Lehrstuhl für Physiologie
Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt
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Intercellular communication during in vitro maturation of bovine cumulus oocyte complexes

Martin Fritz Schönfelder

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"Neue Wege zeigen erfordert erst,
daß die alten gekannt werden.
Diese alten Wege wenigstens soweit zu kennen,
daß man in neue Gebiete eindringen kann, erfordert Zeit."

Rhoda Erdmann (1870-1935)

Für meine Frau
und meine Eltern

Table of contents

Abbreviations

AC	adenylate cyclase
ACC	accession number of EMBL data base
ActR	activin receptor
AG	aminoguanidine hemisulfate salt
ALK	activin receptor-like kinase
App	appendix
ARO	aromatase P450
AT	annealing temperature
bFGF	basic fibroblast growth factor
bHABP	biotinylated hyaluronan binding protein
BMP-15	bone morphogenetic protein-15
BMPR	bone morphogenetic protein receptor
BSA	bovine serum albumin
cAMP	cyclic adenosine mono phosphate
Cc	cumulus cell
CD	clusters of differentiation
cDNA	copy desoxyribonucleic acid
COC	cumulus oocyte complex
Co-SMAD	common-mediator SMAD
CP	crossing point
Cr	corona radiata
Cx	connexin
DDT	dichloro-diphenyl-trichloroethane
DNA	desoxyribonucleic acid
dNTP	desoxi nucleiotide triphosphate
DTT	dithiothreitol
E2	estradiol-17β
eCG	equine chorionic gonadotropin
ECM	extra cellular matrix
ED	effective dose
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EIA	enzyme immuno assay
EMBL	european molcular biology laboratories
eNOS	endothelial nitric oxide synthase
ERK	extra cellular-regulated-kinase
EtOH	ethanol
FA	formaldehyde
FCS	fetal calf serum
Fig.	figure

flk	fetal liver kinase
flt	fms(feline sarcoma virus)-like tyrosin kinase
FSH	follicle stimulating hormone
GAG	glycosaminoglycan
GC	granulosa cell
GD-VEGF	glioma-derived vascular endothelial growth factor
GDF-9	growth and differentian factor-9
GMVB	germinal vesicle breakdown
H1	histone 1
HA	hyaluronan
HARE	hyaluronan associated receptor for endocytosis
HAS	hyaluronan synthase
hCG	human chorion gonadotropin
HGF	hepatocyte growth factor
HIS	histidine
HRP	horseradish peroxidase
HSD	3-beta-hydroxy-steroid-dehydrogenase
ICAM	intercellular adhesion molecule
ICSI	intracytoplasmic sperm injection
IGF-1	insulin like growth factor-1
IHABP	intracellular hyaluronan binding protein
IMAC	immobilized metal affinity chromatography
iNOS	inducible nitric oxide synthase
IPTG	isopropyl-beta-D-thiogalactopyranoside
IVC	in vitro culture
IVM	in vitro maturation
kD	kilo Dalton
KDR	kinase domain region
KGF	keratinocyte growth factor
KL	kid ligand
lCc	luteinizing cumulus cell
LH	lteotropic hormone
L-NAME	N-omega-nitro-L-arginine methyl ester
LYVE	lymph vessel endothelial hyaluronan receptor
MAD	mother against dpp
MAPK	mitogen activated protein kinases
MgCl2	magnesium chloride
MI/II	metaphase I/II
MMLV	Mouse Moloney murine leukemia virus
MOPS	3-[N-Morpholino] propanesulfonic acid
MPF	maturation promoting factor
MPM	modified Parker´s medium

mRNA	messenger ribonucleic acid
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
Oo	oocyte
P4	progesterone
PACE	paired basic amino acid residue cleaving enzyme
PBS	phosphate buffered saline
PCB	polychlorinated bisphenyls
PCDD	polychlorinated dibenzodioxine
PCR	polymerase chain reaction
PGE_2	prostaglandin E_2
QT	quantification temperature
RACE	rapid amplification of cDNA ends
rec	recombinant
RHAMM	receptor for hyaluronan mediated motility
RIA	radio immuno assay
RNA	ribonucleic acid
R-SMAD	receptor-activated SMAD
RT	reverse transcription
SAF	streptavidin fluorescein
SCC	side chain cleavage enzyme
SD	standard deviation
SDS	sodium dodecylsulfate
SMA	similar to MAD
SMAD	SMA and MAD related
SNAP	S-nitroso-L-acetyl penicillamine
SNP	sodium nitroprusside
Tab.	Table
TAO	translumination-aspiration of ovary
TBT	tributyltin
T	testosterone
TGF-beta	transforming growth factor-beta
TIC	theca interna cell
UDP	uridine diphosphate
VEGF	vascular endothelial growth factor
ZP	zona pellucida

1. Abstract

Although the in vitro maturation is an established technique for assisted reproduction, a lack of detailed information about intercellular communication between the oocyte and the surrounding somatic cells is still present. Additionally, differing results between various species demand their critical interpretation. Therefore, a lot of effort has been made to determine the important systems participating in this fundamental signalling process. It has been recognized that the intercellular communication plays a crucial role in the timing of the right events necessary to produce fertilizable oocytes and to coordinate embryo genesis.

Therefore, the aim of this study is to evaluate the communication systems during the artificial maturation of bovine cumulus oocyte complexes (COC) which represents the key to optimal development. Additionally, these results were compared to those from cultivated bovine granulosa cells (GC).

The investigations on the two related cell culture systems were done by using the sensitive real-time RT-PCR to detect minimal alterations in mRNA levels. Enzyme immuno assay (EIA) as well as radio immuno assay (RIA) were employed to measure specificly hormone and protein concentrations in the culture media. Additionally, to support mRNA data immunohistological techniques were used to detect protein localization in the tissue.

In the present study it could be shown that maturing COC posses the ability to produce steroid hormones independently of the natural follicular environment. Furthermore, it could be proven that the environmental pollutant tributyltin (TBT) is able to interfere with the steroidogenic systems of both the maturing COC and the cultured GC which underwent luteinization during cultivation. A distinct dose dependant effect of TBT on estradiol-17β as well as progesterone synthesis could be demonstrated.

A set of different connexins, important cellular channel proteins, could be detected by mRNA analysis showing dependency on the maturational status of the COC. In addition to this direct transmembrane communication by gap junctions, two isoforms of nitric oxide synthases (NOS), the inducible and the endothelial NOS, could be found which produce the small inorganic signal molecule nitric oxide (NO). Both enzymes were varied in COC and GC, respectively. A direct influence of the two gonadotropins FSH and LH on the mRNA levels of the NOS could be displayed in cultured GC.

As indicators for the active participation of the oocyte in the regulation of cellular behavior of the surrounding somatic cells, the presence of the mRNA of two different oocyte derived factors,

the growth and differentiation factor-9 (GDF-9) and the bone morphogenetic protein-15 (BMP-15), could be proven. Additionally, it was possible to sequence almost the whole mRNA of the bovine GDF-9, which was introduced into an E. coli expression system. Unfortunately the resulting recombinant protein of the mature growth factor did not show bioactivity as described in literature. However, with the existence of three potential GDF-9/BMP receptors (BMPR-1A, BMPR-1B and BMPR-2) in COC, GC and theca interna cells (TIC) respectively a functional follicular GDF-9/BMP-system can be assumed.

Furhtermore, the extra cellular matrix (ECM) was subject to dramatic alterations during maturation. It could be demonstrated by histochemistry that the gel-like matrix surrounding the oocyte consists of hyaluronan (HA) which was possibly the product of the highly expressed HA synthase 2 (HAS2). The importance of the ECM could be proved by the coexistence of the two HA receptors CD44 and RHAMM (receptor for HA-mediated motility). Protecting the oocyte from mechanical forces, the HA rich matrix could also serve as a reservoir for diverse growth factors, able to bind to the ECM. One such member is the vascular endothelial growth factor (VEGF), which could be detected enriched in the ooplasm, in the Zona pellucida as well as in the follicular fluid. Its supplementation to the maturation medium significantly influenced the subsequent development of bovine embryos.

In summary, a very complex system of diverse cellular communication systems could be detected, pointing out that all these systems would possibly interfere with each other which indicates their importance for the optimal development of the oocyte. Furthermore, a distinct local steroidogenic system could possibly proof suitable screening for endocrine disrupting agents.

2. Zusammenfassung

Im Rahmen der künstlichen Reproduktion nimmt die in vitro Reifung eine etablierte Rolle ein, doch fehlen immer noch Detailinformationen über die zelluläre Kommunikation zwischen der Eizelle und den umliegenden somatischen Zellen. Des weiteren erfordern die abweichenden Ergebnisse zwischen verschiedenen Spezies deren kritische Betrachtungsweise. Aus diesem Grund wird mit Nachdruck an der Aufklärung wichtiger Signalprozesse geforscht. Es wurde erkannt, dass die interzelluläre Kommunikation offensichtlich eine wichtige Rolle in der zeitlichen Koordination diverser Prozesse spielt, welche für die Generierung einer befruchtungsfähigen Eizelle und die Embryonalentwicklung erforderlich sind.

Deshalb war das Ziel der vorliegenden Arbeit, die verschiedensten Kommunikationswege während der künstlichen Reifung boviner Kumulus-Oocyten-Komplexe (COC) zu untersuchen, da diese den Grundstein der weiteren Entwicklung darstellen. Des weiteren wurden die gewonnen Erkenntnisse mit den Ergebnissen von kultivierten bovinen Granulosazellkulturen (GC) verglichen.

Die Auswertung der genannten Zellkultursysteme erfolgte mittels der sensitiven Real-time PCR, welche auch geringste Abweichungen der spezifischen mRNA-Gehalte zu detektieren erlaubt. Zum spezifischen Nachweis von Hormonen und Proteinen in den Kulturmedien kamen sowohl der Enzym-Immuno-Assay (EIA) als auch der Radio-Immuno-Assay (RIA) zum Einsatz. Zur Bestätigung der mRNA-Ergebnisse wurden immunhistologische Techniken verwendet, um die Lokalisation der Proteine im Gewebe zu belegen.

In der vorliegenden Untersuchung konnte gezeigt werden, dass die reifenden COC unabhängig vom intakten Follikel Steroidhormone produzieren können. Des weiteren konnte ein direkter Einfluß des Umwelttoxins Tributylzinn (TBT) auf die Steroidsynthese der reifenden COC als auch die der luteinisierenden GC-Kulturen nachgewiesen werden. Hierbei war ein dosisabhängiger Einfluß des TBTs sowohl auf die Östradiol-17β- als auch auf die Progesteron-Synthese zu verzeichnen.

Die Existenz einer Reihe von Connexinen, wichtige zelluläre Verbindungsproteine, konnte auf Basis von mRNA-Daten belegt werden, die zum Teil eine eindeutige Abhängigkeit vom Reifestatus der COC zeigten. Neben der transmembranen Kommunikation via Gap Junctions, war es ebenfalls möglich zwei Stickoxidsynthasen (NOS), die induzierbare und die endotheliale NOS, zu detektieren, welche das membrangängige anorganische Signalmolekül Stickoxid (NO) produzieren. Beide Isoformen dieser Enzymfamilie wiesen eine unterschiedliche Regulation in den COC im Vergleich zu den kultivierten GCs auf. Ebenso konnte ein direkter Einfluß der Gonadotropine FSH und LH auf die mRNA-Expression in den GC-Kulturen nachgewiesen werden.

Die aktive Teilnahme der Eizelle an der Regulation von Zellfunktionen der umliegenden somatischen Zellen konnte durch das Vorhandensein spezifischer mRNA-Transkripte des Wachstums- und Differenzierungsfaktors-9 (GDF-9) und des Knochenwachstumsproteins-15 (BMP-15) in der Eizelle belegt werden. Obendrein war es möglich nahezu die vollständige bovine mRNA-Sequenz von GDF-9 zu entschlüsseln, welche im Anschluß als Basis eines E. coli-Expressionssystems diente. Zunächst zeigte aber das rekombinante Protein des nativen Wachstumsfaktors nicht die beschriebene Bioaktivität. Aber die Anwesenheit von drei potentiellen GDF-9/BMP-Rezeptoren (BMPR-1A, BMPR-1B und BMPR-2) sowohl in COC,

GC als auch in Zellen der Theka interna läßt auf ein funktionelles GDF-9/BMP-System in bovinen Ovarfollikeln schließen.

Des Weiteren obliegt auch die Extrazelluläre Matrix (ECM) einer massiven Umgestaltung. Mittels histochemischer Methoden konnte gezeigt werden, daß die gelartige Matrix um die Eizelle zum Großteil aus Hyaluronsäure (HA) besteht, die möglicherweise als Syntheseprodukt der hoch-expremierten HA-Synthase-2 (HAS2) anzusehen ist. Die Wichtigkeit der ECM wird durch die Co-Existenz der beiden HA-Rezeptoren CD44 und RHAMM (Rezeptor für HA-vermittelte Bewegungen) unterstrichen. Neben dem Schutz vor mechanischer Belastung der Eizelle dient diese HA-reiche Matrix möglicherweise auch als Bioreservoir für diverse Wachstumsfaktoren, welche eine hohe ECM-Affinität aufweisen. Hierzu zählt unter anderem der endotheliale vaskuläre Wachstumsfaktor (VEGF), welcher in hohem Maße im Ooplasma, in der Zona pellucida als auch in der Follikelflüssigkeit angereichert war. Seine Supplementierung zum Reifungsmedium hatte einen signifikanten Einfluß auf die nachfolgende bovine Blastocysten-Entwicklung.

Zusammenfassend kann man sagen, daß eine Vielzahl wichtiger zellulärer Kommunikations-systeme nachgewiesen werden konnte. Des weiteren bestehen mögliche Interaktionen zwischen diesen Systemen, denen eine fundamentale Rolle bei der Eizell-Entwicklung zugeschrieben wird. Das lokale Steroidogenese-System dieser Zellkultursysteme könnte möglicherweise auch als Testverfahren für „endokrin entkoppelnde“ Substanzen herangezogen werden.

3. General Introduction

The production of functional female gametes is essential for the propagation of all vertebrate species. The growth of oocytes within ovarian follicles and their development to mature eggs have fascinated biologists since many years, and scientists have realized the importance of the ovarian cross talk between follicle's somatic cells and the oocyte. This intercellular communication leads through oogenesis, folliculogenesis and finally the ovulation of a fertilizable oocyte. Recent studies have revealed key roles of the oocyte in folliculogenesis and established that bi-directional communication between the oocyte and the companion somatic cells is essential for development of an egg competent to undergo fertilization and embryogenesis. A further challenge for the future is to identify the factors that participate in this communication and their mechanisms of action.

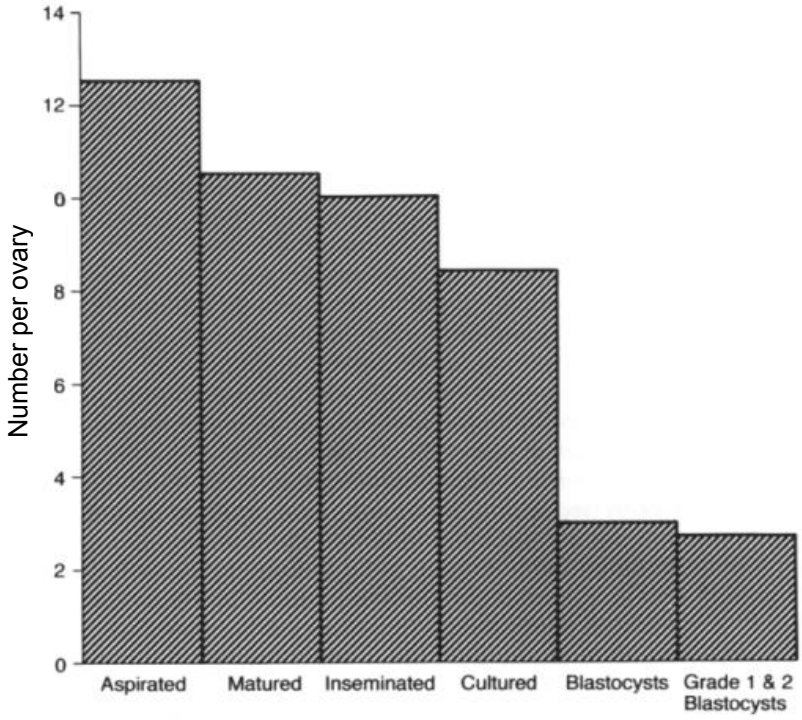
The most important system in ovarian communication is the pituitary-ovarian axis, consisting of the gonadotropins FSH and LH as well as the ovarian steroid hormones estradiol- 17 β (E2) and progesterone (P4). But beneath this endocrine communication several paracrine and autocrine systems are known which are believed to participate in the ovarian development as well. The easiest way of information exchange is made possible directly through the plasma membranes of the neighboring cells by either gap junctions or by small molecules, able to penetrate the membranes by free diffusion like the inorganic ion nitric oxide (NO). Recent research has focused on the most variable cross talk represented by the diversity of peptide hormones such as cytokines or growth factors and their corresponding receptors, for example the vascular endothelial growth factor (VEGF) system or the large super family of transforming growth factors beta (TGF-beta). Additionally, these signaling systems are influenced by or take part in the modulation and reorganization of the extra cellular matrix which in turn could influence cell behavior as well.

Recently, the in vitro production of bovine embryos has become a fundamental technique including intracytoplasmic sperm injection (ICSI), nucleus transfer, sex imaging as well as preimplantation diagnostics leading towards assisted reproduction. Therefore the experimental work on in vitro maturation (IVM) and in vitro fertilization (IVF) of bovine oocytes as well as in vitro culture (IVC) of bovine embryos may connect aspects of developmental biology with the practical work in breeding and industrial meat production.

Back to history, the first reports of IVM with mammalian oocytes were made by Pincus and Enzmann [1]. They observed that rabbit primary oocytes resumed meiosis spontaneously when

they were liberated from vesicular oocytes of large follicles of unmated rabbits. Further studies on the development of sea urchin eggs, sperm capacitation, IVF of oocytes from mouse and other mammalian species led to the first birth of a bovine calf after IVM followed by IVF in 1986 [2].

Figure 1: Efficiency of IVM and IVF of isolated COC per ovary in cattle [2]



This was a first mile stone in artificial production of bovine embryos. Although the knowledge about the development of mammalian oocytes is still increasing, the success to get viable blastocysts is still lower than 20 % (Fig. 1). Statistically, it is necessary to isolate 10 well developed oocytes to receive 1.5 calves. What is the reason of such low efficiency in assisted reproduction? It is undisputable that an efficient IVM, generating qualitatively high oocytes, represents the basis of IVF and at least embryo production [3-5].

3.1. Oogenesis and follicle development

Oogenesis subsumes all events starting with the formation and migration of primordial germ cells, their mitotic progeny and at last the final maturation to fertilizable oocytes [6;7]. The pool of oocytes in the mammalian ovary is fixed early in life; thus in the cow, the oogenesis starts in the early embryonic stage. During this process about two million germ cells will be generated by mitotic division. But senescence leads to a reduction of 95 % until the date of birth. At this point approximately 2 x 10^5 oocytes will have survived the natural occurring reduction [8;9]. Together

with a single layer of flattened mesenchymal ovarian cells, the oocytes - measuring 30-50 µm in diameter - will form the primordial follicles [10].

The first meiotic division begins with a long prophase I, subdivided into leptotene, zygotene, pachytene and diplotene, whereafter the oocytes will arrest for a certain time [11]. This resting phase will not be disrupted until the preovulatory induction. Nevertheless, protein synthesis in the oocyte still occurs through this phase of apparently nuclear quiescence [12].

The follicle population of one animal consists of a resting pool of primordial follicles and a wave-like growing pool of primary, secondary and tertiary follicles. During the initial recruitment, intraovarian factors stimulate certain primordial follicles to grow, whereas the rest of the follicles remain quiescent for months or years [9]. Initial recruitment is believed to be a continuous process that starts just after follicle formation, long before pubertal onset. After initial recruitment, the growing oocytes have to expand and undergo differentiation. The surrounding flattened follicle epithelia becomes cubic and together with the oocyte forms the so called primary follicle. After proliferation, the epithelia builds a multi-layered capsule of GC surrounding the oocyte. This process is associated with the formation of the Zona pellucida (Zp) by the production of specific glycoproteins named ZP1-3. These glycoproteins supposed to be essential for sperm-oocyte interactions [13]. In ruminants the follicular epithelia consists of about ten cell-layers, whereby the secondary follicle appears to be oval due to irregular cell division [14]. At the same time the theca layer will be formed by transformation of mesenchymal ovarian cells under the control of diverse cytokines [15-17]. The current hypothesis involving mesenchymal-epithelial cell interactions is illustrated in Fig. 2 [15]. In detail, GCs of developing follicles produce kid ligand (KL) which can act on theca and stroma cells as well as on the oocytes [18]. Differentiated theca cells, undifferentiated stroma cells and developing oocytes express the receptor c-kit [19]. The basic fibroblast growth factor (bFGF) is important in regulating a wide range of ovarian functions including GC mitosis [20], steroidogenesis [21], differentiation [22], and apoptosis [23]. It has been localized to the oocytes of primordial and primary follicles of many species [24-26]. The receptors for bFGF have been reported in rat and bovine GC. Further paracrine factors in the cell-cell interaction are the

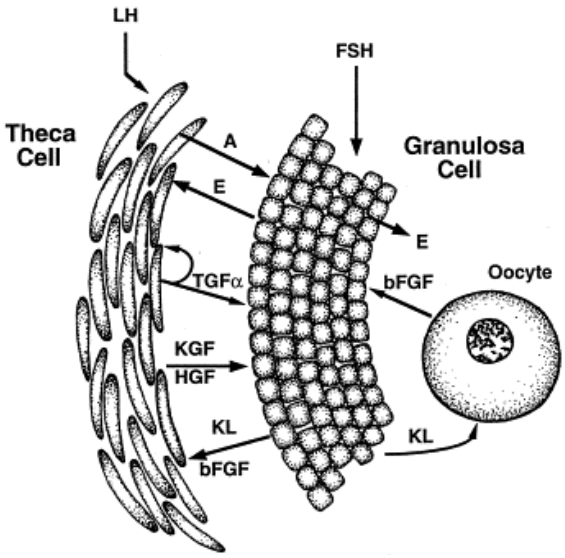


Figure 2. Aspects of antral follicle intercellular control [15].

keratinocyte growth factor (KGF) and the hepatocyte growth factor (HGF) secreted by theca cells and acting as regulator of GC function and growth [27]. In addition, KGF and HGF are suggested to be under the influence of gonadotropins and steroid hormones.

By the time, the antrum formation begins with the production of a hyaluronan- and protein-rich fluid (so called liquor follicularis), the follicle is named now tertiary or antral follicle. In vitro the process of follicle growth and antrum formation is enhanced by FSH, epidermal growth factor (EGF), and insulin like growth factor-I (IGF-I) [28]. The development from primordial up to tertiary and the preovulatory follicle (or Graafian follicle) is characterized by an initial and cyclic recruitment of ovarian follicles leading to two or three follicle waves within one estrous cycle in the cow [29;30]. In cattle, the growth of ovarian antral follicles from approximately 300 µm in diameter to a size of 3-5 mm - detectable by transrectal ultrasound scanning - takes more than 30 days [31]. The follicle growth wave is specifically FSH-dependent, and the simultaneous emergence of a cohort of small (3-5 mm) follicles is always associated with transient FSH rises [32;33]. At day 2 or 3 of the estrous cycle (determined by day 1 after ovulatuion) one single follicle of the cohort (6 up to 24 small antral follicles) will grow independent of the decreasing FSH levels, while the remaining cohort members become static and undergo atresia via apoptosis [29;34-36]. The selected dominant follicle enhances growth and steroidogenesis and is responsible for production of ovarian estradiol, which in turn represses in cooperation with inhibin the pituitary FSH secretion [37]. In this way the dominant follicle prevents any other cohort growth [38]. But the time of dominance usually lasts only several days, because the developing corpus luteum with its progesterone (P4) production represses LH pulse pattern in such a manner that the LH-dependent dominant follicle becomes atretic and does not reach ovulation [39;40]. After the loss of dominance between day 7 and 9 of estrous cycle another transient FSH rise recruits a new cohort, while the preceding undergoes regression. If luteolysis occurs during the the second follicle wave, possibly the second dominant follicle will ovulate [41]. However, if the corpus luteum is still producing P4, the second dominant follicle will undergo atresia and a third wave will begin the run for success. This negative feedback mechanism of P4 on the LH secretion is essential for the right timing of ovulation [42].

