21.5-22 h after GnRH injection, further blood samples were collected every hour between administration of GnRH and anti-eCG, and then every 4 h until ovariectomy. In addition, a blood sample was taken during ovariectomy of each animal.

Collection of follicular fluids, COCs, follicular walls and bovine endometrium

COCs from all follicles > 8 mm, which were presumed to be preovulatory, were obtained by aspiration using a winged infusion set (18 G needle, tubing length 30 cm, volume 0.44 ml, Terumo Europe N.V., Leuven, Belgium) connected to a pump (Air compressor, MagneTek type SP, Thomas Industries Inc., Monroe, LA, U.S.A.) keeping the pressure between 0.2-0.4 bar. After aspiration the tubing of the equipment was flushed with 0.9% (w/v) NaCl and follicular fluid (FF) and flush fraction were collected in separate tubes for each follicle. After recovery of the COCs from FF or flush fraction, 50 IU heparin (10 µl) was added to the FFs before storage of the FFs at –25°C until RIA.

Samples of the follicular walls (± 0.25 cm^2 per follicle) were collected in Eppendorf tubes. Immediately after collection 500 µl of Ultraspec ^{TM} (Biotecx Laboratories Inc., Houston, TX, USA) was added to the samples and after brief vortexing, specimens were stored at –80°C until RNA isolation. From the heifers ovariectomized 21.5-22 h after GnRH administration, follicular walls were collected from 3 at random selected follicles > 8 mm per cow. The walls of small follicles (< 4 mm, n=9) were dissected from ovaries

obtained at the slaughterhouse. In general, follicular walls and fluids were stored within 2 h after OVX or slaughter of the animals.

Samples of bovine uterine wall were collected from cows undergoing caesarian section. The endometrium, to be used as positive control for mRNA of IGFBP2, -3, -4 and -5, was dissected from the rest of the wall, rapidly frozen in liquid nitrogen and subsequently stored at –80°C. Prior to mRNA isolation, 500 µl Ultraspec^{TM} (Biotecx Laboratories Inc.) was added to the samples.

Immunoassays for LH, progesterone and estradiol-17ß

Peripheral progesterone concentrations to establish normal cyclicity were estimated by enzyme immunoassay (van de Wiel and Koops, 1986).

During the superovulatory treatment concentrations of progesterone and estradiol-17ß in peripheral blood samples were estimated by solid-phase ^{125}I RIA methods (Coat-A-Count TKPG and TKE, respectively: Diagnostic Products Corporation, Los Angeles, CA, USA) with modifications as described by Dieleman and Bevers (1987). The sensitivity was 0.15 nmol l^{-1} and 7.5 pmol l^{-1}, and the interassay coefficient of variation 11% and 8.9%, respectively.

Concentrations of LH were estimated by a validated RIA method as described by Dieleman et al., (1983, 1986). The sensitivity of the RIA was 0.4 µg l^{-1} NIH-LH-B4 and the intra- and interassay coefficients of variation were <9%, respectively.

In FF of follicles from which a usable oocyte was obtained, estradiol-17ß concentrations were estimated by solid-phase ^{125}I RIA method (Coat-A-Count TKE) according to the manufacturer. Aliquots of FF were diluted (707 times) with 0.02 M borate buffer (pH 8.5) in 0.9% (w/v) NaCl, The sensitivity and the interassay coefficient of variation of the assay were 0.07 nmol l^{-1} and 14.1%, respectively.

In vitro maturation (IVM), -fertilization (IVF) and -culture (IVC) of COCs

COCs with a multilayered compact cumulus investment were selected for IVM and only heifers (n=15) with > 5 selected COCs were included in the experiment. COCs from one heifer were allocated to one particular well of a 4 well culture plate containing 500 µl collection medium (HEPES buffered M199 (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (FCS, Gibco BRL). The average recovery rate of selected oocytes was 62.4 ± 14.3 (SD) % (n=15 heifers). Maximally 3 h after OVX, COCs were transferred to 500 µl maturation medium (M199 supplemented with 10% FCS, 0.01 units/ml porcine FSH (Sigma, St Louis, MO, USA) and 0.01 units/ml equine LH (Sigma)) and cultured for 22 h at 39°C, in 5% CO_2 in humidified air.

IVF and IVC were carried out as described previously (Izadyar et al., 1996). All COCs from one cow were fertilized and subsequently cultured in one well. Briefly,

COCs were rinsed twice with 2 ml HEPES buffered M199 medium per well before transfer to fertilization medium as described by Parrish et al. (1988) with slight modifications (Izadyar et al., 1996). Fertilization was then carried out during 18 - 20 h with $0.5x10^6$ spermatozoa/ml of a bull with proven in vitro fertility. COCs were freed from cumulus cells and transferred to a well containing 500 µl M199 supplemented with 10% FCS and a monolayer of Buffalo rat liver cells. At day 4 of IVC, non-cleaved structures were removed, and at day 4 and 8 of culture embryos were transferred to fresh co-culture wells. At day 7, 9 and 11 numbers of blastocysts and hatched blastocysts were scored. Day 1 of culture was defined to start immediately after IVF was finished.

IVP conditions were checked routinely throughout the experimental period each week (n=9) with 70 oocytes (2 wells with 35 oocytes) recovered from 2-8 mm sized follicles from ovaries obtained from the slaughterhouse. These cultures were performed by the same investigators under similar conditions. Blastocyst formation and hatching rates after 11 days of IVC were 27.1 ± 5.2 (SD) % and 21.9 ± 5.0% relative to the number of COCs at day 1 of IVC, respectively, indicating that the conditions during IVP were constant throughout the experimental period.

Isolation of total RNA and reverse transcription

Total RNA was isolated essentially according to Biotecx's recommendations (Biotecx's, bulletin no 27, 1992); after thawing of the sample 100 µl of chloroform (purity > 99%, Sigma) was added, the samples were homogenized by vortexing for 15 sec and the homogenate was stored for 5 min on ice. The homogenate was then centrifuged at 13,000 *g* for 15 min and about 4/5 of the aqueous phase was transferred to a new Eppendorf tube. RNA was precipitated by addition of an equal volume of isopropanol (purity > 99%, Sigma), incubation overnight at –80°C, and subsequent centrifugation for 30 min at 13,000 *g*. The supernatant was removed and the pellet was washed once by addition of 0.2 ml of ice-cold 75% ethanol with gentle inversion of the tube, and centrifugation for 15 min at 13,000 *g*. After complete removal of the supernatant, the pellet was left to dry for 10 min in air atmosphere. The pellet was resuspended in 10 µl of water produced by deionization (MilliQ water; MilliQ Plus, Millipore B.V., Etten-Leur, The Netherlands) to which 80 U/ml RNAsin nuclease inhibitor (RNAsin, Promega, Leiden, The Netherlands) was added. Subsequently, RNA purity and concentration were determined from the ratio of absorbance at 260 nm/280 nm (A 260/280 ratio) and absorbance at 260 nm, respectively (RNA/DNA calculator, GeneQuant II, Pharmacia Biotech, Roosendaal, The Netherlands). Only RNA samples with A 260/280 ratios >1.6 were used. RNA samples were stored at –80°C. To check for DNA remnants after the used RNA isolation procedure, amplification was performed of a sample of total RNA isolated from endometrium tissue with the same primer pairs (for IGFBP2, -3, -4 and -5) as used for amplification of the produced cDNAs from follicular tissues (see below).

Prior to the reverse transcription reaction, aliquots of 1 µg RNA, diluted to 10 µl with MilliQ water containing 8 units RNAsin, were incubated for 5 min at 70°C, vortexed for 5 sec and chilled on ice. Reverse transcription was done in a total volume of 20 µl, containing the 10 µl of the sample RNA, 4 µl reverse transcriptase buffer (5X; 250 mM Tris HCl, (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$; Gibco, BRL), 8 units RNAsin, 150 units Superscript^{TM} RNase H^- Reverse Transcriptase (Gibco, BRL), 1.2 µg at random primer (Gibco, BRL) and final concentrations of 10 mM dithiothreitol (DTT) and 1 µM of each deoxynucleotide triphosphate (dNTP). The mixture was incubated for 1 h at 42°C, followed by 5 min at 90°C and thereafter stored at –20°C.

Amplification of cDNA for ß-actin, IGFBP2, IGFBP3, IGFBP4 and IGFBP5

PCR reactions were performed as described before (Izadyar et al., 1998) with slight modifications. Briefly, PCR reactions were carried out in 200 µl tubes (Eurogentec, Seraing, Belgium), using 2 µl of the follicular samples with cDNA as a template in 50 µl volume, containing PCR buffer (final concentration 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, and 0.5 µM of each primer and 1.25 units of TaKaRa Taq polymerase (Boehringer Ingelheim, Alkmaar, The Netherlands). Primers used for PCR are presented in Table 1. Prior to amplification of cDNA for IGFBPs, in each sample the mRNA expression of ß actin was analyzed.

All PCRs were performed in a 24–well thermocycler (Perkin-Elmer, Gouda, The Netherlands). After initial denaturation for 5 min at 94°C, cDNA was amplified during thermal cycles of 15 sec at 94°C, 30 sec at 60°C and 45 sec at 72°C. Final extension was for 10 min at 72°C.

Table 1. Primers for PCR analysis of mRNA in walls of preovulatory follicles defined on the basis of sequences published for bovine tissues (IGFBPs) and for murine tissue (ß-actin).