3.2. Maturation of oocyte

To reach the full competence for fertilization, the final maturation of the oocyte is required. Two distinct events can be distinguished: the meiotic and the cytoplasmic maturation.

Next to the induction of ovulation, the preovulatory LH surge is not only responsible for the induction of ovulation but also for the resumption of meiosis of the oocyte leading to the

germinal vesicle breakdown (GVBD) and the extrusion of the first polar body [43]. Furthermore, LH modulates mRNA transcription and protein synthesis in the oocyte [12;44], in GC as well as in cumulus cells [45]. The morphological changes during the meiotic maturation of almost all vertebrates take place in a similar way. After the abrogation of the meiotic block the nucleus disintegrates (GMVB), chromosome condensation occurs and a spindle apparatus is build [7;44;46]. The GMVB is completed 4-8 h after the LH-surge and the first meiotic division will be finished after 19-20 h. At this time the oocyte enters directly metaphase II (MII) of the second meiotic division and arrests until the sperm will penetrate the oocyte.

Along with the meiotic alterations additionally cytoplasmic events occur: observable changes are the centralization of the mitochondria in the ooplasm, the reduction of the Golgi apparatus and the arrangement of the cortical granules beneath the oolemma [47].

The regulation of this complex events during maturation has been not elucidated in detail, yet some key proteins were found, which play an essential role during the GMVB and the entry into a new cell cycle. Two predominant factors influencing maturation are the maturation-promoting factor (MPF; also called M-phase promoting factor or mitosis-promoting factor) and the mitogen-activated protein kinases (MAPK).

MPF is a protein kinase that drives both the mitotic and meiotic cycles in all eukaryotic organisms. MPF consists of a small subunit, the cdc2 kinase (cell-devision-cycle-gen2), and a large subunit, the cyclin B. Its pre-form is activated by dephosphorylation of different amino acid residues of the cdc2, when cells enter the metaphase of the cell cycle [48]. MPFs functional specifity is to add phosphate groups onto distinct proteins causing chromosomal condensation [49] and depolymerization of nuclear envelop followed by its breakdown [50]. Furthermore, MPF inhibits transcription during mitosis [51] and inactivats the cytoplasmic myosin during cell division until the chromosomes have separated [52]. Once the oocyte maturation is initiated e.g. by P4, MPF activity shows a biphasic progression peaking in MI and MII depending on the cyclin degradation [53]. In MII the oocyte arrests until fertilization or artificial stimulation.

Another important factor during the resumption of meiosis is MAPK which transduces mitogenic signals from the cell membrane to the nucleus [54]. MAPK is a low abundance serine-threonine protein kinase, transiently activated in many cell types by a variety of extra cellular mitogens, e.g. P4 [53] and insulin [55]. Therefore MAPK 42 and MAPK 44 are also called ERK1 and ERK2, which means extra cellular-regulated-kinase 1 and 2, respectively. During oocyte maturation the MAPK is activated by enzyme cascades [56;57]. This activation of MAPK is responsible for its increasing activity during 24 hour IVM in bovine oocyte. Experiments on the tetracycline cycloheximide, an inhibitor of protein synthesis [58;59], showed direct dependence

of the activation of both MPF [60] and MAPK [61] on functional protein synthesis. In bovine oocytes both enzymes are activated about the time of GMVB [62]. It is assumed that MPF is involved in the process of chromosome condensation and nuclear membrane disassembly, whereas the MAPK are responsible for interphase prevention during the transition from MI to MII [63;64]. They possibly influence other signal cascades of e.g. BMP-15, a member of the transforming growth factor beta super family (TGF ß) [65;66].

3.2.1. Oocyte maturation in vitro (IVM)

The principal task of IVM is to simulate conditions for the oocytes to reach the same developmental stage as occurring naturally. It seems that the time-dependent interplay of the different maturation events as indicated above are partly deficient in IVM. Oocytes, which show an early extrusion (16-20 hours after IVM) of the first polar body, developed sooner to blastocysts as oocytes with delayed meiotic maturation [67]. Additionally, low developmental capacity of IVM could base on insufficient functional and biochemical events in the ooplasm [61].

3.2.2. Spontaneous induction of oocyte

The spontaneous induction of oocyte maturation in rabbits after the isolation out of the natural surrounding is also observed in cattle [68], horse [69], and rat [70]. This in vitro effect is supposed to be dependent on the lack of a specific inhibitor present in vivo.

Oocytes of domestic animals such as pigs, sheep and cattle do not resume meiosis when both mRNA and protein synthesis are repressed by the presence of inhibitors [71;72]. Further, the key role played by cAMP in the control of meiosis is well established. Early studies have demonstrated the inhibitory effect of cAMP on meiotic resumption of mouse oocytes [73]. It has been also suggested that a part of cAMP could be synthesized by the cumulus cells and transferred via gap junctions to the oocyte, preventing oocyte maturation [74]. Likewise cAMP, the adenylate cyclase (AC) of the bacterium *Bordetela pertussis*, reversibly maintains bovine oocytes in meiotic arrest [75;76]. The treatment of mouse oocytes with forskolin, an extract of the herb *Coleus forskohlii*, increases the ooplasmic cAMP level, inhibiting a spontaneous resumption of meiosis in a dose-dependent manner during the first 5 hours of culturing. But an increased cAMP level in cumulus cells can activate stimulatory signal of maturation, and thus this signal could overcome the inhibitory effect of high cAMP level in the oocyte [77]. Further functions in controlling the resumption are ascribed to the follicular fluid as well as for the follicular cells. The follicular fluid is comparable to an exudate of the blood plasma enriched

with diverse proteins, glycoproteins and steroids [2]. These supplements show differences in concentration relating to estrous cycle and to follicular development [78-80]. In 1974, an oocyte maturation inhibitor (OMI), a protein less than 2 kD in molecular weight, was isolated from porcine follicular fluid [81]. OMI is a factor preventing oocytes from resumption of meiosis and has been shown for other species as well: cattle [82], rabbit [83], and human [84]. The OMI-levels were influenced by gonadotropins, and were dependent on the follicular stage. LH caused a decrease, whereby FSH stimulated the observable OMI levels [85]. Additionally, concentrations of OMI decreased with increasing follicle diameter, independent of the stage of the estrus [86]. These results suggest that this inhibitory factor is produced by the granulosa cells and/or theca cells of the follicular wall.

Co-culture of rat oocytes with follicular constituents was adopted in order to test the role of follicular components in the control of meiosis. Such studies demonstrated an inhibitory action of granulosa cells as well as granulosa-cell conditioned medium upon the spontaneous maturation of co-cultured oocytes. In contrast, theca tissue was without effect on meiosis. The addition of LH to co-cultures of rat granulosa cells and rat oocytes induced resumption of meiosis, as it did in vivo as well as in vitro in follicle-enclosed oocytes [85].

In conclusion, it is quite likely that the ovarian follicle uses inhibitory as well as stimulatory regulators to ascertain that the ovulated oocyte will be at the right nuclear status for its meeting with the spermatozoa. The molecular principles as well as the exact trigger of the interactions between somatic cells, especially the cumulus cells and the oocyte is still uncertain [71].

3.3. Factors influencing IVM success

The basis of IVM is that competent bovine oocytes must reach at least a minimum diameter of 110 µm [87]. In addition to this, the external conditions have to be adapted, including accurate classification of the follicles, choice of the most suitable collection method and culture media including the right supplementations.

3.3.1. Oocyte classification

The selection of bovine oocytes for IVM on the basis of visual assessment of morphological features was first examined by Leibfried and First [88]. In the following years numerous reports were published dealing with classifications of isolated oocytes and COC. The main features, which were used, are divided into characters of the oocyte (homogeneous or dark clustered ooplasm) and characters of the cumulus (single- or multi-layered cumulus, expanded or compact

cumulus) [89-91]. The existence of a clear relationship between oocyte morphology and embryo yield after IVM/IVF is well established [92].

3.3.2. Indicators of follicle and oocyte quality

The key of IVM success is the ability to identify the quality of follicles, whether they are atretic or non-atretic. Moor and coworkers were the first who classified ovine follicles by macroscopic criteria. They evaluated the results by steroid hormone determination to separate atretic from non atretic follicles [93]. Further classifications were established for cattle by Kruip and Dielman [94] as well as McNatty and coworkers [95]. All three schemes consist of nearly the same criteria, with only minor differences [2].

In the case of isolation technique two common methods have become accepted: follicle aspiration and ovary slicing. Slicing is the most effective technique, leading to higher yield of oocyte recovery, but in contrast aspiration is less time consuming. As shown for cattle, these techniques are also useful for other species as horses [96], buffalos [97] and camels [98].

To increase the oocyte yield by 50 % per ovary the new method of transillumination-aspiration ovary (TAO) would be applied. This technique enables to isolate oocytes from deeper situated follicles which are difficult to identify during regular oocyte aspiration [99].

4. Literature review of investigated components

4.1. Steroidogenesis and endocrine disruption

It has been recognized that sex steroids fundamentally influence the maturation process in different ways: E2 promoted alterations in the reactivity of the calcium liberation system during cytoplasmic oocyte maturation [100] influencing the typical calcium oscillation during fertilization [101]. Additionally, the presence of both aromatizable and non-aromatizable androgens during bovine IVM increased oocyte cleavage rates without affecting embryo development. In contrast, P4 inhibited bovine oocyte maturation leading to reduced embryo development after cleavage [102].

Steroidogenesis within the follicle occurs depending on its developmental stage and gonadotropin status. In preantral follicles, P4 is produced by cells of the theca interna (TIC), indicated by high expression rates of 3-beta-hydroxy-steroid-dehydrogenase (HSD) and cholesterol side chain cleavage enzyme (P450scc). In contrast, in antral follicles predominantly androgens are synthesized and converted by granulosal aromatase P450 (ARO) into E2 [103]. Biosynthesis of steroids in both cumulus and GC has been demonstrated by several authors for different species: e.g. cattle [104;105]; humans [106], rats [107], and pigs [108]. Therefore steroidogenesis should be expected in cultured bovine COC and GCs as well.

The fine-tuned steroidogenic system, involved in normal sexual differentiation and reproduction, can be dramatically influenced by so-called endocrine disrupting agents. Kavlock and Ankley [109] devised the following definition: “An endocrine disruptor is defined as an exogenous agent that interferes with the synthesis, secretion, transport, metabolism, binding, action or elimination of natural blood-borne hormones in the body which are responsible for homeostasis, reproduction and developmental processes”. To date, approximately 60 chemicals have been identified as endocrine disruptors, which could be classified into three main types of endocrine-disrupting mechanisms: mimic of naturally produced hormones, blocking of hormones, and triggering of hormone-like pathways [110] by interfering for example with steroid hormone receptors [111] or interacting with steroidogenic enzymes [112]. The uptake or exposure of such substances can occur in a variety of ways; organisms can be exposed involuntarily to endocrine disruptors as a result of drinking contaminated water, breathing contaminated air, ingesting food or coming into contact with contaminated environment.

Often such disruptors are manmade synthetic compounds such as herbicides, fungicides, insecticides, nematodicides and other industrial chemicals. About half of these compounds are chlorinated, including dioxins (PCDDs), polychlorinated bisphenyls (PCBs) and organochlorine

pesticides such as DDT [110]. For pesticides also trialkyl organotin compounds are broadly in usage. One member of these substances is the environmental pollutant tributyltin (TBT), which has been used since the 1950s as a biocide for wood preservation, marine antifouling paints, antifungal action in textiles and industrial cooling systems, paper mills, and breweries. The effects on the steroidogenic system are widly spread and depend on the concentration and exposure time. In spite of restrictive usage, the ecological effects of TBT are still persistent.

In adult females, the reproductive cycle is a complex process characterized by distinct phases of gametogenesis and embryo genesis. Therefore, various sites of action of interference with the reproductive function are possible: hypothalamus, pituitary gland, ovary and reproductive tract [113;114]. How such reproductive toxicants can affect ovarian function is not yet well understood in detail, but it is known that sewage overflows influence fertility of dairy cows [115]. Additionally, IVM experiments of bovine COC indicated, that very low amounts of PCBs (here Aroclor) were sufficient to disrupt bovine oocyte maturation, its fertilization and developmental competence [116].

4.2. Gap junctions

The ovarian folliculogenesis requires complex regulatory mechanisms involving both extrinsic and intrinsic (endocrine) signaling pathways. Communication between cells is not exclusively mediated by secreted factors, such as steroids or signal peptides. Additionally, signaling and metabolic cooperation between cells could occur directly by transmembran channels, so-called gap junctions. These junction sites are constructs of two hemi channels. The hemi channel of one cell is termed connexons showing a hexagonal arrangement of six protein subunits named connexins (Cx). Cx proteins vary greatly in size and were named according to the molecular weight, ranging from 26kD (Cx26) up to 60kD (Cx60) [117;118]. At present, 12 different genes, encoding more than twenty homologue proteins of the connexin family, have been identified [119]. They consist of both highly conserved and divergent regions. The amino-terminal domains and four membrane-spanning regions are highly conserved, whereas the length and the primary structure of the two intracellular domains, named cytoplasmic and carboxyl-terminal domains, respectively, vary greatly in individual Cxs. The proteins are almost ubiquitous in tissues of multi-cellular organisms and are distributed both tissue- and cell-specific. For the ovarian tissue numerous types of the great Cx-family were found, e.g. Cx26, Cx30.3, Cx32, Cx37, Cx40, Cx43 and Cx45 [120;121].

Multitude studies have demonstrated the existence of gap junctions in ovarian follicles of different species: cow [122], sheep [120], human [123], mice [124], rat [125], rabbits [126].

These Cxs allow inorganic ions, second messengers, and small metabolites <1kD, such as calcium ions, cAMP, inositol 1,4,5-triphosphate, to pass from cell to cell [127]. The diversity of the Cxs is extended by homomeric and heteromeric composition of different Cx within one connexon. Additionally, several connexons could be coupled both homotypic and heterotypic. This multiplex assembly of the gap junctions mirrors its complex selectivity and regulation of intercellular coupling [128;129].

Cx43 is highly expressed in large antral follicles in rats, correlating with high serum FSH levels followed by a drop after the preovulatory LH surge [130]. These results were also demonstrated by exogenous administration of FSH and hCG as well as cell culture experiments [131]. The physiological significance of Cx43 during follicle development is demonstrated by knockout studies in mice, whereby, in absence of Cx43, the follicles arrest in the preantral stage and show morphological abnormalities, like a poorly developed zona pellucida of the oocyte and vacuolated cytoplasm of both GC and oocyte [132]. Additionally, the oocytes were retarded, unable to undergo meiosis and could not be fertilized. By immunohistochemistry and Western Blot analysis of bovine follicles at different developmental stages, from primordial up to large antral, the three predominant connexins Cx43, Cx32 and Cx26 could be detected in context of healthy and atretic behavior of the follicle [122]. Similar results were shown for other species: rat [133], mouse [134], sheep [120], and pig [135]. These experiments reinforce the notion that expression of multiple connexins may be a mechanism of regulating development and regression of follicles.

4.3. Nitric oxide

One of the potential signaling factors during follicle developmental coordination is the inorganic free radical nitric oxide (NO), which has been demonstraded as an important biological messenger in the last decade. NO is involved in vasodilatation and the regulation of normal vascular tone, inhibition of platelet aggregation, neuronal transmission, and cytostasis. To date, three isoforms of NO synthases (NOS) are known: the neuronal NOS (nNOS), the endothelial NOS (eNOS), and the inducible NOS (iNOS). NO is synthesized from the substrates L-arginine and oxygen by the NOS under influence of different cofactors [136]. The non polar and reactive free radical NO is able to diffuse freely through membranes and can modulate protein activity, e.g. binding to heme of guanyl cyclase or cell surface thiols. Besides epithelia or macrophages, NO is synthesized also in ovarian cells of different species and is postulated to be a major paracrine mediator and important regulatory agent in various female reproductive processes, such as ovulation, apoptosis, implantation, pregnancy maintenance, labor and delivery [137;138].

Furthermore, in the brain, NO activates the release of LH-releasing hormone (LHRH) and participates in FSH signal transduction [139].

Most of the research on the NO-system is done by inhibition of the NO synthases and/or by NO induction. The most useful NOS inhibitors are L-NAME (N-omega-nitro-L-arginine methyl ester) and AG (aminoguanidine hemisulfate salt). In contrast to the inhibition of the synthesis, so-called NO-donor can be applied, which produce NO in a dose dependant manner, such as SNP (sodium nitroprusside) and SNAP (S-Nitroso-L-acetyl penicillamine).

By the use of RT-PCR and immunohistochemistry it was possible to detect eNOS and iNOS in the rat ovary, showing distinct sensitivity on gonadotropin status [140]. Nonstimulated ovaries had the highest levels of iNOS mRNA, which declined straight after gonadotropin injection. In contrast to iNOS, eNOS mRNA levels increased after gonadotropin stimulation and peaked in ovaries containing ovulatory follicles [140]. Zackrission and coworkers revealed by immunohistochemistry, that these two isoforms of NOS were primarily existent in the stroma and the theca cell layers, respectively[141]. Additionally, iNOS mRNA was higher expressed in rat GCs as compared to theca cells, whereby eNOS was mainly existent in theca cells and increasing after hCG injection [142].

Shukovski and colleagues have demonstrated that NOS inhibitors are able to suppress hCG-induced ovulation in the rat in vivo [143]. Thus, if ovulation was induced by increasing prostaglandin E_2 (PGE_2) accumulation in the follicle, it would be possible that NO is able to modulate PG synthesis. Therefore, in vitro experiments with cultured COC were carried out, showing an inhibition of PGE accumulation by NO-inhibitors in vitro [143]. These results were comparable to preovulatory follicles prior to ovulation in vivo [144]. Later studies showed that the produced NO stimulated the synthesis of both PGE_2 and $PGF_{2\alpha}$ from the cyclooxygenase pathway enhancing the process of follicle rupture [145;146]. This modulating influence on ovulation has also been reported for other species: delayed ovulation caused by the administration of NOS inhibitors in horses [147], and dose-dependent inhibition of hCG-induced ovulation by L-NAME in rabbits [146].

Alongside the potential influence of NO in ovulation processes, strong evidence indicates a significant participation in steroidogenesis. An inhibition of NO production results in a dose-dependent increase of both E2 and P4, as shown for rats [142], cultured swine GC [148;149] and rabbits [146].

This functional diversity of NO itself suggests a possible influence on the maturation of the oocyte. First results dealing with this context were shown by Jablonka-Sharif and coworkers [150]. They have shown in rats that treatment with L-NAME reduced super ovulation rate of

isolated oocytes as well as that the lack of NO-synthesis resulted in severe defects of oocyte maturation. Furthermore, they reported a strong staining for eNOS both in preovulatory ovarian oocytes and isolated oocytes. Further studies on eNOS knockout mice indicated a reduced number of oocytes in the ovary as well as reduced MII-stage and increased MI-stage oocytes, respectively [151;152]. Supplementation of the NO-donor SNP significantly stimulated meiotic maturation to MII and led to increased embryo development in mice [153]. NOS inhibition resulted in suppressed but reversible resumption of meiosis and reduced embryo development [153;154].

In addition to the mammalian ovary NOS, the existence of NOS at the micropyle of minnow eggs has been also demonstrated. The histochemical staining of NOS was blocked by selective NO-inhibitors. Furthermore, the NO-donor SNP enhance sperm motility at low doses, whereby higher doses inhibited sperm motility [155]. Further studies proved that the application of L-NAME was able to inhibit both eNOS activity in human sperms and the acrosomal reaction [156;157]. Additionally, acrosomal reaction and NOS activity were increased by follicle fluid extracts as well as by NO-donors SNP or SNAP.

Summarizing these various studies, NO seems to be a regulatory agent at low concentration, but cytotoxic at higher doses. Furthermore, NO possible participates in cellular behavior in such a manner, that decreased NO concentrations may prevent apoptosis in preovulatory follicles [142;158] and reduce the incidence of murine follicular cysts in response to hCG stimulation [159].

4.4. Growth factors

4.4.1. Growth differentiation factors-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15)

The process of ovarian follicular growth and oocyte development may assume complex interactions between the oocyte and the surrounding somatic cells as well as hormonal signals between the pituitary gland and the ovary [160;161]. In the last decade especially loss of function studies in mice [162] and sheep [163] indicated that the oocyte plays a regulative role on the neighboring somatic cells controlling their growth, differentiation and rate of ovulation. The initiation of follicle growth is characterized by the transition of primordial follicles from quiescent to growth phase, indicated by a visible increase of the oocyte diameter and transformation of flattened to cubical granulosa cells. It is known that the apparently resting oocyte expresses different factors influencing critical events during oocyte maturation, e.g.

cumulus expansion and steroid hormone synthesis [164]. Strong evidence has been provided to show that members of the TGF-ß super family are involved in controlling cellular events. Two of these growth factors are thought to be important for normal follicular development, namely growth and differentiation factor 9 (GDF-9) and the bone morphogenetic protein-15 (BMP-15; also known as GDF-9B) [165], belonging to the subfamily of BMPs which were originally identified by their ability to induce ectopic bone formation [166]. GDF-9-deficient mice [167] as well as sheep [168] are infertile, because the follicular development is arrested at the primary stage. Further events during oocyte maturation are under possible influence of GDF-9: cumulus expansion by increased expression of hyaluronan synthase 2, stimulation of progesterone production by recombinant GDF-9 as well as increased mRNA expression of cyclooxygenase 2. In addition to stimulating functions, GDF-9 blocks urokinase plasminogen activator and LH receptor mRNA expression [160].

In contrast to GDF-9 knockout female mice, BMP-15 null mice are subfertile and usually have minimal ovarian defects [162], whereas homozygote female sheep with inactivated BMP-15 gene (BMP15-/-) are infertile. However, sheep, showing heterozygote gene combination (BMP15-/+), are fertile accompanyied with increased ovulation rate [163]. Additionally, synergistic roles of BMP-15 and GDF-9 were postulated [169].

In addition to the correct protein sequences, the biological action of BMPs/GDFs requires the TGFβ typical dimeric conformation. Both BMP-15 and GDF-9 are synthesized as large precursor proteins. After dimerization, the molecules are proteolytically cleaved by PACE (paired basic amino acid residue cleaving enzyme) yielding themature proteins [166;170;171]. The secreted dimers initiate signaling by binding cooperatively to BMP receptors. The TGF-ß family members initiate intracellular signaling by inducing the assembly of a heterotetrameric complex of two types of transmembran receptors known as TGF-ß receptors type I and type II [172]. Both receptor types consist of a cysteins rich extra cellular domain, and an intracellular serine/threonine kinase domain. Upon ligand binding to receptor type II, it will activate downstream receptor type I by phosphorylation. Several subtypes of TGF-ß receptors II and I have been identified and were alternatively called activin type II receptor (ActR-II for type II) and activin receptor-like kinase (ALK for type I). Diverse receptors were postulated to interact with the two growth factors GDF-9 and BMP-15: BMPR-II, BMPR-1A (or ALK-3), and BMPR-1B (or ALK-6). Downstream signal transduction is performed via different phosphorylation steps; therefore intracellular signaling cascades of SMAD (SMA and MAD related) proteins are activated, modulating gene expression [172;173]. These proteins are able to migrate into the

nucleus to modulate specific gene transcription by interfering with transcription factors or co-regulators.

4.4.2. Vascular endothelial growth factor (VEGF)

It is well established that a variety of growth factors are essential for successful follicle development and oocyte maturation [25;174;175]. Therefore, the search for essential growth factors has started, understanding their developmental context and using the perceptions to improve the most critical points of the development of immature oocytes. In the last few years evidence has been provided, that the vascular endothelial growth factor (VEGF) could be an important factor in regulation of different ovarian functions.

In detail, VEGF is known to act as a potent mitogen for micro- and macro-vascular endothelial cells [176] and as a stimulator of vascular permeability based on its ability to induce vascular leakage [177]. According to its different functions a lot of synonyms circulate in literature: GD-VEGF (glioma-derived vascular endothelial growth factor), Mouse sarcoma 180-derived growth factor, VAS (Vasculotropin), Vascular endothelial cell proliferation factor, and VPF (vascular permeability factor). VEGF protein has been isolated from pituitaries, macrophages, lung and kidney epithelia, aortic smooth muscle cells, and diverse tumor cell lines as well.

Stated at known structural characteristics, the proteins could be divided into several isotypes: VEGF its selves, named VEGF A [176;178] and several VEGF-related factors, classified VEGF B to E [179-182]. VEGF is a homodimeric protein, linked by disulphide bonds and heavily glycosylated (46-48 kDa, 24 kDa subunits). By alternative splicing of the human VEGF mRNA - its genome consist of 8 exons and 7 introns - several molecular variants occur: 121 (VEGF-121), 145 (VEGF-145), 165 (VEGF-165), 183 (VEGF-183), 189 (VEGF-189), and 206 (VEGF-206) amino acids [176;178]. Compared to the human sequence, these splice variants are lacking uniquely one amino acid in cattle and in rat as well. Sequence alignments shows 95 % identity in amino acid progression. The isoforms could be additionally classified according their biochemical properties: VEGF-121, VEGF-145, and VEGF-165 are soluble secreted forms, while VEGF-189 and VEGF-206 are mostly bound to heparin-containing proteoglycans at the cell surface or in the basement membrane. The bio-availability of VEGF is regulated probably at the genetic level by alternative splicing that determines whether VEGF will be soluble or incorporated into a biological reservoir, where it could be liberated proteolytically by plasminogen activation.

The signaling cascade of the different VEGF-isoforms are mediated by tyrosine-kinase receptors, characterized by the presence of seven immunoglobolin-like domains at the extra cellular face

[178]. They are named VEGF-R1 (flt-1, fms (feline sarcoma virus)-like tyrosine kinase), VEGF-R2 (flk-1, fetal liver kinase-1; or KDR, kinase domain region), and VEGF-R3 (flt-4). The interaction of VEGFs with proteoglycans (heparin-sulfate-like molecules) of the extra cellular matrix is required for efficient receptor binding, but the binding to proteoglycans in absence of the tyrosine-kinases do not induce biological responses [178].

According to ovarian function, VEGFs are essential for the development of follicle and corpora lutea in the ovary, and the establishment of vascular structure in the placenta [183-185]. In the cow, all components of a functional VEGF-system in the oviduct were found to undergo specific modulations during the estrous cycle [186]. This suggests that VEGF might be involved in creating an optimal local environment for fertilization and the developing embryo by modulating permeability within the bovine oviduct. In addition, VEGF transcripts continuously increased in granulosa cells accompanied with arising protein concentrations in the follicular fluid during follicular development [185].

4.5. Extra cellular matrix components during oocyte maturation

During the development of ovarian follicles, the granulosa cells form a multi-layered epithelium surrounding the maturing oocyte. At the end of follicular growth, the granulosa cells differentiate into two subpopulations: the oocyte attached cumulus cells and the mural granulosa cells. Several hours after the endogenous LH surge, the COC undergo expansion in cattle [187] and other species [188;189]. This process is thought to be stimulated by FSH or EGF [190-193]. The resulting spongy-form COC matrix possibly facilitates the extrusion of the oocyte at ovulation and prevent the dispersion of the surrounding cells. Furthermore, the matrix is supposed to play a key role during fertilization and sperm capacitating [194].

One of the principal components of the gel like matrix surrounding the COC is the glycosaminoglycan (GAG) hyaluronan (HA), which is also named hyaluronic acid and was first discovered by Meyer and Palmer [195]. This large linear up to 10^7 Da mucopolysaccharide is composed of repetitive disaccharides (2,000-25,000 units) of (1-ß-4) D-glucorunate and (1-ß-3) N-acetyl-D-glucosamin. It contributes to tissue homeostasis [196] and biomechanics [197] and was found to be a component of essentially all vertebrate extra cellular matrices (ECM). In contrast to the other intra-cellular produced GAGs, HA is synthesized at the cell membrane by hyaluronan synthases (HAS) [198], by adding monosaccharides to the reducing end of the chain. The precursors are UDP-glucuronic acid and UDP-N-acetylglucosamine [199]. In mammals the HAS family consists of three known isoenzymes called HAS1-3, which extrude the growing polymer into the extra cellular space [200;201]. Additionally, the different HAS enzymes show

different catalytic activities in HA synthesis and different synthesis products concerning the molecular weight [202]. HA is carried by lymph flow from the tissues and partly taken up and degraded in the lymph nodes. Finally, the residually part is taken up in the endothelial cells of liver sinusoids. The uptake in the liver of high-molecular weight hyaluronan is very efficient and its normal half-life in serum is only in the order of 2 to 5 min [199]. The polysaccharide is rapidly degraded in the lysosomes to the low-molecular weight products, lactate and acetate. The normal concentration of HA in serum is less than 100 µg/l with a mean of 30-40 µg/l.

To support the complex interactions between cells and ECM, different proteins appear to be involved [203]. Recently, receptors for HA have be identified, including the transmembran receptor CD44 (named by clusters of differentiation), RHAMM/IHABP (receptor for hyaluronan mediated motility/intracellular HA binding protein or CD168), LYVE-1 (lymph vessel endothelial HA receptor-1), and HARE (HA associated receptor for endocytosis).

The glycoprotein CD44, probably the most common HA receptor, is characterized by a variety of isoforms caused by alternative splicing and post-translational modifications [204;205]. This receptor, belonging to the hyaladherins, is responsible for cell to cell and cell to ECM interactions [206], inhibition of apoptosis [207], endocytosis of HA [208], augmentation of tumor cell motility and metastasis [209], as well as the stimulation of lymphocytes [210]. Little information is available concerning the regulation of the CD44 gene expression, though recent studies indicate that CD44 may influence fertility and quality of human oocytes [211].

The second known HA-receptor RHAMM, first mentioned by Hardwick and coworkers [212], possesses two potential HA-binding sites [213]. Interactions between HA and RHAMM are thought to be activate protein kinases that modulate cellular behavior. Although it is not clarified in detail whether RHAMM is located inside the cell, at the cell surface or possibly both, it is ensured that RHAMM is able to promote cell movement [214]. Furthermore, RHAMM may be involved in wound healing [215], migration of smooth muscle cells [216], modulation of ciliary beating of airway epithelial cells [217], sperm motility [218], and embryonic development [219]. According to the interaction with the cell cycle, Mohapatra et al. [220] have shown that an over-expression of RHAMM induces cell cycle arrest by suppressing the expression of cdc2/Cyclin B1 (MPF), a protein kinase complex essential for mitosis.

The major receptor for HA in the lymph vessel wall is LYVE-1, which has only been detected here and not in ovary or testis [221]. The deduced amino acid sequence of LYVE-1 shows 41 % similar to the CD44 HA receptor and binds - like CD44 - both soluble and immobilized HA.

The forth HA receptor HARE has been isolated, cloned and characterized from sinusoidal liver endothelial cells, but its existence is not exclusively limited to the liver [222-224]. HARE is

supposed to participate in the removal of both HA and chondroitin sulfate from the circulation by endocytosis, mediated by clathrin-coated pits. Blocking of HARE by antibodies led to an inhibition of HA clearance in perfused liver, indicating its postulated function [225;226].

Some of these HA-binding molecules are of cumulus cell origin [227;228] and have possible effects on COC functionality. During COC expansion in mammalian species, HAS2 has been suggested to synthesize the huge amount of HA which can also be found in ovarian follicles [189;229;230].

5. Aims of the study

According to the lack of information about intercellular communication between the oocyte and the surrounding somatic cells as well as the existence of diverged results between various species, the following study was undertaken to examine different systems of intercellular communication during artificial maturation of bovine oocyte cumulus complexes in comparison to bovine ovarian granulosa cell cultures.

The aims were

- the valuation of potential local steroid synthesis including the detection of steroidogenic enzymes, such as ARO and HSD
- the examination of possible interactions of the environmental pollutant TBT concerning its influence on cell behavior and steroidogenesis
- the mRNA-expression levels of the three most predominant so-called connexins (Cx26, Cx32, Cx43) and the NO producing system (eNOS, iNOS)
- the examination of different oocyte derived factors, their mRNA sequencing and production of the recombinant protein.
- the evaluation of the possible functions of HA and their synthases (HAS1-3) as well as receptors (CD44 and RHAMM)
- immunohistochemical localization, mRNA expression, protein concentration of VEGF should be examined and the potential influence on embryonic development should be tested.

6. Materials and Methods

6.1. Tissue collection and preparation

For cell culture experiments, ovaries from healthy German Simmental (Fleckvieh) cows were collected at a local abattoir within 10-20 min after slaughter and were transported in sterile Ringer solution at 37 °C into the laboratory. The stage of the estrous cycle was defined by macroscopic observation of the ovaries (color, consistency, corpus luteum stage, number and size of follicles) and the uterus (color, consistency and mucus) [231].

6.1.1. COC retrieval and culture conditions

COC were recovered irrespective of stage of the estrous cycle by aspiration of follicles (up to diameter of 8 mm) with a 10 mL single-use syringe (Braun, Melsungen, GER) connected to a 20G needle (Sterican, Braun, Melsungen, GER) and sorted out for grade 1 COC with a compact and multi-layered cumulus oophorus as well as homogeneous ooplasm [91]. To reduce estrous cycle dependent effects, groups of ten COC underwent IVM [25;232]. COC were cultured in 6-well plates (Greiner, Solingen, GER) filled with 400 µL bicarbonate buffered Modified Parker´s Medium (MPM199, pH 7.2, see 6.6.1.) supplemented with 10 % fetal calf serum (FCS, Gibco, USA) and 0.01 IU/mL of bovine FSH (NIH B1 activity) as well as 0.01 IU/mL LH (NIH LH B10) (Sioux Biochemicals, USA).

6.1.2. GC retrieval and culture conditions

GCs were recovered by rinsing antral follicles with PBS (pH 7.4, see 6.6.2.) and a 10 mL single-use syringe connected to a 20G needle, washed three times in MPM199 and seeded into 12-well uncoated tissue culture plates (4 x 10^5 cells / 2 mL MPM19+10 % FCS / well). To minimize the contamination by atretic GCs of large antral follicle, only small follicles (<8 mm) were punctured, showing lower incidence of atresia. Potential influence of simultaneously extracted COC were prevented by sorting out the GC cultures for oocytes. All treatments with FSH, LH, FSH+LH, HA, recombinant GDF-9 (recGDF-9) as well as tributyltin exposure started 1.5 d after seeding (onset), when all GCs were attached to the bottom and well proliferating. Sampling was carried out at the onset, after 4 h and after 24 h by removing culture medium and cell lysis for total RNA-extraction directly in culture dishes.

6.1.3. Follicle dissection

Only follicles, which appeared healthy (i.e. well vascularized and having transparent follicular wall and fluid) and whose diameters were >4 mm, were used for dissection. Since healthy follicles have relatively constant P4 levels in follicle fluid, only follicles with P4 concentrations below 100 ng/mL FF were used for the evaluation to exclude atretic follicles.

For total RNA extraction, the follicles were dissected from the ovary and classified into five groups according the E2 concentration in follcile fluid (<0.5, >0.5-5, >5-20, >20-180, >180 ng/mL). Additionally, they were separated for GC and TIC as previously described [233]. The corresponding size of follicles were in the range of 1) 5-7 mm, 2) 8-10 mm, 3) 10-13 mm, 4) 12-14 mm, and 5) >14 mm. The extracted TIC and GC were snap-frozen in liquid nitrogen and stored at –80 °C until RNA isolation was carried out. The follicle fluid was stored at –20 °C until steroid determination.

6.2. Hormone determination

Concentrations of P4, testosterone (T) and E2 were determined by an enzyme immunoassay (EIA) using the second antibody technique [234-236]. Therefore as enzyme solution progesterone-6β-hydroxy-hemisuccinate-horseradish peroxidase (HRP), testosterone-3-CMO-HRP or estradiol-6-carboxy-methyl-oxim-HRP was used. Each polyclonal antibody was raised in a rabbit against progesterone-7α-carboxyethylthioether-BSA, for T against testosterone-11α-hemisuccinate-BSA or for E2 against 17β-E-6-carboxymethyloxim-BSA. The effective dose for 50 % inhibition (ED_{50}) of the assay was 6 ng/mL for P4, 250 pg/mL for T, and 3.5 pg/mL for E2. The detection limits of the different EIAs ranged from 0.05 up to 25 ng/mL for P4, 10 up to 5000 pg/mL for T, and 2 up to 1000 pg/mL for E2. To enable a correct statistical evaluation of hormone levels, samples, which were calculated below the lowest standard, were set to the half of the lowest standard concentration. According to the charcoal treatment of the FCS, the hormone levels of P4, T as well as of E2 were completely eliminated.

6.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

6.3.1. Total RNA extraction

For total RNA of the matured COC and GCs, all culture media respectively residues of follicle fluid were removed. Prior to cell lysis, COC and GCs were washed twice in sterile PBS and introduced to total RNA extraction performed by spin columns (NucleoSpin RNA II, Macherey&Nagel, Düren, GER) including DNase1 digestion to reduce contaminating DNA. In

contrast, total RNA from TIC was isolated by the single step method of Chomczynski and Sacchi using TRIzol reagent (Gibco BRL, MD, U.S.A.) [237]. Each isolated RNA sample was dissolved in water and its integrity was verified by OD260/OD280 nm absorption (Biophotometer, Eppendorf, Hamburg, GER).

6.3.2. Reverse transcription of RNA

Total RNA (COC: 200 ng, cultured GC: 500 ng, dissected GC and theca interna: each 2 µg) was reverse transcribed with 200U MMLV-Reverse Transcriptase (Promega, Mannheim, GER) using 2.5 µM random hexamer primers (Gibco BRL, USA) and 0.5 mM dNTP´s (Roche Diagnostics, Mannheim, GER) as described earlier [238].

6.3.3. Primer design and PCR evaluation

For specific gene amplification primer sets (see 6.6.3.) were selected based on published EMBL data or were used from literature as indicated. To perform optimal PCR conditions the most efficient annealing temperatures (AT, see 6.6.3.) were established in a gradient cycler (Mastercycler, Eppendorf, Hamburg, GER) and tested for quantification temperature (QT, see 6.6.3.) by a melting point analysis in the LightCycler (Roche, Mannheim, GER). To verify specificity of each gene amplification, PCR products were isolated by gel extraction MiniElute kit concerning user manual (Qiagen, Hilden, GER) and were commercially sequenced (Medigenomics, Martinsried, GER). New bovine sequences which were acquired by primer verification, were made public available by EMBL data base submission (see 6.6.3.).

6.3.4. Standard Block-PCR and gel electrophoresis

For primer evaluation and first gene amplification a conventional block PCR was used. Therefore, standard reaction mix was performed: 2.5 µL cDNA, 18.4 µL PCR water, 2.5 µL dNTP (10 mM), 0.5 µL forward primer (20 µM), 0.5 µL reverse primer (20 µM), 2.5 µL 10x PCR buffer (including $MgCl_2$; Boehringer, Ingelheim, GER) and 0.1 µL polymerase (Boehringer, Ingelheim, GER). The visualization of PCR results was done by 1.5 % agarose ethidium bromide gel electrophoresis. Therefore 2 µL of PCR samples were mixed with 7 µL sample electrophoresis buffer (see 6.6.4.). Gels were run at 90 V for 40 min and digital documented by ultra violet video station (Pharamcia, GER).

6.3.5. Quantitative real-time PCR

For each sample a standard 10 µL real-time PCR reaction mix (Roche Diagnostics, Mannheim, GER) was prepared containing the following components: 1 µL LightCycler mix, $MgCl_2$ (4 mM), primers (0.4 mM) and sample cDNA (COC: 0.33 ng/µL, GC: 0.83 ng/µL, TIC: 1.66 ng/µL). QT was set below individual melting peak of each PCR product. For specific gene amplification a standard protocol of 50 cycles was used in a LightCycler (Roche Diagnostics, Mannheim, GER): after initial polymerase activation at 95 °C for 10 min, primer specific amplification and quantification cycles were run at AT and QT as indicated above. To evaluate specific gene amplification a final melting curve analysis was performed (60-99 °C) under continuous fluorescent measurement. Relative quantification was determined using sample crossing points (CP) analyzed with LightCycler software 3.5 (Roche Diagnostics, Mannheim, GER) by Second Derivative Maximum method. The PCR efficiency (E) of each primer set was determined in a distinct detection range (2.7 pg up to 8.33 ng of cDNA, Fig. 3a). If necessary, the PCR efficiency was calculated according to the equation (1): $E = 10^{[-1/slope]}$ [239]. A standard curve (n=3) was used to examine the slope (s) and the regression (r).

6.3.5. Intra- and inter-assay variation of LightCycler data

To confirm reproducibility and accuracy of real-time PCR, intra-assay precision (Fig. 3a) was performed three times within one LightCycler run as well as inter-assay variation (Fig. 3b) at three different days by using different batches of LightCycler premixes to create a gene specific standard curve as indicated above.

Figure 3: a) Intravariance analysis within one run (n=3); b) intervariance analysis within different runs (n=3).

Fig. 3a

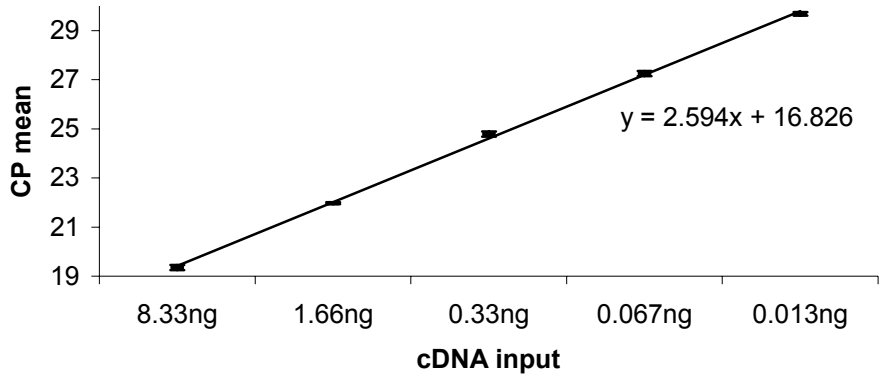
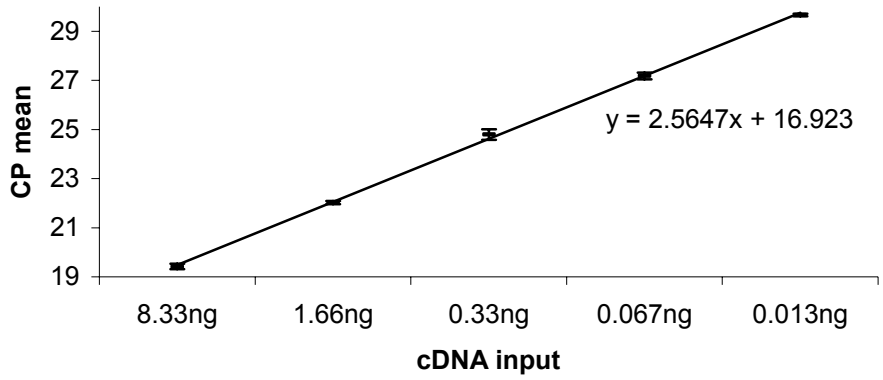


Fig. 3b



Variation tests were carried out with the ubiquitin primer set. Reproducibility (n=3) within one run were calculated by using CP mean variation of 0.35 % (absolutely 6.05 %).
Concerning the inter-assay variation a mean variation of 0.48 % (absolutely 8.40 %) could be detected. The calculation of test precision and variability is based on the standard deviation of CP mean values additionally converted by (($E^{\text{standard deviation}}$)-1)x100 % into absolute data.

6.4. Cloning of growth and differentiation factor 9

6.4.1. Partial cDNA sequencing

Sequencing of the bovine GDF-9 cDNA was performed using the SMART-RACE cDNA-Kit (Clontech, CA; for detailed description see manufacturer guidelines) introducing total RNA of bovine COC. 1 µg total RNA and 200U MMLV-reverse transcriptase (Promega, Mannheim, GER) was used for first strand synthesis in 5'- as well as 3'-direction. Resulting cDNA library was subsequently used for amplification of specific PCR products by introducing the described GDF-9 primers (see 6.6.3.) in a thermal cycler (Master Gradient Cycler, Eppendorf, Hamburg, GER) performing 25 cycles: denaturation at 94 °C for 5 sec, annealing 68 °C for 10 sec and elongation at 72 °C for 2:30 min. PCR products were sequenced commercially (TopLab, Martinsried, GER). Acquired bovine GDF-9 mRNA sequence was published in EMBL data base (AJ302697).

6.4.2. Plasmid generation

The cloning of the mature GDF-9 sequence (starting at the PACE restricting site up to stop codon TGA) was performed by using the pCR TOPO T7/TN cloning Kit (Invitrogen, Karlsruhe, GER). For generating the specific PCR-product (407 bp) of the mature GDF-9 protein the

matGDF-9 primer set (see 12.3.) was used in a touchdown block PCR including 2.5 µL of oocyte cDNA (2.5 ng/µL). The touchdown PCR was performed by graded annealing steps starting with 3 cycles at 64 °C, followed by 3 cycles at 63 °C. Final amplification was generated by 35 cycles at 61 °C. The resulting PCR product was cleaned up by Qiagens MiniElute Kit, following the user manual. Isolated DNA (68.8 ng/µL) was verified photometrically by OD 260/280 (OD=1.7). The TOPO Cloning reaction was performed with 250 ng specific PCR product, pCR T7/TN TOPO plasmid (containing HIS-Tag in front) and Invitrogens OneShot 10F cells (see user manual as indicated). 50 µL of transformed bacteria solution were plated on standard LB agar (1.5 %, see 6.6.5.) including 100 µg/mL ampicillin (C-0378, Sigma, Steinheim,) and cultured over night at 20 °C. After 12 h single colonies were recovered, inoculated in 5 mL of liquid LB medium including ampicillin as indicated and cultured at 37 °C. After 8 h cells were harvested and plasmid extractions were done by E.Z.N.A. Plasmid MiniPrep Kit I (PeqLab, Erlangen, GER). The sense orientation of the PCR product in each plasmid was verified by a control PCR by using the following primer set: T7-TOPO forward and matGDF-9 reverse (see 6.6.3.). If the sequence was integrated correctly in sense direction, a PCR product of 592 bp was generated. Afterwards, plasmids were additionally sequenced (Medigenomics, Martinsried, GER) to evaluate potential base mismatches.