Target gene	Sense	Primer sequence (5'→ 3')	Start position	Fragment size	Reference
b IGFBP2	s	AAC GGC GAG GAG CAC TCT GA	400	286	Upton et al.,
	as	GGA AGG CGC ATG GTG GAG AT	686		1990
b IGFBP3	s	TGG CAG TGA GTC GGA AGA AG	527	266	Spratt et al.,
	as	TTG AGG TGG TTC AGC GTG TC	793		1991
b IGFBP4	s	GGA CAA GGC GTG TGC ATG GA	493	347	Moser et al.,
	as	GAC AGG CTC ACT CTC GGA AG	840		1992
b IGFBP5	s	TCG TGC GGC GTC TAC ACT GA	92	200	Moser et al.,
	as	AGG TCT CCT CTG CCA TCT CG	292		1992
m ß-Actin	s	TGA ACC CTA AGG CCA ACC GTG	408	396	Tokunaga et
	as	GCT CAT AGC TCT TCT CCA GGG	804		al., 1986

b = bovine, m = murine, a(s) = anti(sense).

To determine the range of exponential amplification of the genes to be analyzed, PCR reactions for each of the genes were performed for 20, 25, 30, 35 and 40 cycles (n=5 follicular cDNA samples). For IGFBP3, and -5 a measurable signal was achieved after 30 cycles that was increased after 40 cycles. To assure that for each sample the PCR product was analyzed in the exponential range, two step PCR was performed for amplification of cDNA for IGFBP3, and -5; after 30 cycles a 10 µl sample was removed from the PCR reaction-mixture and thereafter the PCR was continued until 40 cycles were completed. Frequently, accurate reading after CCD imaging (CCD camera, Appligene, B&L Systems, Maarssen, The Netherlands) of the 30-cycle product for IGFBP2 and -4 was not possible and therefore for these genes only the 40-cycle product was analyzed.

For IGFBP3 and -5, 12 samples from follicles with low (n=6) and high (n=6) estradiol-17ß concentrations in FF, respectively, were amplified at the same time and similarly 22 for IGFBP2 and -4 (11 low and 11 high) were analyzed. During the first run of PCR reactions for IGFBP2 and -4 the same samples were analyzed as during the first and second run of PCR reactions for IGFBP3 and -5; samples of the second run for IGFBP2 and -4 corresponded to those of the third and fourth run for IGFBP3 and -5. During the third (IGFBP2 and -4) and fifth (IGFBP3 and -5) run the remaining samples were amplified. With each run of PCR reactions, identically treated positive (bovine endometrium) and negative (MilliQ water) control samples were included. The samples of follicles < 4 mm (n=9) were amplified at the same time as three samples of follicles with low estradiol-17ß concentrations in FF. The 18 cDNA samples of eCG stimulated preovulatory follicles collected just before ovulation were amplified during two runs of PCR reactions (n=12 and n=6) for each gene.

Quantification of RT-PCR products

Ten µl of the 30 cycle and 40 cycle PCR product (for IGFBP3, -5) or of the 40 cycle PCR product (for IGFBP2, -4) were analyzed by gel electrophoresis (1% agarose gel containing 0.4 µg/ml ethidium bromide, (2x15 lanes)) after adding 2 µl of loading buffer (25% w/v sucrose, 0.05% w/v bromophenolblue, 0.05 w/v xylenecyanol). A 100 bp ladder (Gibco, BRL) was included in each gel as a reference for fragment size. All PCR products generated during simultaneously performed PCR reactions were analyzed on one gel. An image of each gel was taken using a CCD camera.

The digitized images were analyzed using ImageQuaNT software (B&L Systems). The integrated pixel intensity (IPI) of each band in an identically sized rectangle fitting the largest band on that gel was estimated; all the IPIs were corrected for a background IPI measured in an identically sized rectangle on each gel.

Restriction enzyme analysis

To verify the identity of the RT-PCR products, an analysis by restriction endonuclease was carried out on pooled amplified cDNAs of follicles with low and high estradiol-17ß concentrations. Enzymes (Eurogentec) used for cutting of the RT-PCR products and the expected restriction fragments are listed in Table 2. Sequence cutting was done in a total volume of 20 µl containing 10 µl of the PCR product, 1 µl (12 IU) enzyme, 2 µl buffer and 7 µl MilliQ water. Final concentrations with which the assay was performed were 10 mM Tris-HCl (pH 7.5 at 37°C), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 100 mg/ml BSA. The mixture was incubated for at least 1 h at 37°C. 10 µl of the mixture was resolved by 2.5 % agarose gel electrophoresis as described before. A 20 bp ladder (Eurogentec) was included as a reference for fragment size. For RT-PCR products for each of the genes, two fragments with the expected size were detected.

Table 2. Restriction endonucleases used for cutting the RT-PCR products

Product	Enzyme	Expected fragment sized (bp)
bovine IBFBP2	BSED-I	182 and 104
bovine IGFBP3	HIN6-I	144 and 122
bovine IGFBP4	HINi-I	195 and 152
bovine IGFBP5	ALU-I	120 and 80

Statistical analysis

Unless marked differently, all values are mean ± SEM. The percentage of hatched blastocyst formation is calculated as the number of hatched blastocysts at day 11 of IVC relative to the number of (non)-fertilized oocytes present at day 1 of IVC. The correlation between hatched blastocysts formation per heifer and the mean of the estradiol-17ß concentration of the fluids of the follicles from which a COC was recovered within one animal was estimated by Pearson's correlation.

Differences in the average follicular estradiol-17ß concentrations between the heifers with low and high mean follicular estradiol-17ß concentration selected for mRNA analysis were analyzed using two sample t-test.

Differences in average IPIs of RT-PCR products per gel between follicles of heifers with low and high mean follicular estradiol-17ß concentration (Figure 2) and between preovulatory follicles and small follicles < 4 mm (Figure 3) were analyzed using two sample t-test. $P<0.05$ was considered significant.

The average IPI of RT-PCR products in eCG-stimulated follicles collected just before ovulation is calculated from 18 samples, analyzed on two different gels.

RESULTS

Superovulation procedure

All heifers showed luteal regression (i.e., peripheral progesterone concentration <3.2 nmol l^{-1}) within 28 h after PG administration. The LH concentration in peripheral blood of the heifers ovariectomized 1.5-2 h after removal of the ear implant, did not exceed 0.7 µg l^{-1} in the samples collected 3 h before and at OVX. In the 6 heifers ovariectomized shortly before ovulation, administration of GnRH always resulted in a preovulatory LH surge with a maximum 2 h later.

Follicular estradiol-17ß concentrations and hatching of blastocysts during IVC

A positive correlation (r=0.60, P=0.019) was found between the hatched blastocyst formation rate per cow and the mean of the estradiol-17ß concentration of the fluid of the follicles from which an oocyte was cultured (Table 3). As indicated in Table 3, Heifers 7891, 9902 and 7920 were selected for mRNA analysis on the basis of lowest and heifers 5259, 1626 and 7212 on the basis of highest mean follicular estradiol-17ß concentration per heifer with respective groups averages of 3.04 ± 2.0 and 6.00 ± 0.4 µmol l^{-1} (P<0.05). The estradiol-17ß concentrations of individual follicles of the heifers with low mean estradiol-17ß concentration were < 4.41 µmol l^{-1} (except 2: (4.6 and 4.9 µmol l^{-1}) and of heifers with high mean estradiol-17ß concentration > 4.41 µmol l^{-1}. The average rates for hatched blastocyst formation for COCs from the low and high estradiol-17ß group were 5.6 and 46.2%, respectively; for COCs from intermediate estradiol-17ß producing heifers it was 32.2%.

Isolation of total RNA and reverse transcription

After isolation of total RNA, the RNA concentration of twelve samples (n=2: heifer 7891, n=4: heifer 9902, n=5: heifer 1626 and n=1: heifer 7212) was too low to obtain an accurate reading of the RNA concentration. Therefore, these samples were only used for qualitative- but not for semi-quantitative analysis. Amplification of ß actin in each cDNA sample resulted in a clear band of the expected fragment size. Amplification of total RNA, isolated from endometrium tissue, did not result in visible bands; amplification of the cDNA of this sample resulted in clear bands of the expected fragment sizes for the respective four IGFBPs.

Table 1. Mean estradiol-17ß concentration in the fluid of preovulatory follicles from which cumulus oocyte complexes were tested for development into hatched blastocysts. Follicles were collected shortly before the occurrence of the LH surge from eCG-stimulated heifers.

Heifer code	Number of preovulatory follicles	Number of selected oocytes	Estradiol-17ß (µmol l^{-1})[3)]	Number of hatched blastocysts at day 11 of IVC
7891[1)]	22	7	2.63 ± 0.29	0
9902[1)]	28	15	3.24 ± 0.35	0
7920[1)]	12	6	3.25 ± 0.60	1
5178	17	7	3.27 ± 0.89	4
9904	18	7	3.40 ± 0.27	3
1628	22	8	3.45 ± 0.40	1
5346	19	8	3.65 ± 0.74	2
7343	20	10	4.17 ± 0.45	3
8038	18	6	4.37 ± 0.61	1
9442	20	16	4.80 ± 0.28	8
5572	15	10	4.86 ± 0.55	2
7896	32	14	5.12 ± 0.25	5
5259[2)]	8	6	5.19 ± 0.32	2
1626[2)]	32	13	6.13 ± 0.35	5
7212[2)]	9	6	6.65 ± 0.53	4

[1, 2)] Heifers with lowest (1) or highest (2) mean follicular estradiol-17ß concentration used for analysis of mRNA for IGFBPs in follicular fluid. [3)] Mean ± SEM.