6.4.3. Expression of recombinant protein

For protein expression, BL21(DE)pLysS OneShot E. coli cells (Invitrogen, Karlsruhe, GER) were transformed using 10 ng plasmid DNA (see user manual). Transformed cells were plated on LB-Agar (1.5 %, see 6.6.5.) including ampicillin (100 µg/mL), chloramphenicol (34 µg/µL) and 1 % glucose and were cultured over night. Separated colonies were rescued and transferred into 5 mL liquid LB medium including indicated antibiotics and glucose and were cultured for 12 h. For protein expression cells were diluted 1:50 in 50 mL fresh liquid LB medium and grown up to mid log phase (OD_{600}=0.4). Protein expression was induced by adding IPTG (Sigma, Steinheim, GER) to a end concentration of 1 mM. The protein expression dynamics were monitored up to several hours.

6.4.5. Isolation of recombinant natGDF9-protein by IMAC

After 4 h of IPTG induction the expression cells were harvested, centrifuged (10,000 g; 4 °C; 10 min) and the cell pellets were stored at –80 °C until protein extraction. In front of protein extraction, cell pellets were thawed on ice, resuspended in Buffer B (see 6.6.6.) and lysed for 60 min at RT until lysate has become clear. Lysate was centrifuged to separate cell debris. For

immobilized metal affinity chromatography (IMAC), 1 mL of the cell lysate supernatant was mixed with Buffer B equilibrated to Ni-TNA resin (Qiagen, Hilden, GER) and incubated for 60 min at 4 °C on a rotary shaker. After binding of the HIS-Tag to the nickel-ion of the agarose resin has occurred, the mixture was mounted on a gravity column. After two washing steps with Buffer C (see 6.6.6.) which removes unbound proteins, the recombinant protein was eluted four times with Buffer D (see 6.6.6.) and additionally four times with Buffer E (see 6.6.6.).

6.4.6. SDS-poly acryl gel electrophoresis

For evaluation of protein purification, the protein samples were separated on a SDS poly acryl gel. Therefore, 14 µL of protein solutions, 5 µL of 4x SDS sample buffer (see 6.6.7.) and 1 µL DTT (1 M) were mixed by vortexing, boiled for 5 min at 95 °C, introduced completely into gel chambers (NuPAGE 4-12 % Bis-Tris gel, Invitrogen, Karlsruhe, GER) and electrophoretically separated for 50 min in MOPS buffer (see 6.6.8.) at 190 V in a Novex gel chamber (Invitogen, Karlsruhe, GER). For visualization of protein bands the gels were stained directly by Coomassie solution (see 6.6.9.) for 1 h, bleached over night in a methanol:acetic acid solution (see 6.6.10.), and digital imaged as indicated. For more sensitive stainings, gels were blotted to PVDF membranes at 30 V for 50min in the Novex gel chamber and transfer buffer (see user manual). Additionally, membranes were air-dried for 1 h, rehydrated in PBS-TWEEN (0.1 %), stained with acetic 0.1 % India ink solution in PBS-TWEEN (0.05 %) for 1 h at RT and decolorized in Ultra pure water over night at 4 °C.

6.4.7. Dialysis of protein solution

The denaturating condition with 8 M urea during protein extraction and isolation requires a dialysis of the protein extract. Therefore, dialysis membranes (cutoff 3.5 kD, Reichert Chemie, GER) were cut into peaces (3x3 cm), boiled 30 min in 0.5 M EDTA, washed three times and finally stored in ultra pure water at 4 °C until use. For dialysis, 1.5 mL tubes containing protein solution were locked with the prepared dialysis membranes. Solutions were adapted to PBS (pH 7.2, see 6.6.2.) over night at 4 °C. After dialysis, total protein concentration were quantified by BCA test protocol (Sigma, Steinheim, GER) according to BSA standards by using Biophotometer (Eppendorf, Hamburg, GER).

6.5. Histological examinations

6.5.1. Tissue preparation

To support the results of mRNA analysis, histological examinations were done. Therefore different tissues or cell types have to be prepared. Immature and cultured bovine COC and ovary were embedded in TissueTek (Miles Inc., Torrance, CA) and snap-frozen in liquid nitrogen. Serial cross sections (COC: 14 µm, ovary 7 µm) were performed by a cryotom (HM 505E, Microm, Walldorf, GER) and mounted on preheated (37 °C) HistoBound glass slides (Marienfeld, Lauda-Königshofen, GER). GCs were grown and treated directly on sterilized uncoated glass slides provided with flexiPERM chambers (Vivascience AG, Hannover, GER). Prior to specific staining, all cryosections as well as cultured GCs were air dried for 30 min at 37 °C. For HA localization all tissues were fixed with ethanol for 5 min at –30 °C, whereas samples for immunohistochemistry were fixed for 30 min with 3.7 % formaldehyde (FA) in PBS.

6.5.2. Localization of hyaluronan

For HA localization all tissues were incubated for 30 min with 5 ng/mL biotinylated HA binding protein (bHABP; Cat. 385911, Calbiochem, CA) diluted in PBS. Unbound bHABP was removed by washing twice in PBS. To detect bound bHABP, slides were treated 30 min with a streptavidin fluorescein conjugate (SAF, 1 µg/mL; Cat. 189734, Calbiochem, CA), washed twice in PBS, counterstained 5 min with propidium iodide in PBS (2.5 µg/mL), and mounted in antifading solution Citiflour AF1 (Agar Scientific, UK). Negative controls were performed either without bHABP or without SAF.

6.5.3. Immunolocalization of different antigens

Immunolocalization of bovine HA receptor CD44 was accomplished by using rat-anti-porcine CD44 IgG (dilution 1:100 in PBS; SM488, lot 130900, DPC Biermann, Bad Nauheim, GER) as first antibody and horseradish peroxidase (HRP) labeled goat-anti-rat IgG (dilution 1:200 in PBS, R1378HRP, lot 6563, DPC Biermann, Bad Nauheim, GER)

The presence of VEGF was detected by specific anti-VEGF rabit IgG (dilution 1:2000 in PBS; a kindly gift from Prof. Schams, Technical University of Munich) and visualized by HRP-labeled goat-anti-rabbit IgG (dilution 1:200 in PBS; Sigma, Steinheim, GER).

After fixation in FA, samples were washed twice in PBS and incubated for 30 min in 1 % H_2O_2 to block endogenous peroxidases. Non specific background staining was reduced by treatment

with 10 % normal goat serum for 30 min. First antibody incubation was done over night, followed by three washing steps in PBS and incubation with HRP-labeled IgG for 1 h. Specific staining was visualized by 0.05 % diaminobenzidine (DAB, Sigma, Steinheim, GER) in 0.01 % H_2O_2. Additionally, samples were counterstained by Mayers Haemalaune (Merck, Haar, GER), dehydrated with graded ethanol solutions and at least embedded in Eukitt (Kindler, Freiburg, GER). Specificity of the immunodetection was proved by several negative controls: a) replacement of the first antibody, b) replacement of secondary antibody, c) incubation with DAB alone to show efficiency of blocking endogen peroxidases and additionally d) pre-absorption of the first antibody involving the respective antigen VEGF165 [184].

6.5.4. Visual documentation of histological examinations

All samples were observed with an Axioscope microscope (Zeiss, Jena, GER) and documented by digital imaging (AxioCam MR with AxioVision software; Zeiss, Jena, GER) or color slides (Kodak Elite 100).

6.6. Buffers and primers

6.6.1 Modified Parker´s Medium 199 (MPM199)

Chemicals	**Concentration**	**Provider**
M199	15.0 mg/mL	Sigma, Steinheim, GER
sodium bicarbonate	2.2 mg/mL	Sigma, Steinheim, GER
Gentamycin	50 µg/mL	Selectevet, GER
Pyruvate	0.23 mg/mL	Serva, GER
ascorbic acid	50 µg/mL	Sigma, Steinheim, GER
calcium lactate	100 µg/mL	Merck, GER

MPM199 was adjusted to pH 7.2 by NaOH and HCl and sterilized by vacuum filtration with bottle neck filters (0.22 µm, Millipore, GER).

6.6.2. Phosphate buffered saline (PBS)

Chemicals	Concentration	Provider
KH_2PO_4	0.24 g/L	Merck, GER
NaCl	8.0 g/L	Merck, GER
KCl	0.2 g/L	Merck, GER
$Na_2HPO\ 2H_2O$	1.44 g/L	Merck, GER

PBS was adjusted to the required pH 7.2 by NaOH and HCl.

6.6.3. List of used primers

Target Gene	Forward primer sequences (5´ to 3´) Reverse primer sequences (5´ to 3´)	FL	AT	QT	EMBL* / Ref.+
18S	AAG TCT TTG GGT TCC GGG GGA CAT CTA AGG GCA TCA CA	365 bp	60 °C	82 °C	AF176811
Ubi	AGA TCC AGG ATA AGG AAG GCA T GCT CCA CCT CCA GGG TGA T	198 bp[a]	60 °C	85 °C	Z18245
ARO	CAT CAT GCT GGA CAC CTC TAA C ATG TCT CTT TCA CCA ACA ACA GTC	457 bp	62 °C	80 °C	U18447
HSD	TAC CCA GCT GCT GTT GGA ATG CCG TTG TTA TTC AAG GC	322 bp	60 °C	84 °C	X17614
FHSR	GAG AGC AAG GTG ACA GAG ATT CC CCT TTT GGA GAG ACT GAA TCT T	343 bp	61 °C	81 °C	L22319
LHR	GAT AGA AGC TAA TGC CTT TGA CAA A CCA GAA TGA AAT TAA ATT CAG AGG AG	198 bp	61 °C	79 °C	U41414 / U41413
HAS1	GGT ACA ACC AGA AGT TCC TGG G CGG AAG TAC GAC TTG GAC CAG	184 bp	55 °C	79 °C	AB017803 *AB017803*
HAS2	GGM TGT GTC CAG TGC ATT AGC GGA C CAG CAC TCG GTT CGT TAG RTG CCT G	144 bp	68 °C	80 °C	U54804 *BTA004951*
HAS3	ACA GGT TTC TTC CCC TTC TTC C GCG ACA TGA AGA TCA TCT CTG C	166 bp	60 °C	80 °C	AJ293889 *AJ293889*
cd44	TAT AAC CTG CCG ATA TGC AGG CAG CAC AGA TGG AAT TGG G	221 bp	64 °C	83 °C	X62881
RHAMM	TGT TGA ATG AAC ATG GTG CAG CTC CCT TAG AAG GGT CAA AGT GTT TGA T	249 bp	61 °C	77 °C	AF310973 *AJ439694*
VEGF	TGT AAT GAC GAA AGT CTG CAG TCA CCG CCT CGG CTT GTC ACA	190, 318, 390, 441 bp	58 °C	-	Garrido et al. 1993
flk-1	AGA CTG GTT CTG GCC CAA C GAA GCC TTT CTG GCT GTC C	379 bp	62 °C	-	Gabler et al. 1999
flt-1	CAC CAA GAG CGA CGT GTG AAG AAG TCC TCG GAG AAG GC	351 bp	62 °C	-	Gabler et al. 1999
CX26	GTG CAA GAA TGT GTG CTA CG CAC GGA AGA AGA TGC TGC	254 bp	60 °C	83 °C	U17592

CX32	AGT CCA GGG GAG GGA CTG CTG CCT CAT TCA CAC CTG TG	363 bp	61 °C	86 °C	X95311
CX43	CTT GCA AAA GAG ATC CCT GC TAA TTG GCC CAG TTT TGC TC	381 bp	61 °C	82 °C	NM_174068
GDF-9	ATG CAA CCT GGT GAT AAA AGA GCC AGA G CTC TTG TGG GAG GCC ACC AGA GG	140 bp	69 °C	80 °C	AF078545 *AJ302697*
matGDF-9	GAC CAG GAG AGT GTC AGC TCT GAA T TTA ACG ACA GGT ACA CTT AGT GGC	407 bp	60 °C	-	AJ302697
BMP-15	CAA CTA GTC AGAGCCACT GTG G AGT GTC CAA TGATGA AGT GC	306 bp	62 °C	84 °C	AF236079 *AJ534391*
BMPR-2	CAG AGG TTG GAA ATC ATC TCC CCT ATA GTC AAA GGT	407 bp	59 °C	82 °C	Regazzoni et al. 2001 *AJ534390*
BMPR-1A	TTG CTG TAT TGC TGA CCT G TTT CAC ACA CAC AAC CTC	307 bp	57 °C	80 °C	Regazzoni et al. 2001 *AJ550059*
BMPR-1B	CAC TCC CAT TCC TCA TC TCA CAG CCA CCT TTT C	444 bp	57 °C	82 °C	Regazzoni et al. 2001 *AJ534389*
T7-TOPO.for	TAA TAC GACTCA CTA TAG GG	-	54 °C	-	TOPO Clonino Kit, Invitrogen, GER

***** source for primer design: accession number of EMBL data base, submitted new bovine sequences are indicated by *italic letters*

[+] reference of primer which were adopted from literature

[a] predominant product in real-time PCR (dependent on short extension time in PCR), further fragment lengths: 426 bp or 654 bp

AT = annealing temperature in real-time PCR

FL = fragment length of PCR product

QT = quantification temperature in real-time PCR

All primers were synthesized by MWG (GER) and stored as stock solutions (200 µM) in 10 mM Tris (pH 8.0) at -20 °C. For use stocks were diluted 1:10 in PCR water.

6.6.4. Electrophoresis buffer for DNA gels

Chemicals in Ab	**Concentration**	**Provider**
Bromphenol blue	15 µL	
Glycerol	100 µL	
Ultra pure water	85 µL	

6.6.5. LB medium/agar (Luria-Bertani)

Chemicals in Ultra pure water	Concentration	Provider
Tryptone Peptone	10 g/L	Difco Lab, USA
Yeast extract	5 g/L	Difco Lab, USA
NaCl	10 g/L	Merck, GER
Ampicillin	100 µg/mL	Sigma, Steinheim, GER
Chloramphenicol	34 µg/mL	Sigma, Steinheim, GER

LB medium was adjusted to pH 7.4 by NaOH, sterilized and stored at 4 °C. Antibiotics were added in front of use. If necessary, LB medium were mixed with agar (15 g/L, Difco Lab, USA), autoclaved and plated in culture dishes (90 mm, Greiner, GER).

6.6.6. Buffers for protein isolation

Chemicals in Ultra pure water	Concentration	Provider
100mM NaH2PO4·H2O	13.8g	Merck, GER
10mM TrisCl base	1.2g	Roth, GER
8M urea	480.5g	Merck, GER

For use, the standard lysis buffer was adjusted to different pH by NaOH and HCl: Buffer B (pH 8.0), Buffer C (pH 6.3), Buffer D (pH 5.9) and Buffer E (pH 4.5).

6.6.7. 4x SDS sample buffer for SDS PAGE

Chemicals in Ultra pure water	Concentration	Provider
Sucrose	0.4 g /mL	Merck,GER
Tris Base	68.2 mg/mL	Roth, GER
Tris HCl	66.6 mg/mL	Roth, GER
SDS	80 mg/mL	Roth, GER
EDTA	0.6 mg/mL	Merck, GER
Serva Blue G250 (1 % stock solution)	75 µL	Serva, GER
Phenol Red (1 % stock solution)	25 µL	Serva, GER

Sample buffer was stored at 4 °C and was heated to 30 °C before use to become completely dissolved.

6.6.8. 10x MOPS SDS PAGE running buffer

Chemicals in Ultra pure water	**Concentration**	**Provider**
MOPS, 3-(N-morpholino) propane sulfonic acid	104.6 g/L	Roth, GER
Tris Base, Tris(hydroxymethyl)aminomethan	60.6 g/L	ICN, ???
SDS, sodium dodecyl sulfate	10.0 g/L	Roth, GER
EDTA, Ethylendinitrilotetra acetic acid	3.0 g/L	Merck, GER

10x MOPS buffer was stored at 4 °C and heated to 30 °C before use to become completely dissolved. 1x MOPS should be at pH 7.7. It is not necessary to adjust pH.

6.6.9. Coomassie staining solution

Chemicals in Ultra pure water	**Concentration**	**Provider**
Brilliant Blue R	2.5 mg /mL	Sigma, Steinheim,GER
Methanol	47.5 %	Merck, GER
Acetic acid	10.0 %	Merck, GER
Ultra pure water	42.5 %	

6.6.10. Coomassie bleaching solution

Chemicals in Ultra pure water	**Concentration**	**Provider**
Methanol	34.6 %	Merck, GER
Acetic acid	7.7 %	Merck, GER
Ultra pure water	57.7 %	

7. Results and Discussion

7.1. Steroidogenesis during IVM of bovine COC

In the present study the propagated steroidogenesis in bovine COC during artificial maturation under FSH+LH was examined [240].

Initial experiments using RT-PCR showed remarkable amounts of ARO and HSD. Both key enzymes of the steroidogenic pathway were detectable at all stages of IVM [240]. The initial high levels of ARO declined dramatically to 0.3 % of onset levels after several hours (Fig. 4a). Additionally, the HSD mRNA levels decreased during IVM too, but did not show this excessive drop like demonstrated for ARO [240]. The outcome of steroid hormone detection mirrored mRNA expression dynamics in part (Fig. 4b/c). Consequently, high levels of E2 (Fig. 4c) were detectable shortly after the onset of IVM, which declined slightly in parallel to ARO until the end of IVM. Although the P4 ratios slowly increased during the duration of IVM, no significant levels were detecable (Fig. 4b).

In contrast to the COC, in GC cultures a gonadotropin treatment showed significant alteration in steroid hormone secretion. According to the luteinizing progress of the GC, the untreated control cells started to produce P4 without any exogenous stimuli and indicated a lack of E2 within a time period of 24 hours [240]. Whereas solely treatment with gonadotropin induced a measurable increase in E2, reaching significant levels after 24 hours by treatment with LH as well as with FSH [240]. But supplementation of both gonadotropins togetehr did not cause an additive effect but an inhibition of steroid hormone secretion compared to single treatment. After administration of FSH+LH the accumulation of E2 as well as of P4 was found reduced, whereas P4 was affected at a lower excess. The treatment with LH alone induced the highest P4-synthesis, followed by FSH and the control.

According to T only time dependant efffects could be detected showing slight increases in COC after 12 hours of maturation and after 24 hours in GC cultures of all treament groups [240].

Figure 4: a) m RNA levels of ARO, b) P4- and c) E2-concentrations in IVM media of maturing COC (n=6). Data were analyzed by one way ANOVA with Student-Newman-Keuls Method and depicted as means±SD. Statistical significance is indicated by letters ($P<0.05$). The charcoal treated medium served as control (n.d. = not detectable).

Fig. 4a

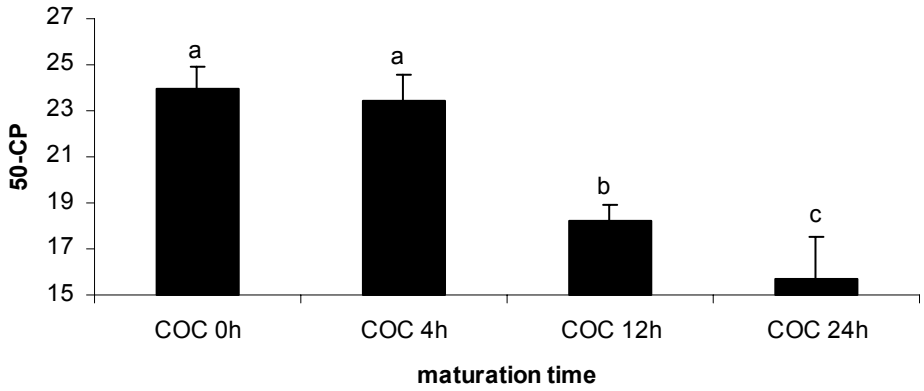


Fig. 4b

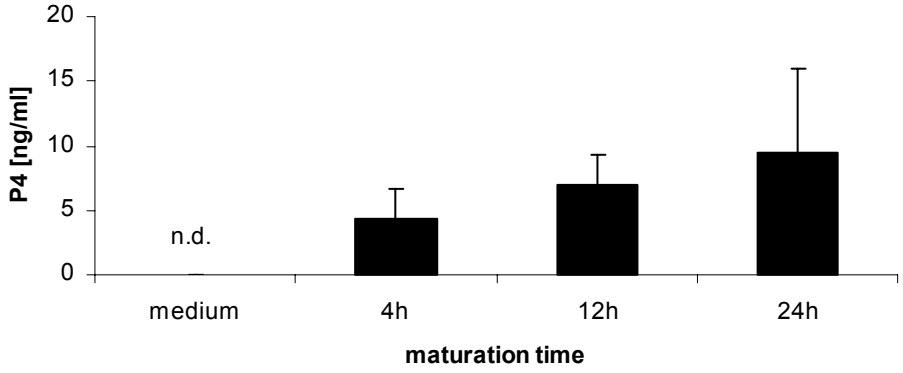
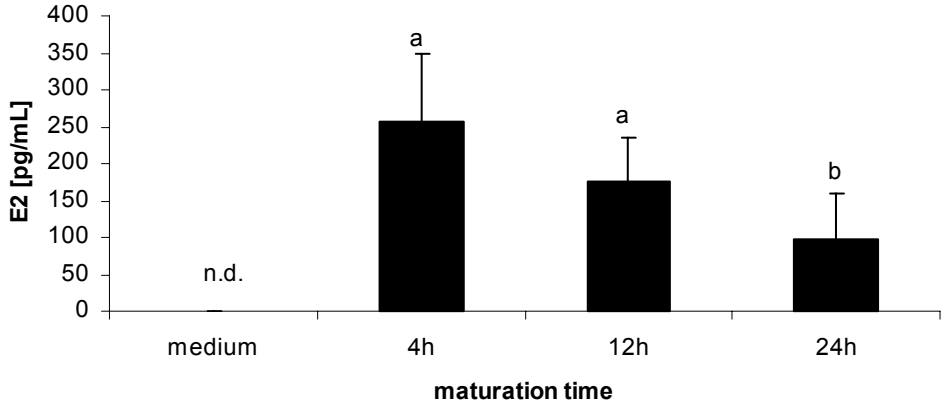


Fig. 4c



All these results suggest that the bovine COC are able to produce their own steroidogenic environment [240] and that it is not categorically required to supplement e.g. E2 to the maturation medium [106]. The importance of the steroids during oocyte maturation were shown by Osborn and Moore using in vitro cultures of sheep follicles [241]. They have shown a direct association of the resumption of meiosis and sequential changes in follicular steroidogenesis. The supplentation of steroid enzyme inhibitors to isolated follicles altered the normal profile of steroids secreted during maturation and induced intracellular changes in the oocytes identified as nuclear abnormalities at fertilization. Additionally, they reported initial high E2 levels, which were followed by a suppression of the secretion. In parallel, a continuous increase in P4 ratios was examined [241]. Similar results were shown for goats as well. Here, 18 h after the administration of hCG to isolated follicles, E2 decreased markedly, whereas P4 remained significantly higher in equine chorionic gonadotropin (eCG) treated follicles [242]. Comparing previously established whole follicle cultures to IVM, the switch in steroid synthesis takes place in COC too [240], which has also been shown in rats. Wojtowicz and coworkers have investigated that rat COC cultures produced predominantly E2 in the first hours, whereas P4 was found increased just in the late culture phase after 24h [243].

Steroidogenesis occurs in vivo within the entire follicle depending on its developmental stage as well as the gonadotropin status. Therefore, the different types of cells, the theca and granulosa cells, fulfill in collaboration the synthesis of P4, T and E2. Data of preovulatory bovine follicles in front of and after the LH surge as well as up to ovulation indicate almost comparable steroid patterns [244] as shown for the IVM in the present study. Additionally, the administration of steroids to the presented cell culture system could cause also unintentional effects, because unbalanced and/or extreme high levels could inhibit oocyte maturation and influence their own steroid hormone production [105].