RT-PCR

In general, mRNA for IGFBP2, -3, -4 and -5 could be detected in tissue of all analyzed follicles regardless of their functionality. Amplification of IGFBP2 and -4 mRNA resulted in moderate to strong bands of the expected fragment size after 40 cycles (Figure 1a). RT-PCR for IGFBP3 and -5 resulted in a band of the expected fragment size of moderate intensity after 30 cycles, which was increased after 40 cycles in all samples (Figure 1b).

Figure 2 shows the mean IPIs per gel of IGFBP3 and IGFBP5 PCR products after 30 cycles of amplification and of IGBP2 and IGFBP4 PCR products after 40 cycles of amplification. The products of the follicles that were not included in the semi-quantitative analysis were analyzed by gel electrophoresis on gel 1 (IGFBP2, -4) and gel 1 and 2 (IGFBP3 and -5). In follicles with high estradiol-17ß concentrations in the fluid the presence of IGFBP2 mRNA was on average higher than in follicles with low estradiol-17ß concentrations, but only in the first gel this difference was significant. No differences in IGFBP3 mRNA levels between groups could be detected. The mRNA level for IGFBP4 was significantly higher in follicles with low estradiol-17ß

concentrations in gel 1, but no differences were observed in gel 2 and 3. Differences between mRNA levels for IGFBP5 varied markedly comparing follicles with low and high estradiol-17ß concentrations although there was a tendency to lower mRNA levels in follicles with low estradiol-17ß concentrations.

Figure 1. mRNA Expression in tissue of preovulatory follicles with low (L) or high (H) follicular estradiol-17ß concentrations collected from eCG-stimulated heifers shortly before the occurrence of the LH surge.
Top panels Representative sample of RT-PCR products for IGFBP4 (after 40 cycles, Figure 1a) or IGFBP5 (after 30 and 40 cycles, Figure 1b) after electrophoresis on agarose gels containing ethidium bromide and CCD imaging; bpl = base pair ladder; + = positive control (bovine endometrium); - = negative control (MilliQ water).
Lower panels Integrated pixel intensity (IPI) readings corresponding to CCD camera image depicted in the top panel.

Figure 2. Expression of mRNA for IGFBP2, -3, -4 and -5 in tissues of preovulatory follicles collected shortly before the occurrence of the LH surge from eCG-stimulated heifers with low (open bars) or high (stippled bars) mean follicular estradiol-17ß concentrations. Relative quantities of mRNA are expressed as integrated pixel intensity (mean ± SEM) by CCD imaging of RT-PCR products after electrophoresis on agarose gels containing ethidium bromide. Numbers above bars indicate the number of analyzed follicles. Bars with different superscripts representing samples within one PCR run are significantly different (*, $P<0.05$).

The expression levels of IGFBP2 and -5 mRNA in eCG-stimulated preovulatory follicles were similar to those in small follicles, and expression of IGFBP3 and -4 was significantly higher in the preovulatory sized follicles (Figure 3).
IPI measurements after 40 (IGFBP2 and -4) or 30 (IGFBP3 and -5) cycles of amplification for IGFBPs in the follicles collected just before ovulation (n=18) were of the same order of magnitude as those from follicles collected just before the expected LH surge.

Figure 3. Comparison between the expression of mRNA for IGFBP2, -3, -4 and -5 in tissue of preovulatory follicles with low estradiol-17ß concentrations collected shortly before the occurrence of the LH surge from eCG-stimulated heifers (n=3, open bars) and in small follicles (< 4mm) dissected from ovaries obtained from the slaughterhouse (n=9, stippled bars). Relative quantities of mRNA are expressed as integrated pixel intensity (mean ± SEM) by CCD imaging of RT-PCR products after electrophoresis on agarose gels containing ethidium bromide. Bars representing the same IGFBP with * are significantly different ($P<0.05$).

DISCUSSION

In cows, stimulation with eCG results in a heterogeneous population of preovulatory follicles with normal and deviant functionality. In deviant preovulatory follicles another pattern of IGFBPs has been observed than in normal preovulatory follicles (van de Leemput et al., 1997). Therefore, eCG stimulated follicles can be used to study the regulation of IGFBPs during preovulatory follicular development. In this study we investigated the presence of mRNA for IGFBP2, -3, -4 and -5 in normal and deviant preovulatory follicles shortly before onset of final maturation in eCG stimulated heifers, using RT-PCR. The functionality of the follicles was established on the basis of estradiol-17ß concentrations in the follicular fluid.

To obtain preovulatory follicles after an identical period of stimulation, a superovulation procedure was used which suppresses release of the preovulatory LH surge by norgestomet ear implant (Vos et al., 1994b). Ovariectomy was performed after removal of the ear implant after a period, which resembles that of normal stimulated

preovulatory development. In previous experiments it has been shown that upon induction of the LH surge with GnRH, at the time of removal of the ear implant, follicles and oocytes undergo maturation similar to that in normally eCG treated animals with regards to steroid synthesis of the follicle and developmental potential of the oocyte at the end of maturation (Vos et al., 1996b). Therefore, it is concluded that the characteristics of the recovered follicles and oocytes in this study resemble that of follicles and oocytes recovered from normally eCG-stimulated animals just before the occurrence of the LH surge.

Follicles were categorized on the basis of follicular estradiol-17ß concentrations. The observed positive correlation between follicular estradiol-17ß concentrations and hatched blastocyst formation rates of the oocytes recovered from these follicles confirms that a high estradiol-17ß concentration indicates normal function of preovulatory follicles vs. a low concentration for deviant follicles.

RT-PCR is predominantly a method for qualitative analysis. In this study, conditions were optimized to be able to perform semi-quantitative comparisons. Since variations in ultimate IPI readings of the amplified product can be introduced during each step of the procedure, only samples were compared of which cDNA amplification and gel electrophoresis were performed simultaneously. In addition, since the most accurate estimation of the original amount of mRNA in a sample is obtained at early stage during PCR reaction and quantitative differences of mRNA are not well reflected when the plateau in amplification is reached (Higuchi et al., 1993), the PCR products were analyzed in the exponential phase of the reaction. After amplification of cDNA for IGFBP3, and -5, in all samples the band intensity of the 40-cycle product was more intense than that of the 30-cycle product confirming that at 30 cycles the plateau of the PCR reaction was not yet reached. However, since for IGFBP2 and -4 only the 40-cycle products were analyzed, it can not be excluded that the plateau of the PCR reaction was reached at this point for some samples with high concentrations of mRNA for IGFBP2 and -4.

IGFBP2, -3, -4 and -5 mRNA could be detected in all analyzed follicles. Interestingly, Armstrong et al. (1998), using in situ hybridization, detected mRNA for IGFBP4 in all healthy follicles > 8 mm collected from ovaries obtained at the slaughterhouse, but IGFBP2 mRNA was only found in 40% of the follicles. Possibly, gene expression for IGFBP2 is at a different level in preovulatory follicles at a specific stage of development (our study) than in general in healthy follicles > 8 mm (Armstrong et al., 1998).

Non (IGFBP3) and occasional differences (IGFBP2, -4, -5) in gene expression of follicles with normal or deviant follicular function were found. IGFBP2 and IGFBP5 mRNA levels were slightly higher in normal than in deviant follicles, and the presence of IGFBP4 mRNA was similar or lower in normal follicles. These differences in IGFBP gene expression can not explain the found differences in the presence of IGFBPs in the follicular fluids of eCG-stimulated preovulatory follicles with normal and deviant steroidogenesis (van de Leemput et al., 1997).

During growth and atresia of follicles of normally cyclic sheep (Monget et al., 1993) and pigs (Mondschein et al., 1991) the decrease and increase in follicular levels in LMW IGFBPs do coincide with changing levels of mRNA for these genes. In sheep, atresia of follicles is associated with an increase in mRNA for IGFBP2, -4 and -5 and growth with a decrease in IGFBP2 and -5 gene expression (Besnard et al., 1996b). Similar changes have been indicated in pigs (Samaras et al., 1993) and in rats (Erickson et al., 1992 a,b,). Therefore, it can be speculated that changes in LMW IGFBPs levels during atresia and growth of follicles during follicular waves in these species are regulated in a different manner than those in preovulatory follicles in the bovine. This is supported by the fact that the level of mRNA expression for IGFBPs in the preovulatory follicles was equal or higher than in small bovine follicles where variable levels of IGFBP2, -3, -4 and -5 are always found (Echternkamp et al., 1994; Funston et al., 1996,).

As for proteins in general, the presence of LMW IGFBPs will be regulated at the level of transcription, translation and exocytosis and degradation by proteolysis. The high levels of mRNA for LMW IGFBPs in both the preovulatory follicles with normal function, collected shortly before the LH surge and the follicles collected just before ovulation, in combination with the absence of LMW IGFBPs in the fluid of these follicles (van de Leemput et al., 1997) might indicate that presence of LMW IGFBPs during preovulatory follicular development is regulated at the level of translation. Also, in situ hybridization studies (cow: Armstrong et al., 1998; sheep: Bernard et al., 1996b) demonstrated that during growth and atresia of follicles individual changes in mRNA presence for LMW IGFBPs occur in granulosa and theca cells. In these two cell types, translation and exocytosis possibly are regulated in a different manner, resulting in a different contribution of the two cell types to the protein level in the follicular fluid. Finally, specific proteases for IGFBPs have been reported in human (Chandrasekher et al, 1995), pigs (Besnard et al., 1997) and sheep (Besnard et al., 1996a) indicating that also at the level of degradation regulation of IGFBPs may take place. In vitro studies in the rat (Fielder et al., 1993) and in the pig (Mondschein et al., 1990) indicate that specific proteolytic activity for IGFBPs can be induced by FSH. During stimulation with exogenous gonadotropins, FSH activity is constantly present at a high level and reduces pulsatile release of endogenous FSH (Bevers et al., 1989) and therefore the regulation of proteolytic activity for IGFBPs might be disturbed. It is possible that an eCG induced imbalance between protein production on one hand and protein degradation on the other hand is responsible for the appearance of LMW IGFBPs in deviant preovulatory follicles.

In conclusion, gene expression for IGFBP2, -3, -4 and -5 was always present in normal and deviant eCG-stimulated preovulatory follicles at the onset of final maturation. The quantitative differences in IGFBP gene expression between normal and deviant follicles can not explain the difference in IGFBP appearance in those follicles. This indicates that, the presence of IGFBPs during preovulatory follicular development is not primarily regulated at the level of gene expression, in contrast to follicles undergoing growth and atresia during follicular waves of the cycle.

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SUMMARIZING DISCUSSION

Assisted reproduction techniques, such as superovulation (SO) and in vitro embryo production (IVP) in combination with transvaginal ultrasound-guided ovum pick up (OPU) can, after subsequent embryo transfer, increase the number of offspring per cow per year since more than one oocyte per estrous cycle can be utilized for embryo production. However, due to losses in reproductive efficiency that can occur during each step involved in the production of a calf, today, only a minor part of the used oocytes (SO: 30% and IVP: 8%) will eventually result in an alive born calf. Deviations in oocyte maturation might contribute substantially to limiting the yield of viable embryos after SO and IVP.

Preovulatory follicular development in normally cyclic cows starts when, during the estrous phase of the cycle, one follicle (± 6-8 mm) is selected from a pool of recruited follicles to become dominant. This follicle continues to grow and finally ovulates 24 h after the occurrence of the preovulatory LH surge.

During SO, firstly the period of preovulatory development is reduced from 60 h to 40 h in comparison to unstimulated cows (Bevers et al., 1987; Dieleman et al., 1986) and secondly, the pattern of pulsatile release of FSH and LH before the occurrence of the LH surge is altered (Bevers et al., 1989). These changes coincide with a heterogeneity in development of the stimulated follicle population (Vos et al., 1994a) and an asynchrony in development of the follicle and its oocyte (de Loos et al., 1991; Hyttel et al., 1991; Dieleman and Bevers, 1993).

During IVP, usually immature oocytes from 3-6 mm-sized follicles are directly subjected to in vitro maturation (IVM). Thus, compared to the situation in vivo, these immature oocytes lack the part of preovulatory follicular development that occurs before the preovulatory LH surge. Furthermore, conditions during IVM are poor and static compared to the wide array of hormones and factors that in changing concentrations are involved in in vivo maturation.

In this thesis concepts of maturation in unstimulated, normally cyclic cows are applied to maturation during SO (chapter 2) and IVP (chapter 3 and 4) in order to gain better understanding of deviations in follicular and oocyte development that occur using these techniques. Also, preovulatory follicular development after SO is used as a model to study maturation in vivo (chapter 5 and 6).

FINAL MATURATION DURING SUPEROVULATION

Effect of prolongation of the period of preovulatory follicular development on the heterogeneity in functionality of the stimulated follicles

Upon superovulation, not all follicles mature synchronously. Therefore, at the time of the preovulatory LH surge, which after treatment for SO is induced at a relatively early time by a group of advanced follicles, possibly not all follicles are prepared to start final maturation yet. Prolongation of the period of preovulatory follicular development to a time that is similar to that in normally cyclic cows might reduce the heterogeneity in functionality of follicles and give more follicles enough time to acquire all features necessary to respond to the LH signal.

Prolongation of preovulatory development can be obtained by temporary postponement of the LH surge. In cows treated for SO with eCG, it is possible to control the occurrence of the preovulatory LH surge using a progestagen block to suppress the endogenous LH surge followed by administration of GnRH to subsequently induce an LH surge (Vos et al., 1994b). The norgestomet ear implant was in 100% of the cases effective in preventing the occurrence of the spontaneous LH surge and subsequent administration of GnRH always resulted in a LH surge within 2-3 h (chapter 2). The period of preovulatory development in the norgestomet/GnRH treated animals was extended with 6-14 h compared to the control animals.

Extension of the period of preovulatory follicular development clearly increased the number of mature preovulatory follicles (experiment A) and also the number of ovulations (experiment B and C). Oocytes collected from the preovulatory follicles (experiment A) showed similar in vitro developmental potential when collected from norgestomet/GnRH or control heifers and also in vivo fertilization rates of the ovulated oocytes (experiment B) were similar for the two groups. These data suggest that heterogeneity of the follicular population was unchanged after prolonged preovulatory follicular development. It cannot be excluded, however, that reduction of heterogeneity of the preovulatory-sized follicular population present at the time at which the spontaneous LH surge would have occurred was a major factor contributing to the improved superovulatory response, and that the increased number of preovulatory-sized follicles observed at ovulation was merely a consequence of more follicles getting stimulated additionally due to extension of the stimulation period. These additional follicles would then contribute the part of the follicular population that is not mature.

Despite the increase in ovulation rate, the yield of embryos at day 7 after AI was similar in heifers with induced and spontaneous LH surges (experiment C). Presumably, early embryonic development was affected by the treatment to extend the period of preovulatory follicular development since the in vitro developmental

potential (experiment A) and in vivo fertilization rate (experiment B) of oocytes from heifers with induced or spontaneous LH surges were similar. This presumption was supported by the recovery of a higher number of stereomicroscopically 'indefinable' embryonic structures from the oviduct after an induced LH surge than after a spontaneous LH surge, which most likely represent fertilized oocytes that prematurely stopped embryonic development (experiment C).

Amongst the wide variety of factors that influence embryo development, products of the oviduct such as oviduct-specific high molecular weight glycoproteins, that have been demonstrated to associate with the developing embryo (sheep: Gandolfi et al., 1991; cattle: Wegner and Killian, 1991; baboon: Boice et al., 1990b), are assumed to play a role in supporting early embryonic development in vivo. In our study, an impaired function of the epithelial cells of the distal ampulla region of the oviduct, that normally produce glycoproteins and other secretory products, is suggested in cows with postponed induced LH surges as indicated by a decreased abundance of PAS positive secretory granules in these cells (experiment B). A lack of oviductal secretory products might have been responsible for the premature arrest of embryonic development.

During the estrous cycle, the secretion of oviductal fluid is supposed to be regulated by hormonal changes in the peripheral blood (bovine: Killian et al., 1989; sheep: Willemse et al., 1975). Although not in the sheep (Gandolfi et al., 1989), in the bovine also the composition of the oviductal fluid is suggested to be regulated by hormonal changes during the estrous cycle (Boice et al., 1990a). In the norgestomet/GnRH treated heifers, the progesterone activity of norgestomet was almost continuously present when high concentrations of estradiol-17ß were produced by the growing follicles whereas in the control heifers the progesterone concentrations were low at that time. Although these differences of the patterns of steroid concentrations were not accompanied by changes in the distribution of progesterone and estradiol-17ß receptors in the epithelium of the oviduct (experiment B), the prolonged norgestomet treatment might have been responsible for the observed reduction in secretory granules of the epithelium of the oviduct.

Whether impaired oviductal function is the only explanation for the disappointing embryo yields remains unclear. It has to be mentioned that also the yield of embryos in the control animals was noticeably reduced (experiment C) compared to previously reported results for the eCG/PG/anti-eCG procedure (Dieleman et al., 1989; Bevers et al., 1993). It is generally accepted that embryo yield after SO can vary between cows (Armstrong, 1993). To ensure SO responses to be as optimal as possible, all heifers in this study were tested for normal cyclicity preceding SO and SO was performed in the absence of a dominant follicle. Nevertheless, repetition of experiment C with cows that have been proven to be 'good' responders to SO, might give better insight in the fate of the fertilized zygotes in cows with postponed induced LH surges.

It was concluded that with the use of the norgestomet/GnRH protocol, LH surges can be exactly timed in and synchronized between eCG-treated cows. Prolongation of

the period of preovulatory follicular development clearly increased the number of mature preovulatory follicles and also the number of ovulations. However, whether the heterogeneity in functionality of the preovulatory follicles was reduced is unclear. Maturation and fertilization were not adversely effected by the treatment but early embryonic development appeared to be impaired which might be due to decreased functionality of the cells of the epithelium of the oviduct.