A lot of efforts were made to improve the developmental capacity of in vitro matured COC, either by co-culture with other cells (such as granulosa, theca or oviduct epithelial cells) or modification of the culture medium by chemically defined ingredients or supplementation of diverse steroid hormones. Tesarik and Mendoza ascribed E2 a beneficial effect on the calcium liberation system during cytoplasmic oocyte maturation in humans, which in turn allows the typical calcium oscillation occurring during fertilization [100]. However, it is still unclear whether E2 should be added or not to the maturation medium for oocyte maturation in vitro, because E2 biosynthesis has been demonstrated in cultures of bovine granulosa cells [104]. Similar results has also been shown for cumulus cell cultures of various species such as humans [106], rats [107;243], rabbits [245] and pigs [108;246;247].

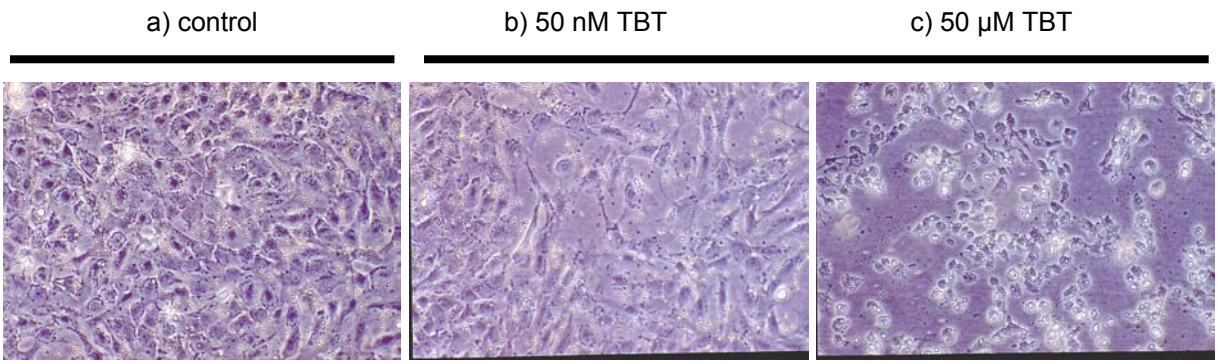
It could be concluded that the isolated COC are able to modulate their artificial surrounding concerning the steroid hormones comparable to an in vivo like manner.

7.2. Effects of TBT on steroidogenesis during IVM and GC culture

The importance of balanced steroid hormone levels is shown by the existence of endocrine disruptors, able to interfere with the endocrine system in almost all animals or humans. With respect to the long half-life of many substances, a lot of manmade chemicals still persist in the environment, although their use has been restricted for many years. A lot of substances, such as PCB or other xenoestrogens affect the hypothamalus-pituitary-gonadal axis as well as directly ovarian functions in ruminants [248;249]. The biocide TBT, cytotoxic in high doses, is able to modulate in very low doses hormonal patterns, leading to imposex syndromes (pseudohermaphroditism) in water snails at doses lower than 5 pg/mL [250]. Additionally, TBT shows immunotoxicity to human natural killer cells causing a decreased binding capacity to tumor cells [251]. Furthermore, induction of apoptosis in rodent thymocytes [252;253] and irritations of skin as well as lung epithelia [254;255] were reported. Although no data are available about a potential accumulation of such substances in the ruminants, the risk of exposure by uptake of contaminated water or nutriments is possible, because TBT is stable for years in sediments of rivers or lakes.

In the present study first results were presented about possible influence of TBT on steroidogenesis in cultured GC and COC during IVM [240]. In order to recover cytotoxic effects of TBT, GC were initially incubated with different TBT-concentrations and were visually characterized. The effects of TBT were shown in GC cultures at a wide range of concentrations (Fig. 5). The control cells (Fig. 5a) were tightly attached to the bottom of the culture dishes and were well proliferated. Incubation with 50 nM TBT (Fig. 5b) caused a visible reduction in cell growth, but the cells were still attached and showed normal shape. Higher concentrations (50 µM) of TBT led to restricted cell growth, indicated by detached cells and unusual appearance (Fig. 5c). Additionally, the following total RNA extraction of treated cells emerged in dramatic decreased amounts of RNA in the cells incubated with 50 µM TBT, whereby GCs treated with 50 nM TBT did not diverge from control (data not shown).

Figure 5: GC cultured a) without TBT (control), b) with 50 nM TBT, and c) with 50 µM TBT



On the basis of these results the TBT concentration was adapted to the different concentrations in the GC culture experiments [240]. First results indicated that 50 pM TBT reduced E2 levels in GC below detection limit, accompanied with increased P4 concentration. In contrast, the double amount of TBT (100 pM) showed slightly decreased E2 concentrations compared to control cells. Additionally, P4 did not differ significantly from control as well. Furthermore, TBT caused neither time- nor concentration-dependant alteration of T-levels when compared to control. Recent studies postlated that TBT inhibits the conversion of androgens to estrogens and therefore T secretion should increase [256;257]. However, in the present system no significant alteration of the T levels was induced by TBT possibly indicating that T is not the predominant substrate for the E2 synthesis. It is known that ARO converts in the same way androstendion to estron. Therefore, additional experiments have to be done to elucidate possible effects of TBT on the androstendion pathway.

The most interesting result of the present study was that 50 pM TBT inhibited completely the production of E2 although ARO mRNA has been detected at higher concentration as compared to the control GC culture. In contrast, P4 concentration was increased by 50 pM TBT despite of lowered HSD mRNA transcripts. If the present results were compared to literature, these data support the findings of Heidrich and coworkers postulating that TBT blocks ARO through only moderate affecting HSD enzyme activity [257].

To further enlighten possible interactions between endogenous hormones and the pollutant, a co-treatment with FSH was introduced. FSH was used for different reasons: a) the known stimulatory effect of gonadotropins on the E2 production in GC [161;240], b) FSH receptor mRNA was much more abundant in GC than the LH receptor (data not shown), and at last c) LH receptor was not detectable in COC before 24 hours of IVM (data not shown).

It was exciting, that the highest TBT treatment (50 nM) led to an increase of all steroids in GC cultures additionally provided with FSH, but the previously measured effects of low TBT levels

were erased by a simultaneous application of FSH. A possible influence of TBT on the FSH receptor expression may be suggested in this context.

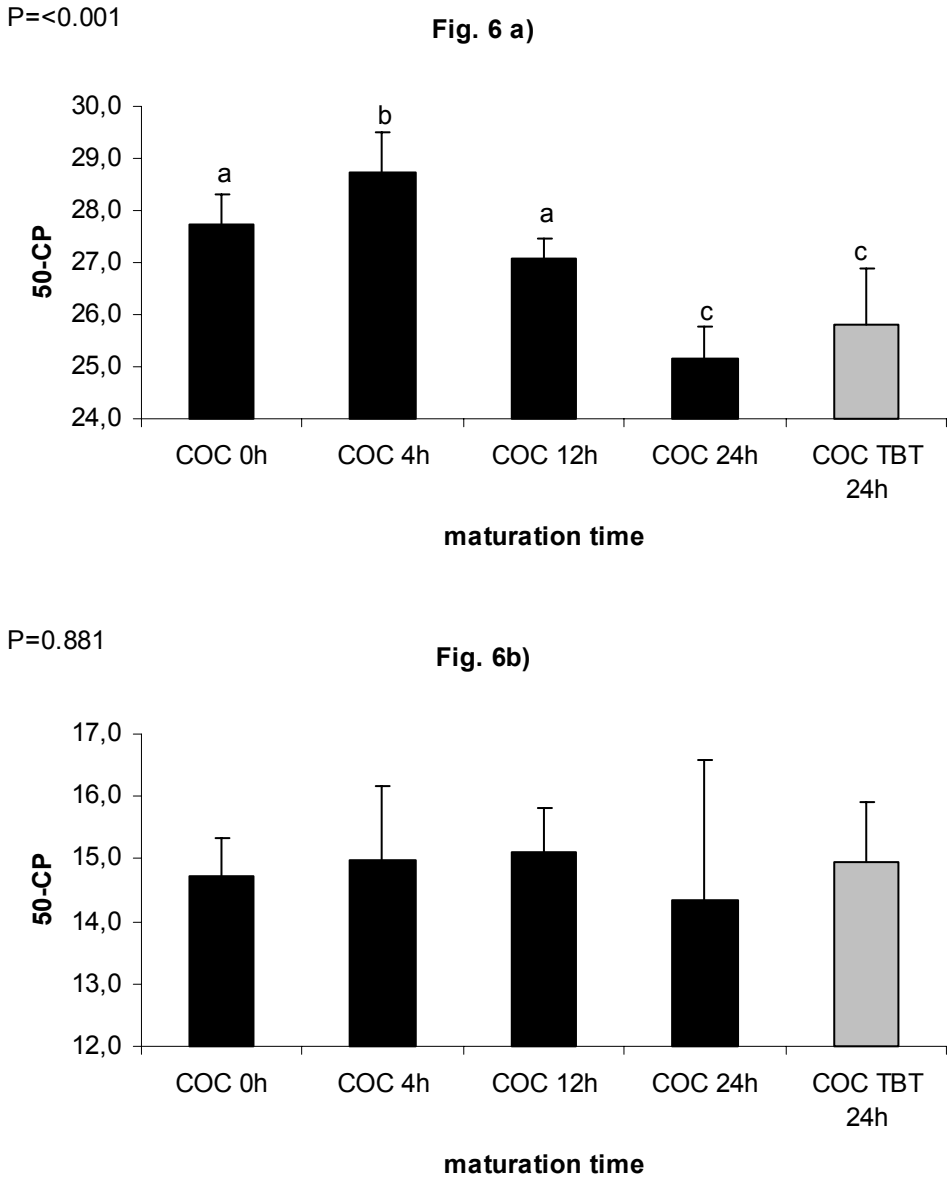
Although TBT alone caused lowered E2-levels at 50 pM, during a co-stimulation with FSH such effects were not detectable. Higher concentrations of TBT (50 nM) were necessary to evoke alterations in steroid ratios. Furthermore, it is possible that the ARO activity is stimulated by FSH-dependant regulatory mechanisms [108;258]. Therefore, our results indicate that in vivo effects of TBT could be much more complex as shown in the isolated cell culture experiments.

In summary, first experiments of administration of 50 pM TBT to IVM medium indicated a slight reduction in E2 concentration accompanied with significantly increased P4 levels after 24 hours. As shown previously these diverged steroid concentrations modulated the steroid synthesis it selves [105], influenced nuclear maturation of the oocyte [241], and affected Ca-liberation essential for successful fertilization [100]. It might be possible that such changing steroid levels not only influence the developing oocytes itself, furthermore the resting pool, limited early before birth, could be damaged irreversibly leading to an earlier dropout of fertility.

7.3. Presence of different connexins during IVM

The importance of the gap junctions is reflected by their appearance in the cell membranes. Almost 20 % of the surface of ovarian granulosa cells is occupied by gap junctions, indicating a significant role of cellular communication during ovarian developmental processes [259,74]. Furthermore, knockout studies of Cx43 revealed the physiological significance, demonstrating follicular arrest in preantral stages and degenerative abnormalities of the oocyte [132]. In vivo studies in cattle have shown dependencies between follicular stage and occurrence of diverse Cx proteins, especially Cx43, Cx32 and Cx26, therefore the mRNA dynamics of these three channel proteins were elucidated during IVM. In contrast to the in vivo studies of Johnson and coworkers [122] just Cx43 and Cx32 were detectable in maturing bovine COC (Fig. 6). Although a functional primer set was generated, no distinct Cx26 mRNA could be amplified by real-time PCR. This lack of Cx26 protein within follicular cells was also shown for toads (Bufo arenarum) [260].

Figure 6: mRNA levels of a) Cx43 and b) Cx32 in maturing COC (n=6). Data were analyzed by one way ANOVA with Student-Newman-Keuls Method and depicted as means±SD. Statistical significance is indicated by letters (P<0.05).



The existence of Cx 43, the most abundant connexin in the ovary, could be verified in the IVM system by RT-PCR too (Fig. 6a). Initial increases were visible about 4 hours after the onset of IVM, followed by a significant drop of Cx43 mRNA (about 8-fold) after 24 hours. It has been shown by functional and morphological studies in cattle that high maturation and development rates were accompanied by prolonged persistence of permeable communication, which is independent from cumulus expansion [261]. The interruption of communication led to lowered maturation efficiencies. Additionally, a decrease in cytoplasmic cAMP levels in cumulus cells caused a premature interruption of the cellular cross talk. The direct stimulation of cAMP on

cellular connection via Cx43 was also examined for cultured human GCs, indicating an increased permeability in presence of high cAMP levels [262]. Taken together with the results of the previous chapter of TBT treatment, the cAMP could be a possible factor affected by the introduced endocrine disruptor. For human natural killer cells a rapid drop in cAMP concentration after TBT exposure could be evaluated, resulting in a rapid decrease in the cytotoxic function [251]. By the way this proposed cAMP breakdown could happen in bovine cumulus cells as well. Therefore, a reduction of cAMP levels in response to TBT could be postulated for the bovine GC potentially causing a loss of the cAMP-triggered permeability of Cx43-channels. In spite of unchanged Cx43 mRNA expression in the COC in presence of TBT (Fig. 6b), additionally, a potential interuption of cellular connection may lead to a premature resumption of meiosis in the oocyte, due to the fact that high cAMP normally hold the oocyte in meiotic arrest [74;263]. This premature meiosis possibly cause an insufficient nuclear as well as cytoplasmic maturation. In contrast, in vivo the cAMP dependent meiotic arrest is naturally abolished by the LH surge, which is responsible for the interruption of the cell-to-cell communication. The analysis of the LH-effects on Cx43 gene expression revealed a significant decrease (45 %) in Cx43 mRNA level 24 h after incubation [130;264].

In vivo data of intact follicles indicate, that Cx43 protein was absent in primordial follicles, but was present in granulosa cells of primary/secondary and antral follicles [122]. Furthermore, Cx43 was present at the borders between granulosa cells and the oocyte. Expression of Cx43 increased in healthy developing antral follicles, but decreased during follicular atresia.

In contrast to Cx43, the Cx32 mRNA (Fig. 6b) was detectable in maturing COC without any significant changes. Compared to the mRNA expression of Cx43, Cx32 mRNA was less abundant. Johnson and coworkers examined that Cx32 was not present in healthy follicles but was present in granulosa cells of atretic antral, and especially small antral follicles [122]. Additinally, Cx26 was present in the oocyte of primordial and primary/secondary follicles, and in the granulosa and/or theca cell layers of healthy antral follicles.

It could be concluded that Cx43 and Cx32 but not Cx26 were present in the COC during IVM. Cx43 showed a time dependent decrease as well as a slight increase after 24 h TBT treatment. But anyhow, according to this discrepancies between in vivo and in vitro, more detailed protein information has to be generated to elucidate these different results between the present study and previous in vivo data of Johnson and coworkers.

7.4. Presence of NO synthases in bovine ovarian cells

In order to show the existence of NOS isoforms in bovine ovarian cells, an established GC culture as well as the IVM of bovine COC were used. Therefore, the GCs were pre-cultured for 1.5 days and treated with LH, FSH or FSH+LH by replacement of the entire culture media. COC underwent common IVM, supplemented with the same gonadotropin concentrations as used for GC culture. In order to show steroidogenic activity of the luteinizing GCs, steroid hormone determination in culture media was performed.

Table 1: Statistical analysis of effects of culture time and gonadotropin treatment on P4 and E2 synthesis as well as iNOS mRNA expression levels, done by two way ANOVA following Student-Newman-Keuls method and depicted as P-values.

	P4	**E2**	**iNOS**
culture time	P<0.001	P<0.001	P<0.001
Treatment	P=0.031	P<0.001	P<0.001
Co-variance	P=0.156	P<0.001	P<0.001

To detect significant influences of both culture time and treatment, a two way ANOVA was performed (Tab. 1), indicating, that the difference in the mean values of both P4, E2 and iNOS mRNA among the different levels of time as well as of the treatment were greater than expected. All three parameters showed high alterations according to both the culture time and the treatment. A significant difference appears in both time and treatment. Additionally, the co-variance analysis indicates significant interaction between the culture time corresponding to which level of treatment was present (Tab. 1).

Figure 7: a) P4, b) E2 hormone concentrations and c) iNOS mRNA-levels in cultured GC in response to gonadotropins. Dashed line indicates initial iNOS mRNA at the onset. Data were analyzed by two way ANOVA with Student-Newman-Keuls Method and depicted as means±SD. Significant differences ($p<0.05$) between groups are indicated by letter and between groups and onset by asterisks (*).

Fig. 7a)

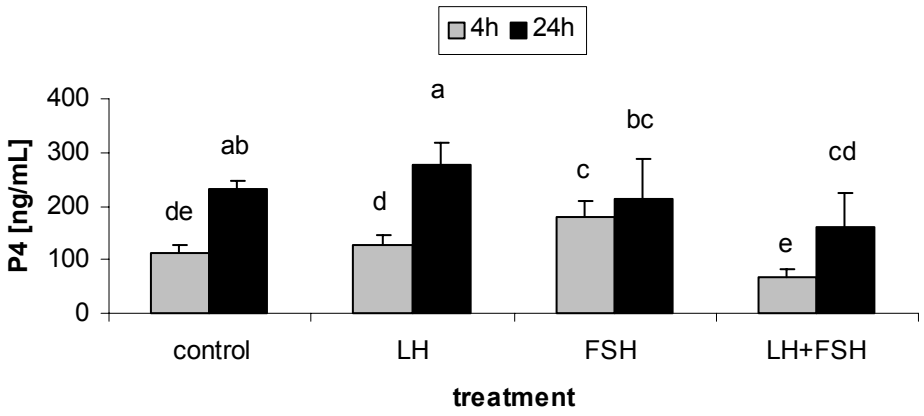


Fig. 7b)

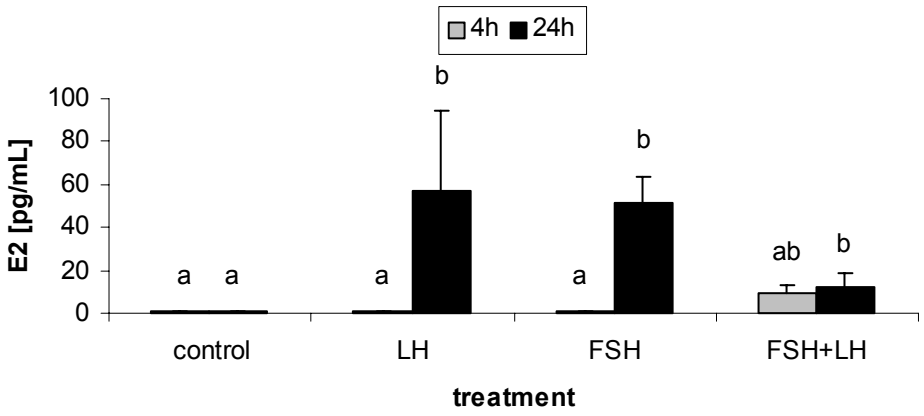
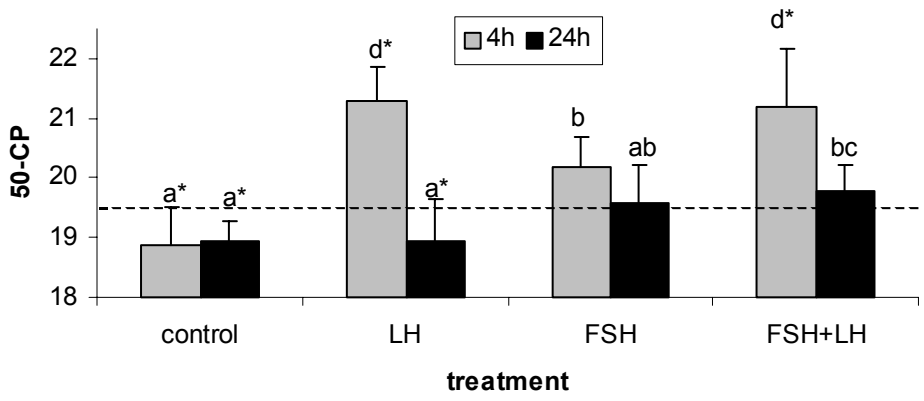


Fig. 7c)



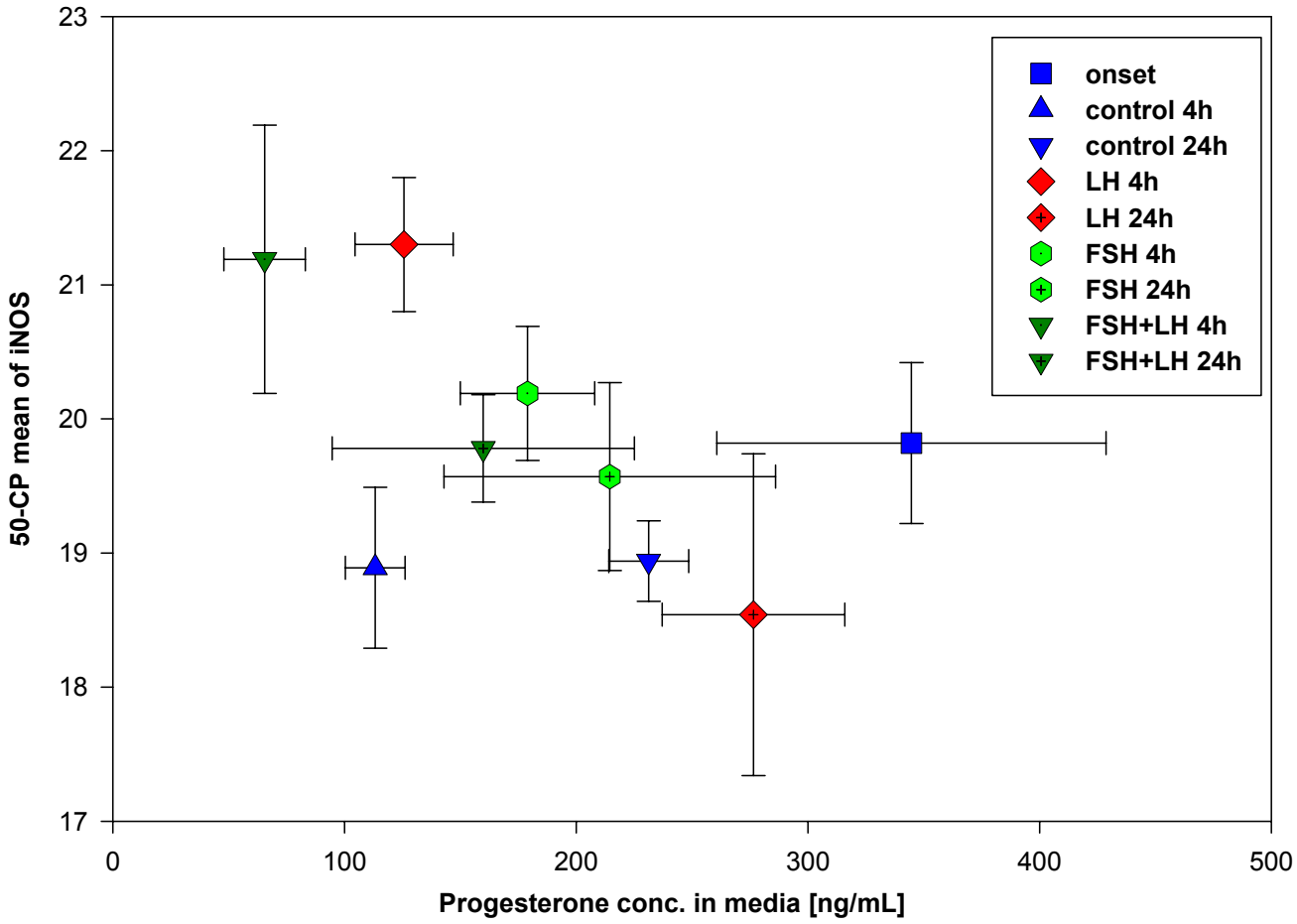
In Fig. 7 the steroid hormone secretion of cultured GC is illustrated in detail. In all cultures a significant increase in P4 was detectable, independent of treatment (Fig. 7a). Just slightly increased P4 concentrations were detectable between the control compared to the gonadotropin treatments. In this case only the supplementation of FSH+LH caused reduced P4 concentrations at any time, whereby LH and FSH alone induced minor increases compared to control. More abundant effects were examined for the E2 secretion (Fig. 7b). Without any stimulus of gonadotropins it was not possible to detect any E2 synthesis in GC cultures. LH and FSH alone caused almost the same increase of E2 after 24h. Additionally, the treatment of both gonadotropins in combination showing lower excess in E2 secretion as when they were applied solely. The iNOS mRNA expression (Fig. 7c) in the control culture was declining after the onset (dashed line) and stayed low up to 24h. Furthermore, without any gonadotropin stimulus the iNOS mRNA did not alter within 24 hours of culture, although P4 levels were increasing significantly, indicating luteinization of GC after their isolation out of the follicles (Fig. 7a). In contrast to the control, any treatment with gonadotropin led to a significant increase after four hours of iNOS mRNA, which peaked in the LH treated group. Treatment with FSH resulted in minor stimulation of iNOS expression, whereby co-stimulation caused neither an additive nor an inhibitory effect on the existence of iNOS mRNA, concerning to LH.