It is clear that the treatment procedure can be used to collect follicles and oocytes at fixed stages in vivo to study the impact and mechanisms of specific steps of development on potential growth into viable embryos (Hendriksen et al., 1998; van de Leemput et al., 1998). Furthermore, although embryo yields were not improved after postponement of the preovulatory LH surge, SO with controlled LH surge might still be able to increase the efficiency of SO procedures; the exact timing of the LH surge facilitates the administration of anti-eCG and also makes estrous detection before AI superfluous. However, studies are needed to investigate pregnancy rates after such applications of the protocol.

IN VITRO MATURATION

The contribution of IVM to the limited yield of embryos after IVP

The production of transferable embryos in vitro comprises three different processes; in vitro maturation (IVM), IVF and IVC. In general, during IVP, immature oocytes collected from 3-6 mm sized follicles are subjected to IVM. The lack of part of the preovulatory follicular development and the artificial conditions during IVM do not seem to effect nuclear maturation of the oocytes, but evidence is growing that cytoplasmic maturation is disturbed under these conditions (Hyttel et al., 1986; Izadyar et al., 1998). It can be speculated that impaired maturation of the oocyte will reduce its ability to be fertilized and to develop into an embryo during IVC and therefore will negatively affect embryo yield after IVP.

That IVM of matured oocytes is indeed a limiting step in embryo yield after IVP is demonstrated in the study described in chapter 3. When in vivo matured oocytes collected from eCG stimulated heifers were used for IVF and IVC instead of in vitro matured oocytes collected from 2-8 mm sized follicles, (hatched) blastocyst formation increased with 100% (chapter 3). During the study, in vivo and in vitro maturation were synchronized to allow simultaneous performance of IVF and IVC for both groups of oocytes. Therefore, experimentally induced artifacts (Marquant-Leguienne and Humblot, 1998) during IVP can not have influenced blastocyst rates. Our data confirm previous speculations on reduced blastocyst formation after IVM. Leibfried-Rutledge et al. (1987) found reduced in vitro fertilization rates after IVM but never succeeded in production of embryos of in vitro matured and fertilized oocytes. Greve et al. (1987) transferred 20 and 6 in vitro produced embryos generated from in vitro

and in vivo matured oocytes, respectively. These transfers resulted in 0 (IVM) and 2 (in vivo maturation) pregnancies.

Effect of maturation conditions on limited embryo yield after IVP

It is unclear whether the conditions during IVM itself or the use of immature oocytes from 2-8 mm-sized follicles are responsible for the found difference in (hatched) blastocyst formation in chapter 3. Therefore, in chapter 4, the in vitro developmental potential of oocytes with identical startcompetence, i.e. oocytes from preovulatory follicles (> 8 mm) of cows treated with eCG, was evaluated after either IVM or in vivo maturation. The significantly higher (hatched) blastocyst formation rate after in vivo maturation clearly demonstrated that conditions during IVM are not optimal yet and can still be improved. Many attempts have been made to improve in vitro maturation conditions (review, Bevers et al., 1997). Since maturation in vivo is a very complex process with the involvement of many hormones, factors and regulatory mechanisms that, in addition, change during the progress of maturation it is very difficult to mimic this situation in vitro. Another main difficulty in the establishment of optimal IVM and also IVP conditions is the metabolic flexibility of the embryo to blastocyse and to hatch even under suboptimal conditions; development into a (hatched) blastocyst does not guarantee the birth of a calf when transferred into a cow, since the quality of the (hatched) blastocyst might be seriously influenced by suboptimal culture conditions. Many different methods are used to judge embryo quality (Lindner and Wright, 1983; Gardner and Leese, 1986; van Soom and de Kruif, 1992; Greve et al., 1993; Hasler et al., 1995; Massip et al., 1995; Overström, 1996; van Soom et al., 1997) but, to date, no reliable method is available other than calving outcome after transfer of the embryo.

Effect of startcompetence of the oocyte on limited embryo yield after IVP

Besides culture conditions, the startcompetence of the used oocytes for IVM can not be neglected as an important factor in successful maturation and further development. In our studies (chapter 3 and 4), oocytes were collected from eCG treated animals. Yield of in vivo produced embryos after eCG treatment varies considerably between cows (Armstrong, 1993) due to, among others, deviations that occur in follicular development (de Loos et al., 1991; Hyttel et al., 1991; Dieleman and Bevers, 1993; Vos et al., 1994a). This eCG-induced variation in follicle functionality is clearly reflected in embryo yield after IVP as demonstrated by the large variation in (hatched) blastocyst formation between oocytes that were treated similarly but were obtained from different eCG-treated cows (chapter 3). In addition, exclusion of oocytes derived from eCG-treated cows with extreme follicular dysfunction on the basis of estradiol-

17ß and progesterone concentrations in the follicular fluid increased the average (hatched) blastocyst formation rate with about 10% (Figure 2, chapter 4). Likewise, a positive correlation was found between the mean of the estradiol-17ß concentration of the fluid of the preovulatory follicles of a cow and the percentage of the oocytes collected from these follicles that develop into hatched blastocysts during IVC after IVF (chapter 6, van de Leemput et al., 1998).

The startcompetence of immature oocytes is among others dependent of the status and the size of the follicle of origin. Larger follicles (> 6 mm) provide COCs that are much more competent to develop into transferable embryos after IVF and IVC (Lonergan et al., 1994) or after cloning (Barnes et al., 1993). One study, using culture of individual oocytes, confirms this increased competence based on exact follicular sizes (Blondin and Sirard, 1995).

Since the increase in developmental competence of COCs from larger follicles is not due to a better nuclear maturation (Ectors et al., 1995) it is probably due to a better cytoplasmic maturation. Hyttel et al. (1997) and Assey et al. (1994) describe that morphologic changes in the cytoplasm of the oocyte of the dominant follicle, so-called 'prematuration', already take place before the occurrence of the LH surge. Interestingly, also oocytes from subordinate follicles, that are impending to undergo atresia, undergo changes in the cytoplasm that resemble the changes that are occurring during prematuration in the dominant follicle ('pseudomaturation', Assey et al., 1994). This finding might explain why oocytes from larger follicles, even if they show light signs of atresia (Hawk and Wall, 1994; Blondin and Sirard, 1995; Hazeleger et al., 1995), are more successful than oocytes from smaller follicles during IVM.

Above mentioned studies provide further evidence for the need of complete preovulatory development in order to achieve competent oocytes. If COCs from small follicles are used for IVM, one has to consider that these COCs lack any form of prematuration. Barnes et al. (1993) suggest establishing prematuration media for oocytes from these follicles, that prevent the oocyte from resumption of nuclear maturation and promotes cytoplasmic maturation. Inhibition of meiotic resumption, can be obtained by culture of COCs in the presence of follicular components, such as, follicular hemisections (Richard and Sirard, 1996a) thecal cells (Richard and Sirard, 1996b, van Tol and Bevers, 1998), thecal cell conditioned medium (van Tol and Bevers, 1998) or granulosa and thecal cells (Kotsuji et al., 1994).

Effect of oocyte maturation on the progress of embryonic development during IVC

The progress of embryo development during IVC after IVF of in vivo matured oocytes was slower when compared to that of in vitro matured oocytes recovered from 2-8 mm follicles (chapter 3). A similar timing of development for in vivo matured oocytes was found in the second experiment (chapter 4), and interestingly, in this study the in vitro matured oocytes, this time recovered from preovulatory follicles (> 8

mm) at the time of the occurrence of the LH surge, developed synchronously with the in vivo matured oocytes (chapter 4). In general, embryo formation in vivo is slower than under in vitro conditions. In vivo, the first cleavages proceed rather fast (8-16 cell within 72 h after insemination, Massip et al., 1983; van Soom and de Kruif, 1992) but subsequent blastocyst formation starts only at the end of the sixth or towards the completion of the seventh cell cycle. During in vitro development of oocytes collected from small and medium sized follicles the first cleavages proceed on average slower than in vivo with peak concentrations of 2, 4, 8 and 16 cell stage embryos are at 36, 42, 60 and 102 h after fertilization (van Soom et al., 1992). On the contrary, blastocyst formation in these embryos starts already during the sixth cell cycle. Unlike the timing of the first cleavages, that are suggested to be regulated by inherited components of the oocyte (van Soom et al., 1992; Yadav et al., 1993; Grisart et al., 1994), timing of blastocyst formation is supposed to be mainly influenced by composition of the media during IVC (van Soom et al., 1997). However, our data suggest that timing of blastocyst formation is certainly also a function of the oocyte, and that this is programmed during the period of preovulatory development that is usually surpassed in oocytes collected for IVM from 2-8 mm sized follicles.

IN VIVO PREOVULATORY FOLLICULAR DEVELOPMENT

The use of preovulatory follicular development after eCG-treatment for SO as a model to study the role of the IGF system in final maturation

Concentrations of steroid hormones in the fluid of an eCG-stimulated preovulatory follicle can be an indicative parameter for the developmental potential of its oocyte (chapter 4, van de Leemput et al., 1998). Follicular function of eCG-stimulated follicles may be considered normal if follicular steroid concentrations are similar to those in follicles of normally cyclic cows at that stage of development (Dieleman et al., 1983a, b); if steroid concentrations are outside the normal range, follicular function can be considered deviant. In this way, SO can be used as a model to study the role certain factors in preovulatory follicular development; differences in the presence of such a factor in the follicular fluid of normal and deviant follicles can be used to speculate on their function (chapter 5 and 6).

The insulin-like growth factor (IGF) system comprises 2 factors (IGF-I and IGF-II), 6 different binding proteins (IGFBPs) and 3 different receptors (IGF-I, IGF-II and insulin receptor) (Sara and Hall, 1990; Rotwein, 1991). In general, during growth and atresia of follicles during follicular waves of the estrous cycle (Echternkamp et al., 1994; Funston et al., 1996) and during preovulatory follicular development (Funston et al., 1996) levels of IGF-I and IGFBP3 in the follicular fluid are rather static. With

respect to the low molecular weight (LMW) IGFBPs, i.e. IGFBP2, -4 and -5, growth and atresia of follicles coincide with a respective decrease and increase in follicular levels of these proteins (Echternkamp et al., 1994; Funston et al., 1996). LMW IGFBPs are absent in dominant follicles with high concentrations of estradiol-17ß in their fluid, but as soon as these follicles show signs of atresia, on the basis of decreased follicular estradiol-17ß concentrations, LMW IGFBPs are found again in the follicular fluid (Echternkamp et al., 1994; Funston et al., 1996). During final follicular maturation no LMW IGFBPs are found in the fluids of normally cyclic cows (Funston et al., 1996).

Speculations on the role of IGF-I in follicular development on the basis of differences in the presence of IGF-I and IGFBPs in fluids of follicles with normal and deviant function

At the onset of final maturation, IGF-I and IGFBP3 were present at similar levels in the fluids of follicles with normal and deviant follicular function (chapter 5). In 35% of the fluids of deviant follicles collected at 12 h before the preovulatory LH surge LMW IGFBPs could been demonstrated (chapter 5). The presence of these LMW IGFBPs is neither found in the fluid of their normal developing counterparts (chapter 5) nor in follicles at this stage of development of normally cyclic cows (Echternkamp et al., 1994; Funston et al., 1996) and could, in homology with other species, indicate a decreased bioavailability of IGF-I in these follicles (rat: Bicsak et al., 1990; Adashi et al., 1992; sheep: Monget et al., 1993; pig: Grimes et al., 1994). Considering that most of the deviant follicles collected at this time were qualified deviant due to too low follicular estradiol-17ß concentrations our data would support earlier hypotheses on the role of IGF-I in preovulatory follicular development before the LH surge. IGF-I would play an important role in the acquirement of follicular dominance by increasing the sensitivity of the dominant follicle to FSH (Driancourt, 1991) and serve an autocrine mechanism by which aromatase activity of granulosa cells during preovulatory follicular development might be stimulated (rat: Adashi et al., 1985; pig: Hsu and Hammond, 1987). Based on this hypothesis, deviant follicles with decreased bioavailability of IGF-I might be less sensitive to FSH and therefore suffer decreased induction of aromatase activity and estradiol-17ß synthesis.

It has to be considered that the follicles in our study were collected from eCG-treated animals in which FSH-like activity in plasma was maintained high until the occurrence of the LH surge. Most likely deviant follicles would have become atretic under normal circumstances but are now ‘rescued’ from atresia by the high levels of eCG. In previous experiments is has been demonstrated that neutralization of eCG just before the LH surge decreased the ovulation rate enormously (Vos et al., 1994a). This indicates that indeed part of the preovulatory follicles after eCG treatment can only continue growth and development due to the constantly high levels of eCG. Possibly

these follicles will never acquire all features of healthy dominant follicles. Therefore, it is not surprising that this part of the follicle population will not shed a competent oocyte.

It would be interesting to know the origin of the LMW IGFBPs in the fluid of the deviant follicles. If they are remnants of the LWM IGFBPs that gradually disappear from follicular fluid during growth of follicles, this would support the hypothesis that these follicles never acquired follicular dominance and that they were 'rescued' from atresia by the exogenous gonadotropins. On the other hand, induction of LMW IGFBPs after that the follicular fluid was cleared from these proteins would indicate that the follicles did undergo normal preovulatory follicular development. Follicular atresia, in the bovine associated with an increase of LMW IGFBPs (Echternkamp et al., 1994), a decrease in aromatase activity (McNatty et al., 1984) and estradiol-17ß concentrations (Kruip and Dieleman, 1982; McNatty et al., 1984) would then have been induced after follicular dominance was acquired.

After the occurrence of the LH surge, at the time that the oocyte undergoes germinal vesicle breakdown and develops to metaphase I, the presence of IGF-I and its binding proteins was rather similar in both normal and deviant developing follicles (chapter 5). This might suggest that the IGF system does not play an important role in final maturation of follicle. However, in vitro, IGF-I has been shown to stimulate cumulus cell expansion and nuclear maturation of the oocyte (Izadyar et al., 1997) and the presence of IGF-I in combination with FSH during oocyte maturation increased blastocyst formation after IVF and IVC (Harper and Brackett, 1992). Perhaps instead of a clear regulatory role, IGF-I has a more permissive function in final maturation. Further studies are needed to prove this.

Interestingly, just before ovulation (22 h after the occurrence of the LH surge) LWM IGFBPs reappear in the follicular fluid of normal follicles and remain absent in the fluids of deviant follicles. Possibly, IGF-I or LMW IGFBPs by themselves or in collaboration with each other have a different function in ovulation or corpus luteum formation than during preovulatory development.

The difference in presence of LWM IGFBPs during preovulatory follicular development between normal and deviant follicles can not be explained by a difference in gene expression for these IGFBPs (chapter 6) and thus regulation has to take place at the level of translation or protein degradation. The existence of specific proteases for IGFBPs have been reported in human (Chandrasekher et al., 1995), pigs (Besnard et al., 1997) and sheep (Besnard et al., 1996). In vitro studies in the rat (Fielder et al., 1993) and in the pig (Mondschein et al., 1990) indicate that specific proteolytic activity for IGFBPs can be induced with FSH. Interestingly, during growth and atresia of follicles in the bovine, granulosa- and theca cell specific changes in mRNA presence occur (Armstrong et al., 1998). In these two cell types, translation and exocytosis possibly are regulated in a different manner, resulting in a different contribution of the two cell types to the protein level in the follicular fluid. An similar mechanism might also exist during preovulatory follicular development.

Role of other growth factors in preovulatory follicular development

Although not analyzed in our study, important roles of other growth factors in preovulatory follicular development can not be neglected. Growth factors such as EGF, TGFα and bFGF have been demonstrated to stimulate granulosa cell proliferation and inhibit granulosa cell differentiation in vitro in the bovine (EGF: Gospodarowicz and Birdwell 1977; Franchimont et al., 1986; FGF: Gospodarowicz and Birdwell 1977; TGFα: Skinner and Coffey, 1988; bFGF: Vernon and Spicer, 1994) suggesting that they might act in vivo by enhancing growth and delaying terminal maturation of follicles. On the contrary, EGF and TGF" have been shown to induce cumulus expansion and promote nuclear maturation of the bovine oocyte (EGF: Coskun et al., 1991; Harper and Brackett, 1993; Kobayashi et al., 1994; Lorenzo et al., 1994; TGFα: Lorenzo et al., 1994).

Conflicting data from in vivo and in vitro studies on the function of growth factors address an important point in the study to the role of growth factors in follicular development. Growth factors are ubiquitously present in cells and organs and belong to complex systems made up by the factors themselves, their receptors and binding proteins. They are often structurally and functionally related (Monget and Monniaux, 1995) and can bind to the same receptors (Pratt and Pastan, 1978; Adashi et al., 1987). Present day knowledge of all the elements of these systems is far from complete. Therefore, although in vitro studies are valuable, the balance between the different systems might be completely disturbed under culture conditions and influence the obtained results. The mechanism of action of IGF-I and other growth factors is, to date, mainly based on in vitro studies on different species and therefore has to be considered mostly hypothetical with respect to extrapolation to the in vivo situation in the cow. In vivo studies on not only the function of growth factors but also on functions of individual IGFBPs and on the interactions of the two components are necessary. The increasing availability of components such as recombinant human IGFBPs (Spicer et al., 1997) and IGF-I analogues (Adashi et al., 1992) in combination with intrafollicular sampling (Ginther et al., 1997) and injection techniques that do not interfere with follicular development can be very useful in further investigations.

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SUMMARY

The use of assisted reproduction techniques can generate up to 27 (superovulation, SO) or 50 (in vitro embryo production, IVP) calves per cow per year instead of only one calf per cow per year after normal mating. That is due to the possibility to use more than one oocyte per estrous cycle; namely on average 15 (SO) and 60 (IVP). However, using these techniques, the efficiency per used oocyte decreases dramatically. Instead of the efficient use of oocytes during natural mating, only 30% efficiency is reached with SO. Furthermore, IVP generates only 8 calves per 100 used immature oocytes. This loss in efficiency might be due to deviations that occur during the final maturation of the oocytes.

In this thesis concepts of final follicular maturation in unstimulated normally cyclic cows are applied to maturation during SO and IVP in order to gain better understanding of deviations in follicle and oocyte development using these techniques. Also preovulatory follicular development after SO is used as a model to study maturation in vivo.

Chapter 1 introduces the subject. Deviations in preovulatory follicular development as induced using SO or as a result of oocyte collection and maturation during IVP are discussed against the background of the course of follicular maturation in unstimulated normally cyclic cows. In cows treated for SO the period of preovulatory follicular development is reduced compared to this period in unstimulated normally cyclic cows. Also, after SO an asynchrony of development is found within the population of stimulated follicles as well as between the follicle and its oocyte. For IVP, oocytes are usually collected from 3-6 mm follicles. These follicles lack part of the processes of follicular growth and selection. Furthermore, during in vitro maturation (IVM) of the collected immature oocytes, deviations in cytoplasmic maturation are frequently found.