If iNOS mRNA is compared directly to the P4-levels, a possible negative correlating effect of P4 and E2 on the iNOS mRNA could be presumed. Therefore a correlation test between iNOS and the two steroid hormones was performed, indicating no confirmation of the presumption (Tab. 2).

Table 2: Spearman rank order correlation of iNOS versus P4 and E2.

	P4	**E2**
Correlation coefficient	-0.224	-0.0140
P value	0.0626	0.909

Figure 8: Scattered blot of iNOS mRNA [50-CPmean] versus P4 concentration [ng/mL]. Data are depicted as means±SD in both directions.



Thus, correlation between P4 and iNOS failed short of significance in the P-value, the data were prepared for a scattered plot to clarify the assumption of a negative correlation (Fig. 8). This kind of diagram depict the dynamics of both measurements in direct comparison.

Focused on the control samples (blue characters) it is obvious, that the iNOS mRNA levels were just slightly decreased after 4 hours according to the onset and stayed unchanged until the end of the experiment. In contrast, after 4 hours any treatment with gonadotropin led to an initial increase in iNOS expression, whereby the P4 concentrations did not diverge from control. After 24 hours iNOS levels in all groups reached almost the same extend. Additionally, all P4-levels were almost balanced after 24 hour. Thus, this depiction indicate, that the increase in iNOS was predominantly caused by the treatment with the gonadotropins and not by the low P4 values evoked by the replacement of the medium before onset of the experiment.

Concerning eNOS, the constitutive NOS isoform, no reproducible results could be generated for the GC culture by established primer sets in real-time RT-PCR (data not shown). Therefore, no or very low expression of eNOS mRNA may be expected for the present culture.

Dependency of iNOS mRNA expression on gonadotropins was also shown by other authors [265]. Nitrate/nitrite concentration are metabolic indicators for active NO synthesis and could be detected in follicular fluid of rats. These metabolites were decreasing significantly in the follicular fluid 5 and 10 hours after hCG injection in the rat, accompanied with reduced iNOS mRNA levels [142]. However, endothelial NOS (eNOS) mRNA expression was detected mainly in the theca cell layers and further increased 5 and 10 h after hCG injection but remained low in GCs. In connection with hCG stimulation, initial increases, comparable to the present results, of iNOS protein concentration as well as mRNA levels were described for experiments on rats [141;265]. Therefore, a similar gonadotropin dependent effect on NO synthesis in bovine GC could be postulated, which is consistent with other authors. Additionally, a possible role in ovulation could be ascribed to iNOS too, which was shown earlier by others [266;267]. Likewise, Lee and colleagues demonstrated that hCG could stimulate iNOS in non-ovarian cells as well [268].

Compared to the results of GC cultures, in artificial matured COC both iNOS and eNOS were detectable (Fig. 9). Here, in bovine COC both iNOS (Fig. 9a) and eNOS (Fig. 9b) mRNA were detectable by real time PCR. The iNOS mRNA levels (Fig. 9a) were initially high but declined significantly (about 8-fold) during maturation. Compared to the GC culture, such an initial increase after 4 hours was not detectable. Additionally, the excess of reduction in iNOS mRNA in COC were higher as in GC. Focusing on eNOS, a continuous presence of mRNA in maturing COC was detectable. Furthermore, a significant elevation of eNOS after 4h and 12h of IVM was examined.

As indicated in chapter 7.1. the bovine COC were able to produce steroid hormones as well under the influence of FSH+LH showing continuously increased P4 concentration, whereby E2 reached initial high levels after experimental onset [240]. In comparison to steroid synthesis of the COC, the expression of iNOS showed inverted characteristics to P4 concentrations. Therefore, a direct steroid dependency could be expected. But this context is controversely discussed in literature. For example, Yamagata and coworkers examined a negative correlating effect between the NO-inhibition and steroidogenesis, indicating increased P4- and E2-levels accompanying with decreased NO concentration in the follicular fluid in the rat [142]. Additionally, significantly reduced NO metabolites (nitrate/nitrite) in human follicles paralleled with higher fertilization rates of the mature oocytes [269]. Other authors postulate for the human system, that there is no significant difference in nitrate/nitrite concentration between different follicle stages indicating no NO alteration [270]. Furthermore, a positive correlation between nitrate/nitrite were examined for human [271]. According to this discrepancies between different

species, differential regulations may be assumed. Therefore, further studies have to be performed to compare these different species.

Figure 9: iNOS/eNOS mRNA-levels in COC during a 24 hours maturation. Data were analyzed by one way ANOVA followed by Student-Newman-Keuls Method. a) iNOS- and b) eNOS mRNA levels respectively were shown according to the maturation time. Data were depicted as means±SD. Significant differences ($p<0.05$) between groups are indicated by letters.

Fig. 9a)

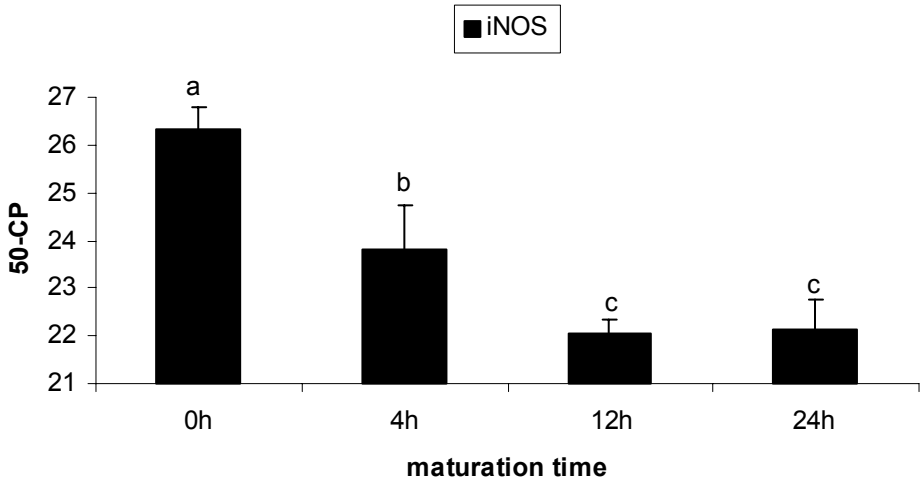
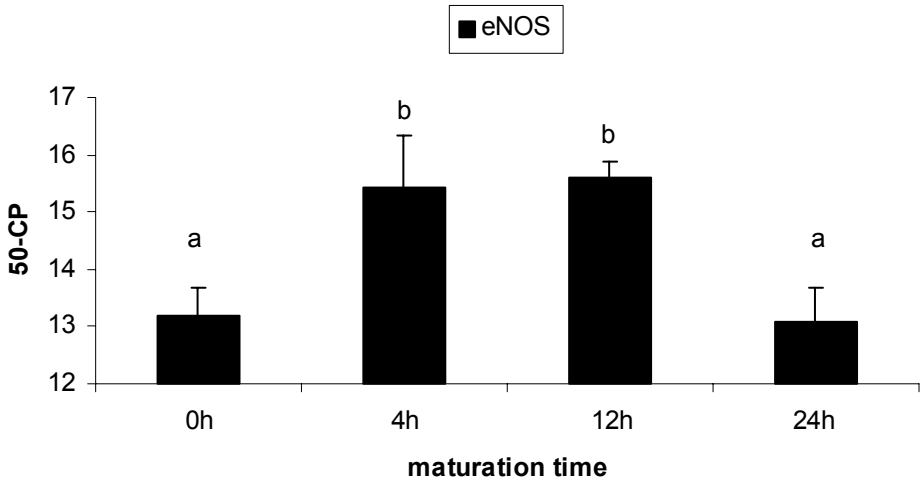


Fig. 9b)



In contrast to GC, the existence of the two isoforms iNOS and eNOS could also be shown by immunohistochemistry in mouse MII-oocytes as well as developing embryos [272]. The protein distribution of both was evenly or as fine granules in the ooplasm. Furthermore, NOS activity in oocytes was shown by the NADPH diaphorase assay, reflecting the distribution of the enzyme

activity in the whole ooplasm [272]. Additionally, Sengoku and coworkers reported a reduced resumption of meiosis in oocytes when exposed to selective NO-inhibitors [153].

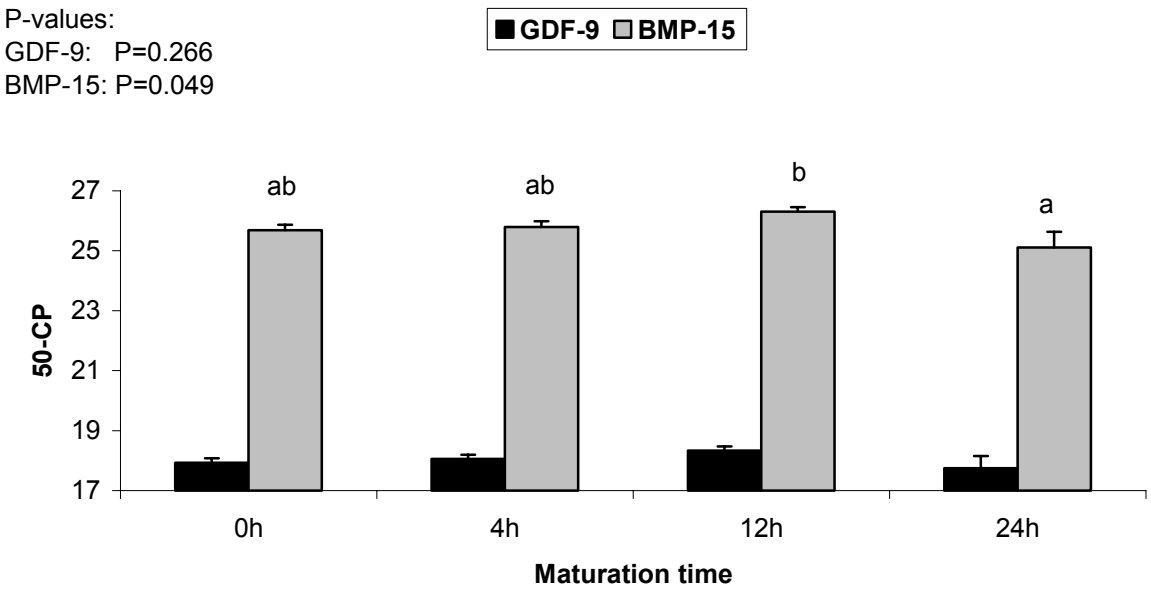
The present results indicate a maturation dependent regulation of the NOSs in the bovine system, but to demonstrade the protein activity and NO dependence additional experiments have to be performed. In summary, due to the decreasing iNOS mRNA expression a decrease of NO and its metabolites respectively is possible. Additionally, the up-regulation of eNOS (between 4 and 12 hours of IVM) in temporal context to the meiotic resumption and extrusion of the first polar body (between 6 and 10 hours of IVM) could indicate a mutual coherence of these two events.

7.5. GDF-9 and BMP-15 in maturing bovine oocytes

7.5.1. Expression of GDF-9 and BMP-15 in maturing COC

Currently, no data are available about the existence of GDF-9 and BMP-15 during artificial maturation of oocytes. Therefore, these two oocyte specific factors of the TGFbeta family as well as their potential receptors have be elucidated during IVM.

Figure 10: GDF-9 and BMP-15 mRNA-levels in maturing COC during 24 hours. Data were analyzed by one way ANOVA followed by Student-Newman-Keuls Method. Data are depicted as means±SD. Significant differences ($p<0.05$) between groups are indicated by letters.



Shortly after aspiration of COC out of the follicle, a basic level of GDF-9 (Fig. 10) could be detected lasting up to 24 hours of IVM without any changes (P=0.266). Likewise to GDF-9, BMP-15 mRNA levels stayed constant (Fig. 10) but were obviously higher expressed. Just a slight increase ($p<0.05$) of BMP-15 expression was recognizable after 12 hours of IVM. At any other time point of IVM, no changes in BMP-15 levels were observable compared to the onset. Faced together these two growth factors, the BMP-15 niveau exceeded the levels of GDF-9, and if the expression levels were correlated, a highly significant positive coherence (Tab. 3) between these two genes were observable.

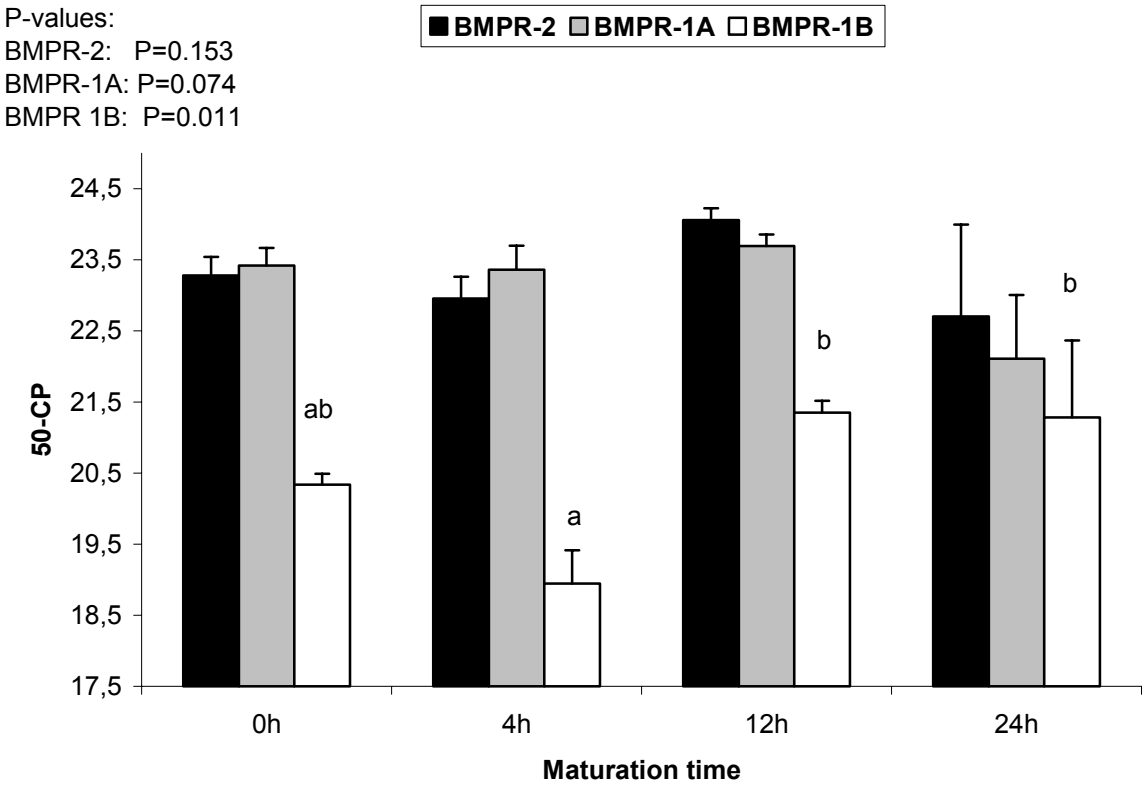
7.5.2. Expression of BMP receptors in maturing COC

Beside optimal processing and dimerization of the two described growth factors, the cascade of signaling requires additional a functional set of two receptor types named BMPR-1 and BMPR-2. Therefore mRNA levels of three potential receptors were quantified by real-time RT-PCR including the two subtypes BMPR-1A and –1B as well as BMPR-2. As indicated in Figure 11, BMPR-2 and 1A showed a parallel progression concerning the time course of IVM. These results were supported by high significant positive correlations (Tab. 3). In contrast, BMPR-1B showed lower mRNA levels as well as significant time dependent variations, showing a minimum after 4h of IVM.

Table 3: Correlations between target genes generated by Spearman Rank Order Correlation (r=correlation coefficient, P=P-value)

Matured COC	BMP-15	BMPR-2	BMPR-1A	BMPR-1B
BMPR-1B			r=0.555;P=0.005	
BMPR-2			r=0.873;P<0.001	r=0.759, P<0.001
BMP-15		r=0.730, P<0.001	r=0.683, P<0.001	r=0.618, P=0.001
GDF-9	R=0.927, P<0.001	r=0.737, P<0.001	r=0.729;P<0.001	r=0.603;P=0.002

Figure 11: BMPR-2, BMPR-1A and BMPR-1B mRNA-levels in maturing COC during 24 hours. Data were analyzed by one way ANOVA followed by Student-Newman-Keuls Method. Data are depicted as means±SD. Significant differences ($p<0.05$) between the groups of one target gene are indicated by letters.



7.5.3. Expression of BMP receptors in granulosa and theca cells

During final follicle development, the GCs as well as the TICs are under influence of both gonadotropins and steroid hormones and possibly showimg different gene expression patterns. Therefore the different follicular cell types were separated into GC and TIC at different stages of follicle development, indicated by the E2 concentration in the corresponding follicular fluid. Additionally, isolated GC were cultured and exposed to LH, FSH, and to a combination of both.

In the present study it could be demonstraded that in vivo all three receptors were present in the TIC as well as in GC. In TIC BMPR-2 and BMPR-1B showed unchanged levels at all stages of follicle development (Fig. 12). Only BMPR-1A (Fig. 12) displayed a significant alteration, peaking in large follicles (E2 >180 ng/mL). These result was underpinned by a positive correlation between BMPR-2 and 1B. In contrast, no significant coherence between the BMPR-1A was detectable to the two other receptors types (Tab. 4).

Figure 12: BMPR-2, BMPR-1A and BMPR-1B mRNA-levels in theca interna cells at different follicle stages. Data were analyzed by one way ANOVA followed by Student-Newman-Keuls Method. Data are depicted as means±SD. Significant difference ($p<0.05$) between the groups of one target gene are indicated by letters.

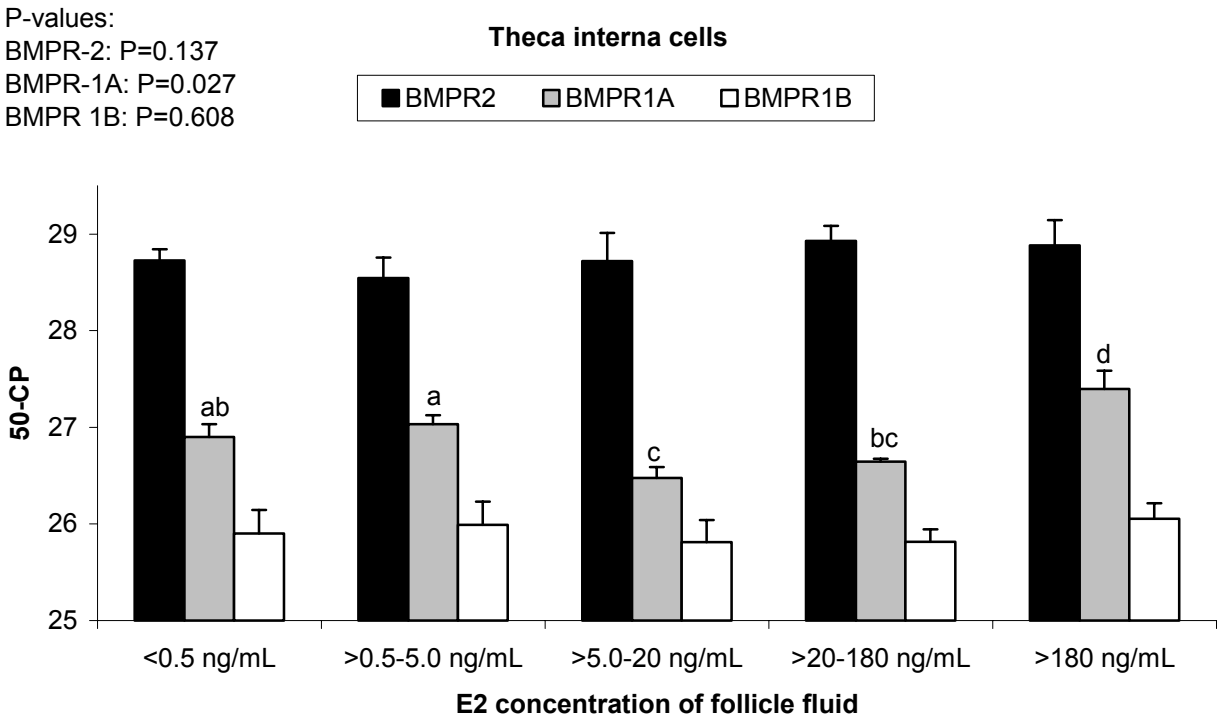


Figure 13: BMPR-2, BMPR-1A and BMPR-1B mRNA-levels in isolated granulosa cells at different follicle stages. Data were analyzed by one way ANOVA followed by Student-Newman-Keuls Method. Data are depicted as means±SD. Significant differences ($p<0.05$) between the groups of one target gene are indicated by letters.