In chapter 2, the effect of prolongation of the period of preovulatory follicular development after SO on the heterogeneity of the population of preovulatory follicles and their oocytes with respect to the potential to mature, to ovulate, to be fertilized and to develop into embryos was investigated. In eCG-stimulated heifers, the spontaneous occurrence of the LH surge was suppressed with a norgestomet ear implant and at a later time a LH surge was induced using GnRH. The protocol resulted in a LH surge at the desired time in 100% of the cases. Prolongation of the period of preovulatory follicular development from 42.4 to 53.8 h increased ovulation rates with 25%. It was suggested that the heterogeneity of the follicular population, as is present in normally stimulated heifers at the time of the spontaneous LH surge, was reduced. The increased ovulation rates did not coincide with an increased number of embryos at day 7 after fertilization. The treatment with norgestomet did not adversely affect

final maturation and fertilization. However, the treatment might have disturbed early embryonic development by altering the secretory activity of the cells of the epithelium of the oviduct.

The superovulation protocol with LH surge induction (chapter 2) was used in the studies described in chapter 3 and 4 to obtain oocytes at a fixed stage of development in vivo. In chapter 3, it was investigated to what extend IVM contributes to limiting yields of viable embryos in currently used IVP programs. The use of in vivo matured oocytes, collected from eCG-stimulated heifers at 22-24 h after the LH surge, instead of in vitro matured oocytes collected from 2-8 mm sized follicles, for IVF and IVC improved blastocyst formation and hatching with 100%. Additionally, also the progress of embryonic development was different for the two groups of oocytes; both blastocyst formation and hatching of blastocysts progressed slower for in vivo matured oocytes than for in vitro matured oocytes.

The decreased embryonic development after IVM (chapter 3) might be due to the maturation conditions per se or to a difference in startcompetence of the oocytes collected from 2-8 mm sized follicles when compared to the oocytes from preovulatory follicles generated in eCG-treated cows. In chapter 4 the significance of the conditions during maturation for efficient IVP was tested using oocytes for in vivo maturation and IVM which presumably had an equivalent startcompetence. Therefore, heifers were stimulated with eCG using the same protocol as described in chapter 2. From part of the heifers, oocytes from preovulatory follicles were collected at the presumptive time of the LH surge and subsequently subjected to IVM. From the other heifers, oocytes from preovulatory follicles were collected 22-24 h after the occurrence of an induced LH surge. Both groups of oocytes were subjected to IVF and IVC. Blastocyst formation and hatching rates were significantly lower after in vitro maturation than after in vivo maturation of the oocytes. It was concluded that the conditions during IVM are an important factor responsible for limited yield after IVP. In this study, the progress of embryonic development was similar for both groups of oocytes and was conform to that as observed for the in vivo matured oocytes in chapter 3. Since in that study in vitro matured oocytes from 2-8 mm follicles developed at a faster rate than in vivo matured oocytes from preovulatory follicles it was suggested that oocytes undergo certain changes during follicular development from 2-8 mm to preovulatory stage at onset of final maturation which may be involved in programming embryonic development.

Both in the studies described in chapter 3 and 4, a large variation in blastocyst formation between individual heifers was found, which is probably due to differences in response to superovulatory treatment. When in the group of heifers donating the in vitro matured oocytes (chapter 4) heifers with exceptional follicular function on the basis of estradiol-17ß and progesterone concentrations in the follicular fluid (FF) were excluded, rates of blastocyst formation increased indicating that the steroid producing capacity of the follicular wall influences oocyte quality.

In chapter 5 and 6 preovulatory follicular development after eCG-treatment for SO was used as a model to study the possible role of the insulin-like growth factor (IGF)

system during final maturation in vivo. Earlier results from in vitro studies suggested a stimulatory role of IGF-I on growth and differentiation of granulosa cells and on final maturation of the oocyte. IGF binding proteins (IGFBPs) are suggested to decrease bioavailability of IGF-I but also individual functions of these IGFBPs on follicle and oocyte maturation can not be excluded.

In chapter 5, levels of IGF-I and IGFBPs in the FF of eCG-stimulated follicles with 'normal' and 'deviant' follicular function on the basis of steroid hormones in the FF were compared at several times during final maturation. Final maturation in eCG-stimulated preovulatory follicles with 'normal' function was characterized by rather constant levels of IGF-I and IGFBP3 in the FF. Low molecular weight IGFBPs (LMW IGFBPs, i.e. IGFBP2, -4 and -5) were absent in FF at the time the oocyte undergoes germinal vesicle breakdown (GVBD) and reaches metaphase I (MI). Just prior to ovulation, in 15% of the FF of the follicles with 'normal' function, LMW IGFBPs were found. While the levels of IGF-I and IGFBP3 in the fluid of 'deviant' follicles were not different when compared to eCG-stimulated follicles with 'normal' function, the presence of LMW IGFBPs was different in these follicles; at the onset of final maturation, 35% of the 'deviant' follicles contained LMW IGFBPs. All these follicles, except one, were classified 'deviant' due to too low estradiol-17ß concentrations, probably a sign of atresia. During final maturation, LMW IGFBPs were occasionally found (GVBD, MI) or absent (just prior to ovulation) in 'deviant' follicles. It was concluded that only a permissive role of IGF-I in final maturation can be expected. It was also concluded that, as in unstimulated, normally cyclic cows, low estradiol-17ß concentration in the FF of large follicles (> 8mm) coincided with the presence of LMW IGFBPs. The appearance of LMW IGFBPs in the FF of follicles with 'normal' follicular function just prior to ovulation indicated a different role of the IGF system during ovulation and corpus luteum formation.

Because different IGFBP patterns were found in the FFs of eCG-stimulated follicles with 'deviant' follicular function and of concurrently developing follicles with 'normal' follicular function, eCG stimulated follicles were used to study the regulation of the presence of IGFBPs during preovulatory follicular development (Chapter 6). Using reverse transcriptase-polymerase chain reaction (RT-PCR), mRNA levels for LMW IGFBPs were analyzed (semi-quantitatively) in eCG-stimulated preovulatory follicles at the onset of maturation with 'normal' or 'deviant' function, on the basis of estradiol-17ß concentrations in their FF. No or small differences in gene expression were found. Therefore, it was concluded that the earlier found difference in the presence of LMW IGFBPs in FF could not be explained by a difference in gene expression for these IGFBPs in the wall of the follicle. Thus, regulation of the presence of these proteins has to take place at the level of translation or protein degradation.

Finally, in chapter 8, the results of the performed experiments are discussed against the background of the current knowledge and existing hypotheses on final follicular maturation. Possible directions of future research in order to improve the efficiency of assisted reproduction are discussed.

SAMENVATTING

Theoretisch kan het gebruik van moderne voortplantingstechnieken in plaats van één kalf per koe per jaar na natuurlijke dekking, wel 27 (superovulatie, SO) of 50 (in vitro embryoproduktie, IVP) kalveren per koe per jaar genereren. Dit is mogelijk doordat bij SO en IVP veel meer eicellen dan de ene eicel die normaal vrijkomt gedurende de oestrische cyclus van het rund, gebruikt worden voor embryoproduktie; namelijk gemiddeld 15 (SO) en 60 (IVP). Echter, de efficiëntie van embryoproduktie per gebuikte eicel daalt van bijna 100% bij natuurlijke dekking, tot 30% na SO en tot maar 8% bij IVP. Een belangrijke oorzaak hiervoor zou kunnen zijn dat t.g.v. een afwijkend maturatieproces van de ovariële follikel een minder goede eicel geproduceerd wordt.

In de studies beschreven in dit proefschrift worden bekende aspecten van follikelrijping tijdens de normale cyclus van het rund geimplanteerd in follikelrijping tijdens SO en IVP, met als doel meer te weten te komen over het belang van de afwijkingen in dit proces tijdens SO en IVP. Tevens wordt, gebruik makend van afwijkingen die door SO in de follikelrijping ontstaan, het proces van follikelrijping tijdens de normale cyclus bestudeerd.

Hoofdstuk 1 geeft achtergrondinformatie over het onderwerp. Afwijkingen in preovulatoire follikelontwikkeling zoals die geinduceerd worden tijdens SO of zoals die gevonden worden als een resultaat van de manier van eicellen verzamelen en matureren tijdens IVP, worden bediscussieerd in relatie tot het verloop van dit proces in normaal cyclische koeien. In koeien die behandeld zijn voor SO is de periode van preovulatoire follikelontwikkeling verkort ten opzichte van die in een normaal cyclische koe. Ook wordt er een asynchronie gevonden in de ontwikkeling van enerzijds de gestimuleerde preovulatoire follikels ten opzichte van elkaar en anderzijds de individuele follikel ten opzichte van zijn eicel. Voor IVP worden eicellen meestal verzameld van 3-6 mm grote follikels. Deze follikels hebben gedeeltes van de ontwikkeling tot preovulatoire follikel nog niet doorgemaakt. Bovendien worden tijdens in vitro maturatie (IVM) afwijkingen in de cytoplasmatische rijping van eicellen gezien.