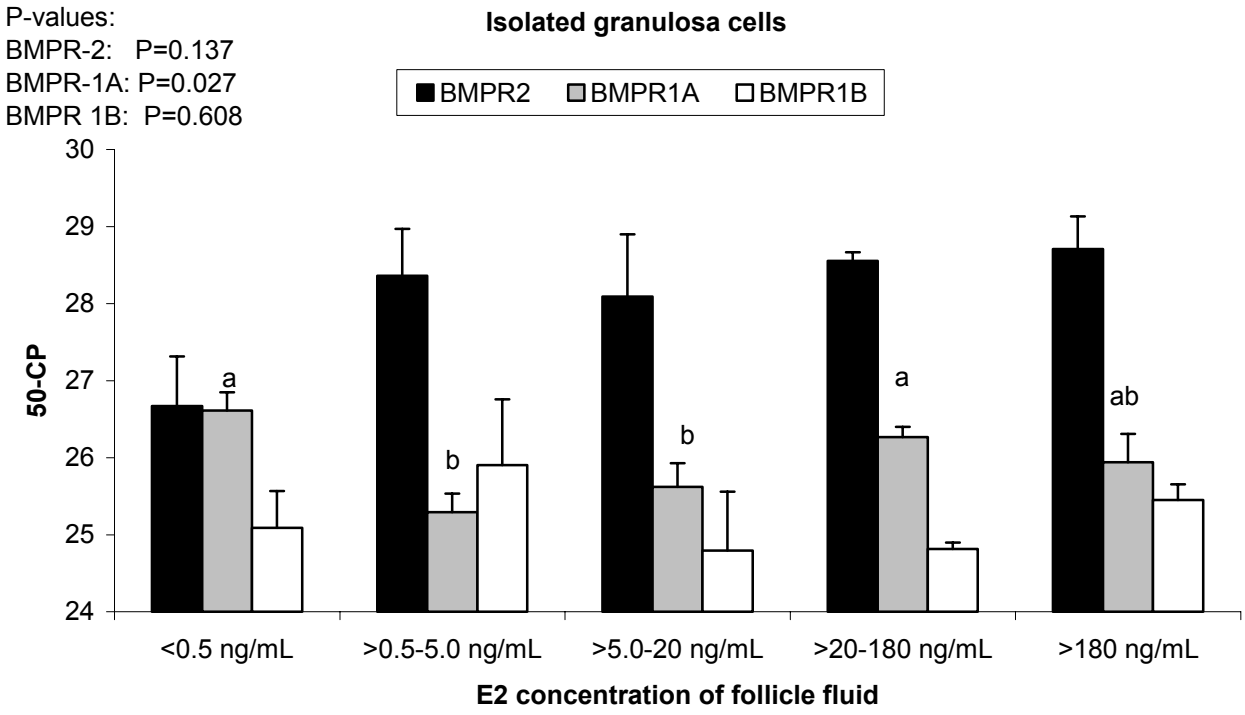
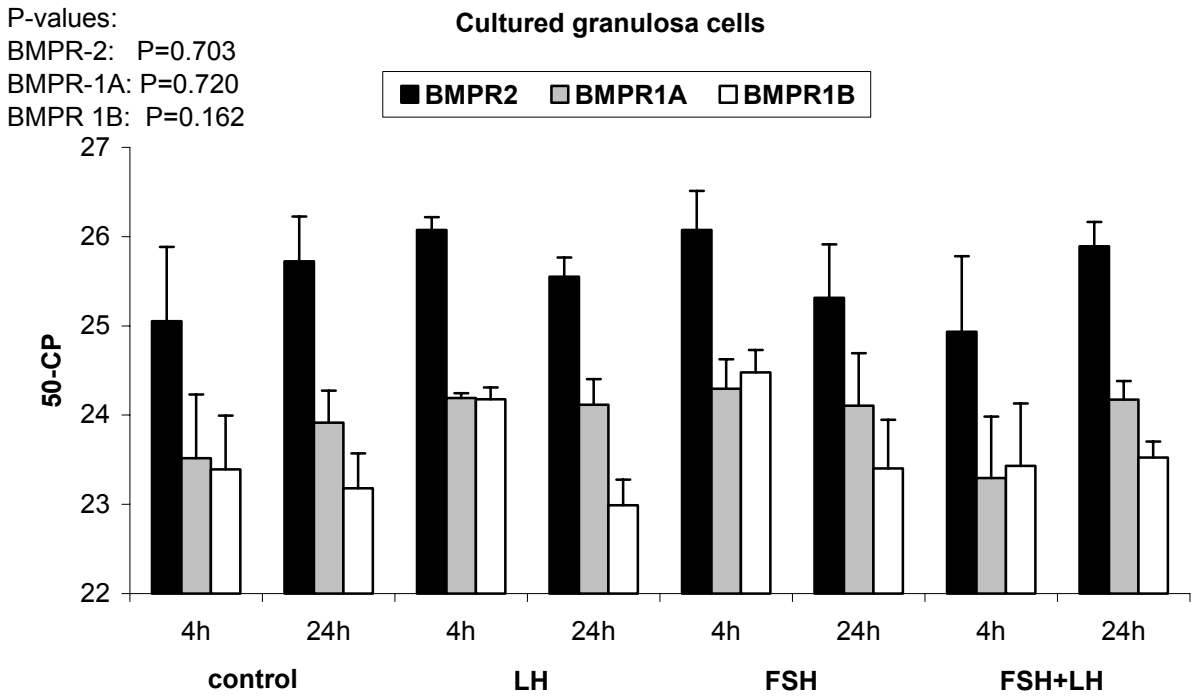


Table 4: Spearman Rank Order Correlation between BMP receptors in GC and TIC (r=correlation coefficient, P=P-value. No significant correlation between two groups is indicated by "-".

TIC	**BMPR 1A**	**BMPR 1B**
BMPR 2	-	r=0.586, P=0.002
BMPR 1A		-
Isolated GC		
BMPR 2	-	r=0.639, P=0.002
BMPR 1A		-
Cultured GC		
BMPR 2	r=0.642, P<0.001	r=0.560, P=0.002
BMPR 1A		r=0.433, P=0.0243

As in TIC, the different BMP receptors in GC showed almost the same mRNA expression dynamics (Fig. 13). Both BMPR-2 and BMPR-1B were present in isolated GC at all stages of follicular development without significant changes in mRNA levels. Additionally, the two receptor types showed again a significant positive correlation which has also been examined for the TIC (Tab. 4). Again, only BMPR-1A expression levels were altered between different follicular stages in GC (Fig. 13) which has been shown before for corresponding TIC (Fig. 12). But neither a direct dependence on E2 levels nor a positive correlation to the other tested BMP receptors could be demonstraded (Tab. 4).

Figure 14: BMPR-2, BMPR-1A and BMPR-1B mRNA-levels in cultured GC during 24 hours treated with gonadotropins. Data were analyzed by one way ANOVA followed by Student-Newman-Keuls Method. Data are depicted as means±SD. Significant differences (p<0.05) between the groups are indicated by letters.



To examine a possible dependence of BMP receptor expression on the administration of different gonadotropins, in vitro studies were done with cultured GC (Fig. 14). The results indicated that BMPR-2 seemed to be the dominant receptor type, likewise in isolated GC and TIC. Neither the treatment with LH, FSH nor the co-treatment of both gonadotropins induced any changes mRNA levels in the tested cell cultures (Fig. 14). In addition, it was not possible to detect any time dependent effects during the duration of the experiment. Additionally, BMPR-2 correlated positively to BMPR-1A as well as to BMPR-1B (Tab. 4).

In the last decade oocyte derived factors, such as GDF-9 and BMP-15, seemed to play an important role during follicle development by controlling cell proliferation of the oocyte surrounding cells. Currently, no data are available about the existence of these two mRNA transcripts during IVM of bovine COC. The present results indicate that in the bovine follicles as well as in COC the postulated BMP receptors are present at mRNA level. But a possible existence of there proteins has to be confirmed by additional experiments e.g. Western Blot or immunhistochemistry. In the last years, several studies have shown the localization of these receptors within the ovary of sheep [273-275], mice [276;277] and rat [278]. In sheep, natural occurring mutants were used to study functionality of these receptors. A mutation in the FecB gene of Booroola ewes was elucidated which posseses a single mutation in the BMPR-1B region, leading to a) hyperprolific follicles, b) less responsiveness of GCs to its natural ligands of the TGFbeta family [274] and c) increased ovulation rates [275]. On basis of these results an impaired function of BMPR-1B in sheep leading to precocious differentiation of GCs and of follicular maturation was postulated. Additionally, Wilson and coworkers have shown a similar expression of BMPR-1B and –2 in GCs of FecB and wildtype sheep, suggesting that BMPR-1B possibly impede premature luteinization of GC in vivo [279]. The mutation likely leads to partial rather than complete inactivation of BMPR-1B.

In contrast, BMPR-1B-deficient mice are infertile, showing irregular estrous cycles and a failure in cumulus expansion [276]. These effects in deficient mice were not exclusively limited to the ovary; these animals also displayed severe skeletal defects [277]. Current reports indicated, that the mRNA expression of all three receptors were detectable by in situ hybridization in the rat ovary, demonstrating a distinct dependency on follicle stage [278]. For BMPR-1B they could show a strong staining of the oocyte as well as of GC and the theca, especially in dominant follicles. Additionally, the expression maintained high in atretic follicles, but was undetectable in CL until luteolysis occurs. BMPR-1A was existent in the oocyte, GC, theca as well as in the CL [278]. The mRNA levels were lowest in primordial follicles, but increased continuously up to late preantral stages [278]. As shown for the BMPR-1B, the BMPR-1A mRNA was existent in

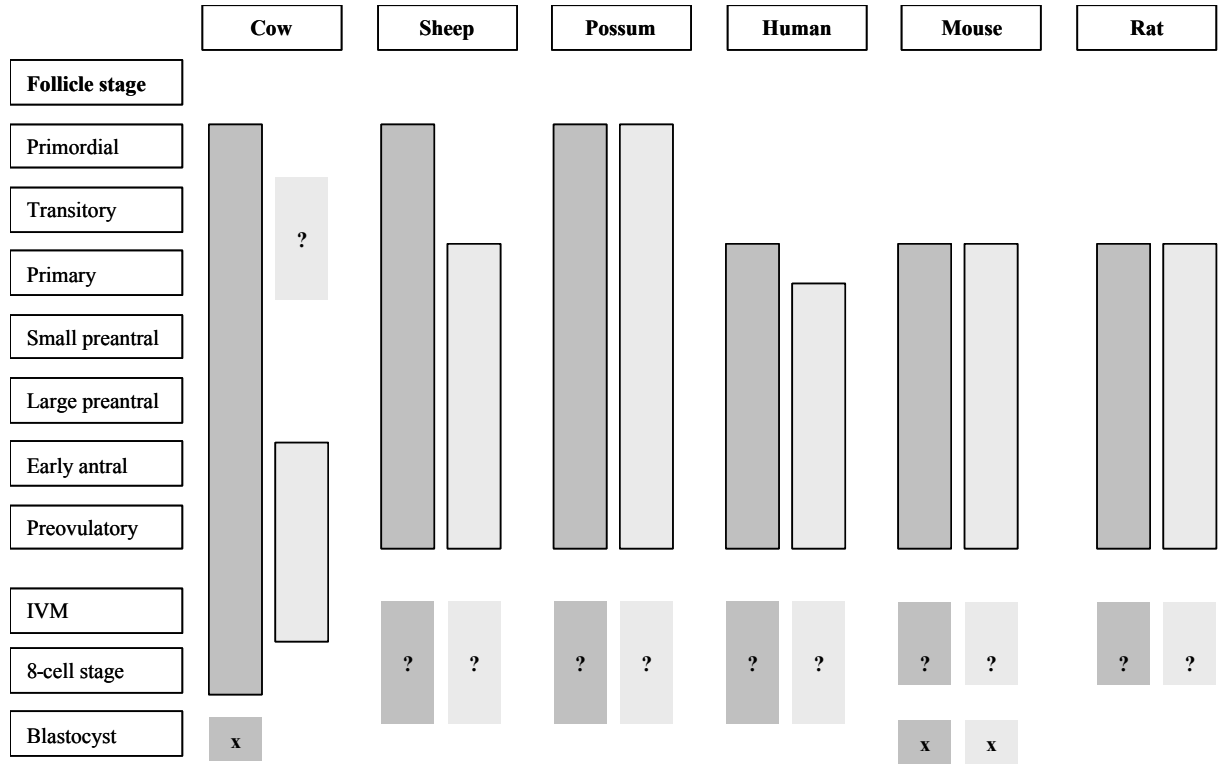
atretic follicles. In the case of BMPR-2, the GCs indicated the highest level in rat ovary in secondary but not in primordial follicles. In cumulus cells the BMPR-2 rapidly decrease after the ovulation [278]. Compared to the expression patterns of the ligand BMP-15 a coherence of the receptors could be assumed. Several authors could elucidate in rats that BMP-15 mRNA was absent in primordial follicles but arised with the oocyte recruitment, correlating with the time point of formation of cubical GCs [280;281]. The levels increased continuously and peaked in follicles containing a minimum of three cell layers of GCs and onward the mRNA maintained high until the stage of ovulation, but was decreased in atretic follicles [278].

In the present study, the existence of the complete BMP system could be shown for the cow too. Nearly all target genes showed constant expression levels at the examined developmetal stage which is comparable with the literature. Furthermore, the expression levels of the ligands (BMP-15 and GDF-9) correlating more or less positively with the receptors in the cultured COC. Additionally, no dependency on gonadotropin treatment of cultured GC could be demonstrated for the indicated BMP receptors. These results assume that the receptors mRNA expression is possibly influenced by its ligand, oocyte stage or other still unknown factors.

According to the in vivo results of isolated TICs and GC from different follicle stages, it could be further postulated that the different steroid hormone levels of diverse follicle stages do not influence receptor expression of BMP-1B and –2 neither in TICs nor in GCs. Only BMPR-1A indicated significant alterations at different follicles stages, but these deviations did not correlate with the increased E2 concentrations in the follicular fluid.

All these results implicate the oocyte a key role of regulating its own environment. New factors may be expected in the near future. In the case of the emerging discoveries, it is essential to compare the various examinations in different species due to the fact of possible variations among the species. Figure 15 summarizes the current knowledge of the existence of GDF-9 and BMP-15 deduced from results derived from cow [171;282], sheep [283], possum [284], human [285], mouse [286;287], and rat [278;280;281]. The overview clarified the species specific occurrence of the two growth factors indicating that both factors are present in human, mouse and rat not until primary follicle stage. In contrast, the ruminates and the possum GDF-9 is expressed in the early stage of follicular development. Furthermore, a different regulation of oocyte recruitment could be assumed among the various species. But to clarify the differences between the species, comparative experiments have to be done, using the same detection systems to ensure equal sensitivity.

Figure 15: Comparative summary of known mRNA data of GDF-9 (dark gray bars) and BMP-15 (light gray bars) in different species at the various stages of follicular development including the results of the present study. The bars marked with a „x" indicate stages of development, when the factors were undetectable. If detailed data are not available, follicle stages are signed by „?".



7.6. Sequencing the bovine GDF-9 cDNA, cloning and production of mature recombinant GDF-9 protein

In order to evaluate direct physiological effects e.g. towards mRNA expression of BMP receptors, a functional recombinant protein should be provided.

7.6.1. Sequencing of GDF-9

Neither the exact bovine GDF-9 mRNA sequence nor a native or recombinant protein was available. Therefore, it was necessary to examine a full length cDNA sequence of the bovine GDF-9 using the established RACE technology.

The sequencing of the bovine GDF-9 mRNA led to a 1212 bases long partial mRNA which was verified by forward and reverse sequencing. At the 5'-end of the sequence about 40 bp are missing towards the start codon ATG verified by alignment of known sequences of other species. Translated into protein, an open reading frame could be identified, encoding 404 amino acids. This examined GDF-9-cDNA sequence has been submitted to a nucleic acid database to permit

public access (EMBL: AJ302697). The deduced open reading frame showed the following homologies to known GDF-9 cDNA as well as deduced protein sequences of other species (cDNA-homology: 96 % sheep, 84 % human, 71 % rat as well as mouse; overall protein homology 65-95 %).

7.6.2. Production of recombinant mature GDF-9

In the present study the examined cDNA was integrated into an expression vector (Fig. 16) and used for protein expression in E. coli.

Figure 16: Partially vector sequence including the DNA- and protein sequence (one letter code) of recGDF-9. The presented sequence starts at the start codon and includes additionally the HIS-tag sequence (amino acid position 5 up to 10). The mature GDF-9 sequence is printed in bold letters, italic letters indicate the sequence provided by the manufacturer.

```
      1 ATGCGGGGTTCTCATCATCATCATCATCATGGTATGGCTAGCATGACTGGTGGACAGCAA     60
      1  M  R  G  S  H  H  H  H  H  H  G  M  A  S  M  T  G  G  Q  Q     20

     61 ATGGGTCGGGATCTGTACGACGATGACGATAAGGATCCAACCCTTGACCAGGAGAGTGTC    120
     21  M  G  R  D  L  Y  D  D  D  D  K  D  P  T  L  D  Q  E  S  V     40

    121 AGCTCTGAATTGAAGAAGCCTCTGGTTCCAGCTTCATTCAATCTGAGTGAATACTTCAAA    180
     41  S  S  E  L  K  K  P  L  V  P  A  S  F  N  L  S  E  Y  F  K     60

    181 CAGTTTCTTTTTCCCCAGAATGAATGTGAGCTCCATGACTTTAGACTTAGCTTTAGTCAA    240
     61  Q  F  L  F  P  Q  N  E  C  E  L  H  D  F  R  L  S  F  S  Q     80

    241 CTGAAGTGGGACAACTGGATTGTGGCCCCACACAAATACAACCCTCGATACTGTAAAGGG    300
     81  L  K  W  D  N  W  I  V  A  P  H  K  Y  N  P  R  Y  C  K  G    100

    301 GACTGTCCCAGGGCGGTCGGACATCGGTATGGCTCTCCAGTTCACACCATGGTGATGAAC    360
    101  D  C  P  R  A  V  G  H  R  Y  G  S  P  V  H  T  M  V  M  N    120

    361 ATCATCCATGAGAAACTTGACTCCTCAGTGCCAAGACCATCCTGTGTACCTGCCAAGTAT    420
    121  I  I  H  E  K  L  D  S  S  V  P  R  P  S  C  V  P  A  K  Y    140

    421 AGTCCTTTGAGTGTTTTGGCCATCGAGCCTGATGGCTCAATTGCTTATAAAGAATATGAA    480
    141  S  P  L  S  V  L  A  I  E  P  D  G  S  I  A  Y  K  E  Y  E    160

    481 GATATGATAGCCACTAAGTGTACCTGTCGTTAA                               513
    161  D  M  I  A  T  K  C  T  C  R  *                               171
```

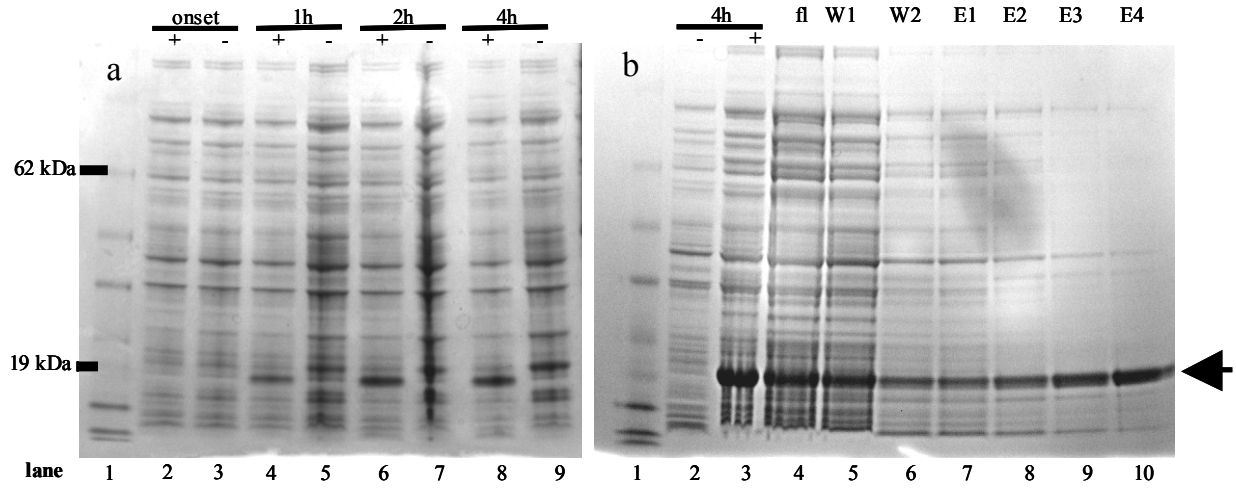
The cDNA sequence, amplified by conventional block PCR, consists of the native GDF-9 protein sequence and was integrated into an expression plasmid vector by TA-cloning reaction. The cloned sequence consisted of 408 bp, encoding for a hypothetical protein of 135 amino acids with 15.5 kDa (Fig. 16). The tagged recombinant protein is enlarged by the HIS-Tag and an antibody recognition site up to 513 bp which encodes for 170 amino acids possessing a calculated weight of about 19.5 kDa (Fig. 16/17). The right plasmid sequence was verified by

commercial sequencing and the sense integration was proved by PCR using T7-forward primer and gene-specific reverse primer (data not shown).

Thus, the lac-promotor of the vector was leaky, the E. coli cultures, used for protein expression were supplemented by 0.8 % glucose to repress protein expression in the bacteria until IPTG induction (data not shown). Additionally, due to the high protein expression, the recombinant protein accumulated in inclusion bodies inside the bacteria (data not shown). Therefore, a total protein isolation by 8 M urea was favored in front of the IMAC isolation of the HIS-tagged recGDF-9.

Figure 17a shows an exemple of a the time-dependant protein expression in E. coli after IPTG induction. After total protein isolation the HIS-tagged recGDF-9 was bound to a gravity column provided with agarose which carries covalently bound nickel ions. After washing with low pH solutions, a protein of about 19 kDa could be eluted (Fig. 17b). This protein solution was dialysed against PBS (pH 7.2) and protein concentration was quantified by BCA-test.

Figure 17: SDS-PAGE of recGDF-9 production in E. coli. a) Time course of protein expression with (+) and without (-) IPTG induction; b) isolation of recGDF-9 protein by IMAC with Ni-TNA resin after 4 h IPTG induction: flow through (fl), washing (W1-2), elution (E1-4, pH 4.5).protein elution. Arrow indicate specific recGDF-9.



7.6.3. Effects of recGDF-9 in GC culture

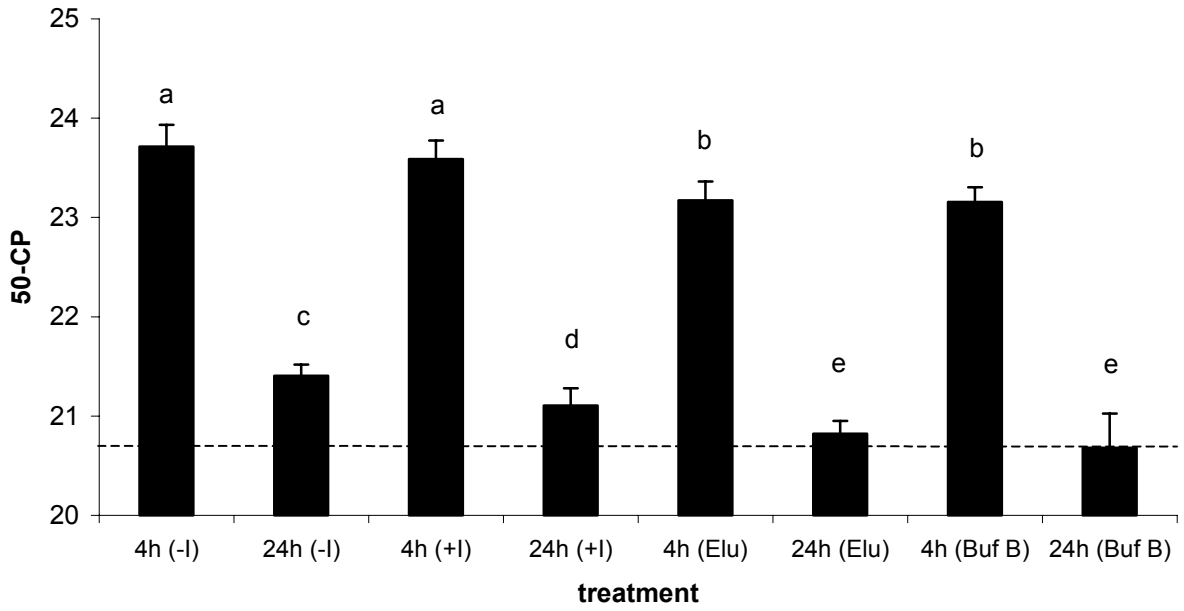
In the literature diverse effects in various species were ascribed to the growth factor GDF-9. Critical events during oocyte maturation are supposed to be under influence of GDF-9: e.g. cumulus expansion through increased expression of HAS2, stimulation of progesterone production by recombinant GDF-9 as well as increased mRNA expression of cyclooxygenase 2. To evaluate the biological activity of the recombinant protein, the recGDF-9 was applied to established GC culture, expressing potential receptors of GDF-9 BMPR-2, -1A, and -1B as shown above. To evaluate a possible bioactivity of the recGDF-9, HAS2 (Fig. 18) was used as an

indicator because its mRNA expression characteristics in cultured GC were known as described by Schoenfelder und Einspanier in cattle [288] as well as in other species.