In hoofdstuk 2 werd het effect van het verlengen van de periode van preovulatoire follikelontwikkeling na SO op de heterogeniteit van de preovulatoire follikels en hun eicellen getest, met betrekking tot het vermogen tot maturatie, ovulatie, fertilizatie en ontwikkeling tot embryo's. In eCG-gestimuleerde pinken werd het optreden van de spontane preovulatoire LH piek onderdrukt m.b.v. een norgestomet oorimplant. Op een later tijdstip werd vervolgens een LH piek geinduceerd m.b.v. GnRH. Dit protocol resulteerde in 100% van de gevallen in een LH piek op het gewenste tijdstip. Verlenging van de periode van preovulatoire follikelontwikkeling van 42.4 uur tot

53.8 uur verhoogde het aantal ovulaties met 25%. Waarschijnlijk was de heterogeniteit van de follikelpopulatie verkleind t.o.v. de heterogeniteit die gevonden wordt in normaal gesuperovuleerde dieren op het moment van optreden van de spontane LH piek. Het verhoogde aantal ovulaties resulteerde niet in een verhoogd aantal embryo's op 7 dagen na fertilizatie. De norgestomet behandeling had geen meetbare invloed op finale maturatie en fertilizatie, maar de behandeling zou wel het proces van vroeg embryonale ontwikkeling verstoord kunnen hebben als gevolg van een negatief effect van deze behandeling op de secretoire activiteit van de epitheelcellen in het oviduct.

Het superovulatie protocol met LH piek inductie (hoofdstuk 2) werd in hoofdstuk 3 en 4 toegepast om eicellen op een vast moment gedurende de ontwikkeling in vivo te verkrijgen. In hoofdstuk 3 werd onderzocht in hoeverre IVM bijdraagt aan de lage opbrengst aan embryo's na IVP. Het gebruik van in vivo gematureerde eicellen, verzameld op 22-24 h na de LH piek uit gesuperovuleerde pinken voor IVF en IVC (i.p.v. in vitro gematureerde eicellen afkomstig van 2-8 mm grootte follikels), verhoogde de blastocyst vorming en hatching met 100%. Opmerkelijk was dat ook de snelheid van embryonale ontwikkeling verschillend was voor de twee groepen eicellen; zowel blastocyst vorming als –hatching verliepen langzamer voor in vivo- dan voor in vitro gematureerde eicellen.

De verminderde produktie van embryo's na IVM (hoofdstuk 3) kan veroorzaakt worden door de maturatie condities enerzijds of door een verschil in startcompetentie van de eicellen, verzameld van 2-8 mm follikels, t.o.v. die verzameld uit preovulatoire follikels (> 8 mm) van met eCG behandelde pinken anderzijds. In hoofdstuk 4 werd het belang van de condities gedurende het maturatie proces voor efficiente IVP getest, door voor zowel IVM als voor in vivo maturatie eicellen met eenzelfde startcompetentie te gebruiken. Daarom werden pinken behandeld met eCG volgens het protocol beschreven in hoofdstuk 2. Van een gedeelte van de pinken werden eicellen uit preovulatoire follikels verzameld vlak voor het verwachte optreden van de LH piek en vervolgens gematureerd in vitro. Van de andere pinken werden gematureerde eicellen verzameld uit preovulatoire follikels 22-24 uur na het optreden van de geinduceerde LH piek. Beide groepen eicellen werden vervolgens in vitro gefertilizeerd en gekweekt. Na IVM werden procentueel minder blastocysten gevormd en trad procentueel minder hatching op dan na in vivo maturatie van de eicellen. Er werd geconcludeerd dat de condities tijdens IVM een belangrijke factor zijn voor de gelimiteerde embryo-opbrengst na IVP. Opvallend was, dat de snelheid van embryo-ontwikkeling na zowel in vivo als in vitro maturatie in dit experiment gelijk was en overeen kwam met die van de in vivo gematureerde eicellen in hoofdstuk 3. Omdat in hoofdstuk 3 de in vitro gematureerde eicellen (afkomstig van 2-8 mm follikels), sneller ontwikkelden dan de in vivo gematureerde eicellen, werd gesuggereerd dat eicellen gedurende de fase van follikelontwikkeling van 2-8 mm tot aan het optreden van de LH piek, bepaalde veranderingen doormaken die een functie hebben in het programmeren van de eicel voor verdere embryonale ontwikkeling.

Zowel in hoofdstuk 3 als 4 werd een grote variatie in blastocystvorming

vastgesteld tussen individuele pinken. Waarschijnlijk werd deze variatie veroorzaakt door verschillen in respons op SO. Wanneer in de groep pinken waarvan eicellen voor IVM waren verzameld (hoofdstuk 4), de dieren met ‘afwijkende’ follikelfunctie op basis van oestradiol-17ß en progesteron concentraties in follikelvloeistof (FF) werden uitgesloten, nam de blastocystvorming toe. Er werd gesuggereerd dat de capaciteit van de follikel om steroiden te produceren de eicelkwaliteit beinvloedt.

In hoofdstuk 5 en 6 werd de preovulatoire follikelontwikkeling zoals die optreedt na behandeling met eCG om SO te induceren, gebruikt om de mogelijke rol van insuline-achtige groeifactoren (IGF’s) gedurende follikelmaturatie te bestuderen. Resultaten van in vitro studies suggereren een stimulerend effect van IGF-I op de groei en differentiatie van granulosa cellen en op de finale maturatie van de eicel. IGF bindende eiwitten (IGFBPs) verlagen mogelijk de biologische beschikbaarheid van IGF-I, maar daarnaast kunnen ook individuele functies van deze IGFBPs op follikel- en eicelrijping niet worden uitgesloten.

In hoofdstuk 5 werden niveau’s van IGF-I en IGFBPs vergeleken in FF van eCG gestimuleerde follikels, met een op steroidproduktie gebaseerde ‘normale’ of ‘afwijkende’ follikelfunctie, op verschillende tijdstippen tijdens de maturatie. Finale maturatie in eCG-gestimuleerde follikels met een ‘normale’ functie werd gekarateriseerd door een vrijwel constant niveau van IGF-I en IGFBP3 in de FF. IGFBPs met een laag molecuul gewicht (LMW IGFBPs: IGFBP2, -4 en -5) werden niet aangetroffen op het tijdstip dat de eicel ‘germinal vesicle breakdown’ (GVBD) ondergaat en ook niet op het moment dat de kern van de eicel zich in metafase I bevindt (MI). Vlak voor ovulatie werd in 15% van de follikels met ‘normale’ follikelfunctie LMW IGFBPs in de FF aangetoond. In de FF van follikels met ‘afwijkende’ functie werden, in vergelijking met follikels met ‘normale’ functie, gelijke concentraties van IGF-I en IGFBP3 aangetroffen, maar de aanwezigheid van LMW IGFBPs was verschillend voor de twee groepen follikels; 35% van de follikels verzameld aan het begin van de maturatie bevatte LMW IGFBPs. Op één follikel na, waren al deze follikels als ‘afwijkend’ geclassificeerd op grond van een te lage oestradiol-17ß concentratie in de FF, hoogst waarschijnlijk een teken van atresie. Tijdens het proces van maturatie werden slechts af en toe (GVBD, MI) of geen (MII) LMW IGFBPs aangetroffen in de FF van de ‘afwijkende’ follikels. Er werd geconcludeerd dat IGF-I hooguit een permissieve rol speelt in het proces van finale maturatie. Tevens werd geconcludeerd dat, zoals in normaal cyclische koeien, lage oestradiol-17ß concentraties in de FF van grote follikels (> 8mm), samengaan met de aanwezigheid van LMW IGFBPs. Het voorkomen van LMW IGFBPs in FF van follikels met ‘normale’ functie, vlak voor ovulatie, duidt op een andere rol voor het IGF systeem gedurende ovulatie en corpus luteum vorming dan gedurende het proces van finale maturatie.

Omdat verschillende IGFBP patronen werden gevonden in de FF van eCG-gestimuleerde follikels met ‘normale’ en ‘afwijkende’ follikelfunctie, werden in hoofdstuk 6 eCG-gestimuleerde follikels gebruikt om de regulatie van de aanwezigheid van deze IGFBPs in FF gedurende preovulatoire follikelontwikkeling te

bestuderen. Gebruik makend van ‘reverse transcriptase-polymerase chain reaction’ (RT-PCR) werden op semiquantitatieve wijze mRNA niveau’s voor IGFBPs vergeleken in follikelwanden van follikels met een ‘normale’ en een ‘afwijkende’ follikelfunctie op basis van oestradiol-17ß concentraties in de FF, aan het begin van het maturatie proces. Er werden geen of slechts minimale verschillen in genexpressie voor de IGFBPs gevonden. Daarom werd geconcludeerd dat het eerder gevonden verschil in aanwezigheid van IGFBPs in de FF van de twee categorien follikels niet verklaard kon worden door een verschil in genexpressie voor deze IGFBPs. De aanwezigheid van IGFBPs in FF op dit tijdstip wordt dus waarschijnlijk door middel van translatie of eiwitdegradatie bepaald.

In hoofdstuk 8 tenslotte, worden de resultaten van de uitgevoerde experimenten bediscussierd tegen de achtergrond van de huidige kennis en opvattingen omtrent final maturatie. Tevens wordt gespeculeerd over mogelijke richtingen van verder onderzoek met als doel de efficientie van geassisteerde voortplanting te verhogen.

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