To test potential influence of endotoxins, which could be present as contaminations in the eluted recGDF-9, several control samples were included into the experimental design: incubation with E. coli cell lysate without IPTG induction and E. coli cell lysate with IPTG. Additionally, dialyzed extraction buffer served as further control. For protein treatment all culture media were replaced by fresh media containing the specific ingredients. All samples were incubated in the same amount of culture media containing equilibrated protein concentrations (100 ng).

The results of HAS2 mRNA expression (Fig. 18) were first analyzed by two way ANOVA, indicating significant effects in time ($P<0.001$), in treatment ($P<0.001$), as well as in co-variance ($P=0.008$).

Figure 18: HAS2 mRNA expression of culture GC (n=3) in response to recGDF-9 treatment over 24 h incubation: Treatments buffer B served as control, E. coli lysate without IPTG stimulation (-I), E. coli lysate with IPTG stimulation (+I), isolated recGDF-9 (Elu). Dashed line indicate onset expression level of HAS2 mRNA. Significant differences ($p<0.05$) between the groups are indicated by letters.



A multiple comparison between the several treatments showed significant differences (Tab. 5).

Table 5: All pair wise multiple comparison procedure between GC culture treated with recGDF-9. Analysis was done by two way ANOVA following Student-Newman-Keuls method and depicted as P-values.

	lysate + IPTG	**eluate**	**Control**
lysate – IPTG	P=0.072	P<0.001	P<0.001
lysate + IPTG		P=0.005	P=0.002
Eluate			P=0.435

This statistical analysis (Tab. 5) visualizes significant effects of the whole cell lysates on the behavior of HAS2 mRNA expression. The purification of recGDF-9 showed no remarkable increase of HAS2 and did not differ at any time from the control, indicating that the recGDF-9 had no effect on HAS2 mRNA expression at this concentration and exposure time. This fact was underlined by no differences between E. coli lysates (with or without IPTG).

The results from statistical analysis are illustraded by Figure 18 showing no statistical variance between control and recGDF-9 treated cell. The significant increases in HAS2 expression might be induced by residual endotoxins or other proteins present in the whole cell lysates.

These results of the first screening for bioactivity of the recGDF-9 indicate, that the produced protein is possibly a) inactive, b) less concentrated, c) active but not on these cells, or d) the assay system is not adequate. Reasons for an inactive protein could be a) artificial protein folding, b) unusual or missing disulfide bounds, or c) the lack of glycosylations (typically for E. coli produced proteins) or dimerization.

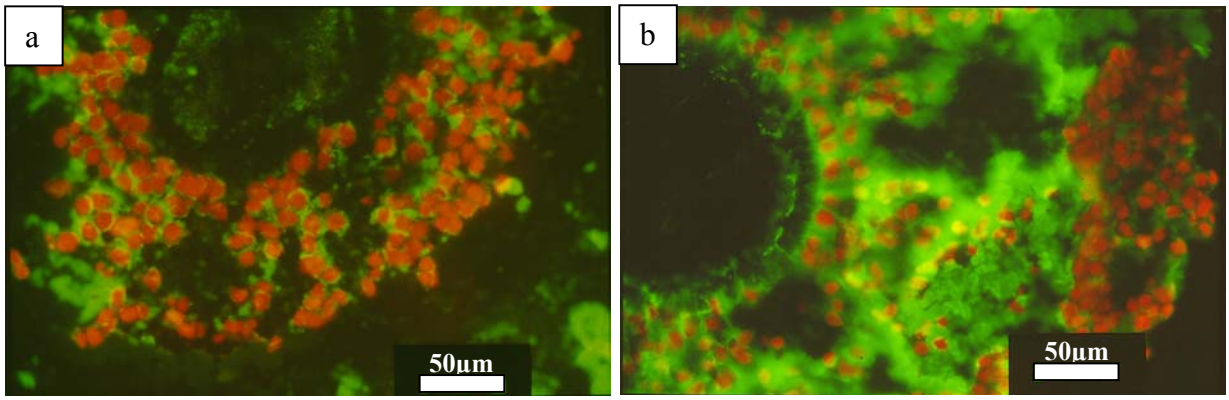
Therefore, additional experiments have to be done to get a bioactive recGDF-9. Possibly one could be the examination of a new expression vector adapted to eukaryotic cells such as yeast to achieve the sufficient glycosylations of the recombinant protein. Another improvement could be the co-transformation of the premature GDF-9 sequence in parallel with the PACE enzyme: This cotransfection possibly enables a correct protein folding of the pre-form of GDF-9 as well as its right processing by the PACE enzyme.

Nevertheless, the present recGDF-9 could be used for the production of antibodies, possibly providing the histological localization of GDF-9 during the IVM of the COC and in the ovary respectively.

7.7. The hyaluronan producing system in bovine COC and GC

In this study a functional hyaluronan synthesizing and binding system was detected during in vitro maturation of bovine oocytes as well as in cultured GC, including the first full-length sequencing of the bovine HAS2 mRNA by use of RACE technology (EMBL: BTA004951) [288]. Only two of the three identified mammalian HA producing enzymes, the HAS2 and HAS3, were observed in the different follicular cell cultures. Their obvious regulation throughout the oocyte maturation may be mediated by gonadotropins. By the use of real-time RT-PCR rapid increases within the first hours of IVM were detecable especially for HAS2 and cd44 mRNA expression [288]. These results indicated extensive alterations of the microenvironment of bovine COC during the cumulus expansion which has been demonstrated additionally by a tremendious increase of HA embedding the oocyte as well as the cumulus cells (Fig. 19).

Figure 19: Histochemical staining for HA during expansion of bovine COC. A biotinylated HA binding protein and streptavidin FITC were used to localize HA on crysections of maturing COC: a) before IVM and b) 24 hours after IVM.



Compared to almost unchanged HAS3 mRNA levels, HAS2 was the most abundantly expressed HA-synthesizing enzyme during bovine COC expansion which has been been reported in mouse cumulus cells as well [289]. But in contrast to the rapid mRNA expression of HAS2 in the cow [288], recent reports of Kimura et al. about porcine IVM showed a significant increase of HAS2 not until 24 h [189]. Additionally, HAS3 mRNA was located exclusively in porcine oocytes and decreased under gonadotropins. Such low amounts of porcine HAS3 expression were comparable to the present bovine system [288]. However, the faint transcript concentration does not exclude HAS3 from being unimportant for the HA production during cumulus expansion [201]. As shown for other species the enzymatic activity of HAS3 is characterized by a higher metabolic rate producing short HA fragments [290]. This low molecular weight HA, produced by HAS3, directly influences the expression of cytokines and cell shape in eosinophiles [291],

but the consistency as well as the effect of the different HA molecules in the bovine cumulus is unknown. Independent of biomechanical forces during ovulation, HA matrix could transmit signals by binding to specific receptor proteins and potentially influence cell functions and properties in a paracrine manner [292]. Although little is known about the regulation of the HA receptors CD44 and RHAMM in reproductive tissue, the few experiments suggest CD44 as playing an important role during human oocyte maturation [211] and preventing apoptosis in human GC [207]. The CD44 protein has been demonstrated by immunohistochemistry for a variety of mammalian epithelial cells, including oviduct, uterus and vagina [293]. Such observations suggest a local communication between the cumulus derived HA and the HA-receptors of the reproductive epithelium [294].

Our results suggest an endocrine influence on the mRNA expression of the HA receptor CD44. Although GCs produced steroid hormones under FSH or LH, the production of progesterone and estradiol lagged behind the initial increase of CD44 mRNA at 4 h [160]. Therefore, the major influence of CD44 induction in GCs is more likely based on gonadotropins rather than steroid hormones.

In contrast to the lack of CD44 modulation in GC by simultaneous treatment of LH and FSH, the same conditions led to a significant increase of CD44 mRNA transcripts during COC maturation. Hence, our results suggest both an endocrine as well as paracrine regulation of CD44 through gonadotropins and probably oocyte factors, respectively. Such modulatory effects of oocyte derived proteins onto the HA system in maturing COC were postulated for rats recently [295].

Although RHAMM remained unregulated by gonadotropins, an important function could be ascribed to this HA receptor in the bovine reproductive tract because continuous mRNA expression were found during IVM. RHAMM may mediate movement and attachment of human cumulus cells [296] or could modulate ciliary beating of oviduct epithelia, as described for the respiratory system [217].

Our in vitro results indicate that a balance of FSH and LH is capable of modulating main components of the HA-system in bovine follicular cells. An exogenous application of HA during the bovine IVM showed beneficial effects on both IVM and in vitro production of embryos [297]. Additionally, Saito and coworkers postulated that the concentration of HA in the follicular fluid could be used as an indicator to estimate oocyte fertilization capacity [230].

7.7.1. Effects of HA treatment on steroidogenesis and specific mRNA expression in bovine granulosa cells

During follicle development in vivo HA will accumulate up to 1 mg/mL in the follicular fluid of rats as a result of HAS activity of cumulus and granulosa cells [188;229]. Therefore it is obvious, that supplementation of HA could influence cellular behavior. In respect to the observations on beneficial effects during artificial embryo production [297], a possible influence of HA onto the presented HA system can be postulated. On this account, bovine GC cultures were exposed to a defined HA-concentration, which has been ascribed beneficial effects on IVM. The GCs were cultured for 24 hours with 1 mg/mL HA, accompanied with mRNA and steroid hormone examinations.

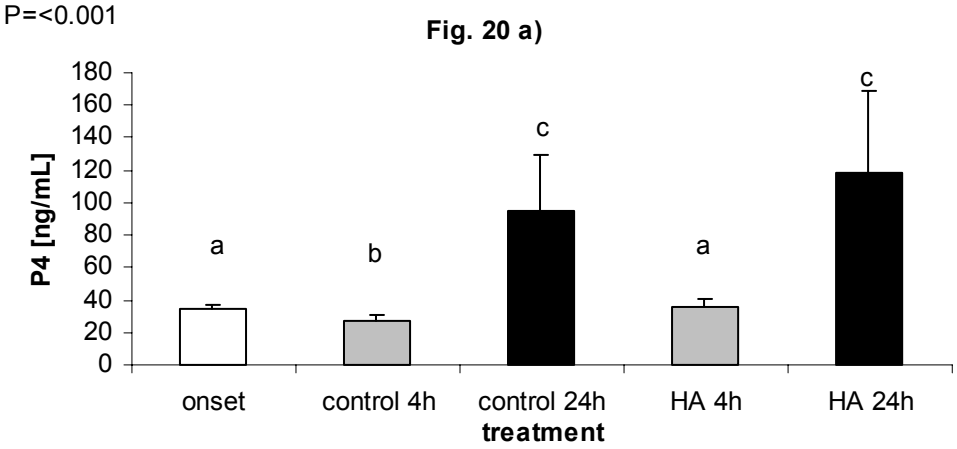
Table 6. Statistical analysis HA treatment on steroidogenesis and mRNA levels of GC performed by two way ANOVA followed Student-Newman-Keuls method. Data are depicted as P-values.

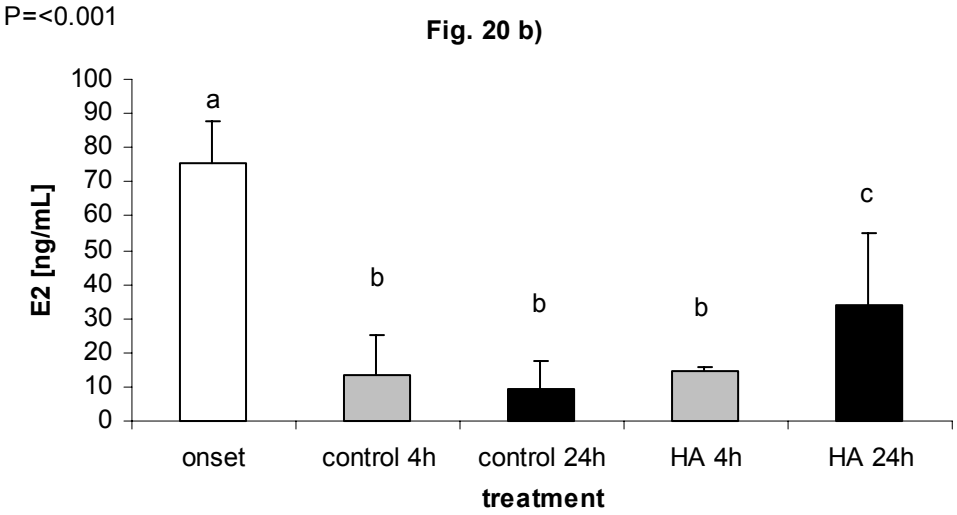
	HAS2	**HAS3**	**CD44**	**RHAMM**	**P4**	**T**	**E2**
time	<0.001	<0.001	0.298	<0.001	<0.001	<0.001	<0.001
treatment	0.011	0.521	0.695	0.147	0.256	0.606	0.072
Co-variance	0.008	0.004	0.945	0.127	0.599	0.356	0.073

The two way ANOVA analysis of HA-treatment experiments showed significant time-dependant mRNA changes of HAS2, HAS3 as well as RHAMM (Tab. 6). 18S rRNA, and ubiquitin indicated no significant alterations (data not shown).

Additionally, steroid hormone analysis indicate time-dependant alteration typical for luteinizing GC showing an increase of P4 (Fig. 20a) and almost unchanged E2 (Fig. 20b) concentrations without any stimulation such as gonadotropins [240]. Although two way ANOVA analysis indicated no significant influence of HA concerning E2 synthesis among all groups, a tendency of increased E2 levels were recorded for HA treatment after 24 hours (Fig. 20 b) when analysed by one way ANOVA.

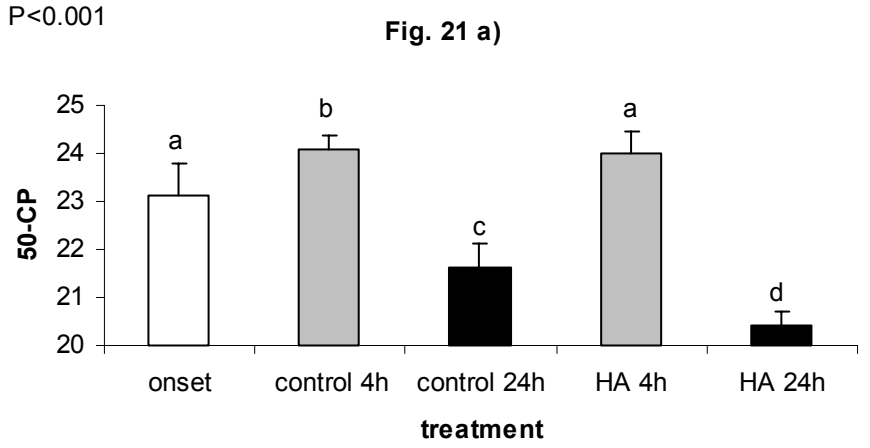
Figure 20: Steroid hormone synthesis measured by EIA in GC after HA treatment (1 mg/mL): a) P4 and b) E2. Statistical analysis were done by one way ANOVA following Student-Newman-Keuls method. Data are depicted as means±SD. Significant differences ($p<0.05$) between the groups are indicated by letters.

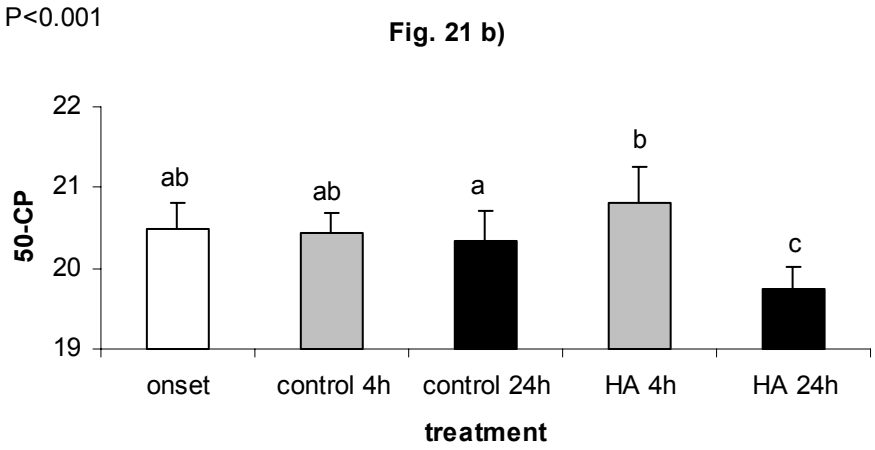


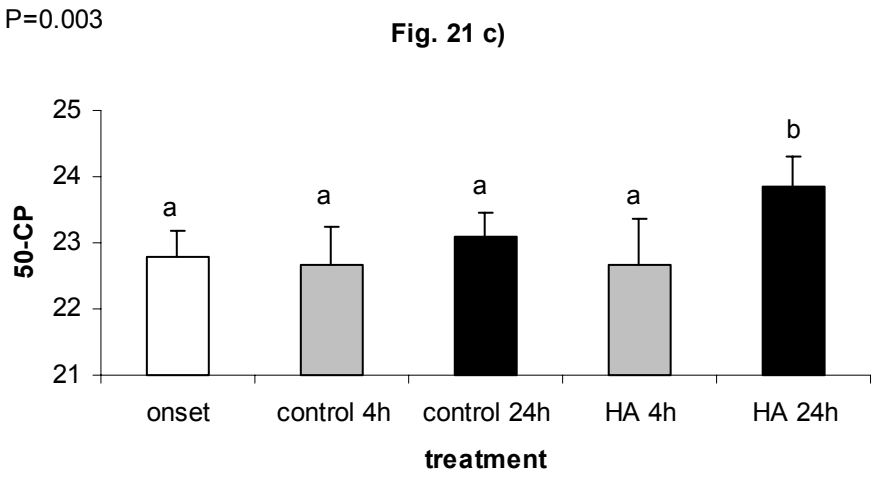


Furthermore, just for HAS2 mRNA expression significant influence of HA supplementation was detectable. Expression analysis by real-time RT-PCR revealed slight influence by supplemented HA, leading to a reduction of both HAS2 (Fig 21a) and HAS3 (Fig. 21b), whereby the intercellular HA receptor RHAMM (Fig. 21c) increased.

Figure 21: Granulosa cell culture (n=6) treated with 1 mg/mL HA. Expression of mRNA were quantified by real-time PCR and depicted as means of 50-CP. a) HAS2-mRNA, b) HAS3-mRNA, and c) RHAMM-mRNA. Significant differences ($p<0.05$) between the groups are indicated by letters.







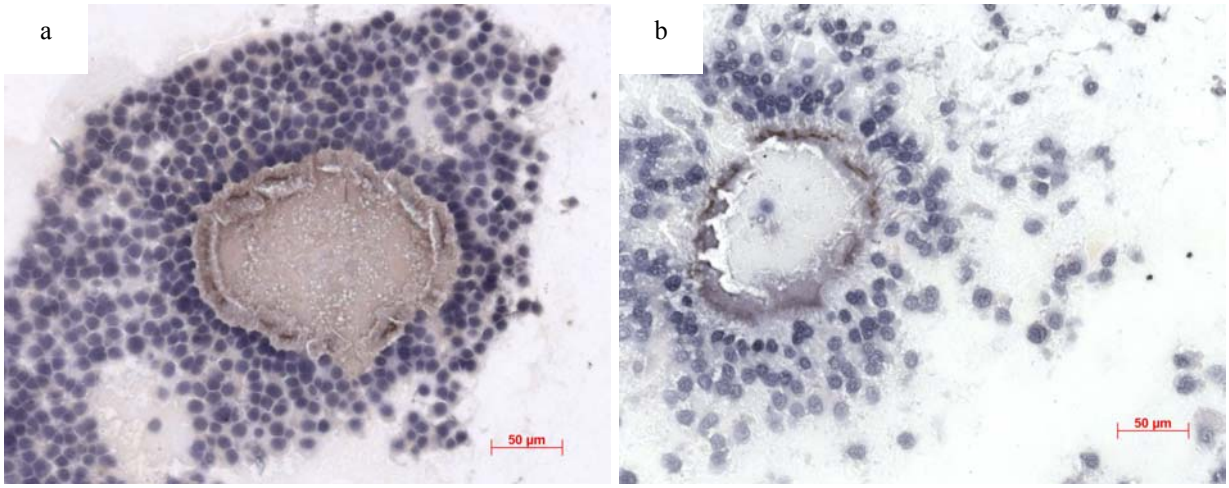
In vivo the accumulation of HA expands the COC and promotes detachment of the COC from the follicular wall to prepare the oocyte for ovulation. During this process some HA produced by the cumulus cells will be released into the follicular fluid and additionally its concentration will increase after hCG injection [188;230]. Saito and coworkers observed a significant positive correlation between the follicular fluid concentration of HA and progesterone [230]. In parts this increased P4 level could be shown in the present study as well: P4 concentration of HA treated GC were slightly increased after 24 hour incubation (Fig. 20a). In contrast, an inverse correlation was observed between the follicle fluid concentration of HA and E2 by Saito and coworkers [230]. This result could not be approved by the present HA treatment. In fact E2 was increased by HA. Similar results of increased E2 in correspondence to high HA concentrations were reported from human, possibly indicating, that follicles with high HA levels may contain mature oocytes. Thus, the role of HA remains to be clarified by additional experiments, including evaluation of effects caused by different amounts and molecular sizes of HA.

7.8. Vascular endothelial growth factor during IVM and embryo production

Nowadays, it is well established that a variety of growth factors is essential for a successful follicle development as well as for oocyte maturation. Due to ovarian function, VEGF is fundamentally important for the development of the follicle and the CL by regulating vascularization. In order to show the potential role of VEGF not only for angiogenesis but also on IVM followed by embryo development, a complete VEGF system consisting of the ligand and two of its receptors has been detected for the first time in the bovine COC [232]. By the use of RT-PCR it was possible to show the expression of the smaller VEGF transcripts and their specific receptors flt and flk changing remarkably in a time-dependant manner in the progress of 24 hour IVM. The specific transcript concentrations of VEGF declined within 24 hours of culture to nearly undetectable levels. After 12 hours almost all transcripts disappeared, whereas both receptor mRNAs were found enriched between 6 and 12 hr of IVM. In the follicular fluid of growing ovarian follicles, immunoreactive VEGF, measured by RIA, increased significantly, reaching highest concentrations immediately before ovulation of the oocyte.

The immunohistochemical localization of VEGF in bovine COC revealed strong signals within the cumulus cell complex clearly extending beyond the ooplasm (Fig. 22 a) at the onset of IVM. Within 24 hours of IVM immunoreactive staining for VEGF disappeared remarkably from cumulus cells and the ooplasm (Fig. 22 b).

Figure :22: Immunohistochemical localization of VEGF in bovine COC a) before and b) after IVM (24 hours)



An exogenous application of VEGF at the beginning of a 24 hour IVM significantly improved cleavage rates of zygotes and their development up to the blastocyst stage. Co-treatment with FSH showed obvious synergistic effects, when compared with untreated control embryos [232]. In addition, the number of blastomeres in deriving blastocysts increased after VEGF supplementation. These results indicate a functional VEGF system controlling important events apart from the known angiogenetic effect during in vivo and in vitro maturation of the bovine COC, possibly affecting the early embryonic viability.

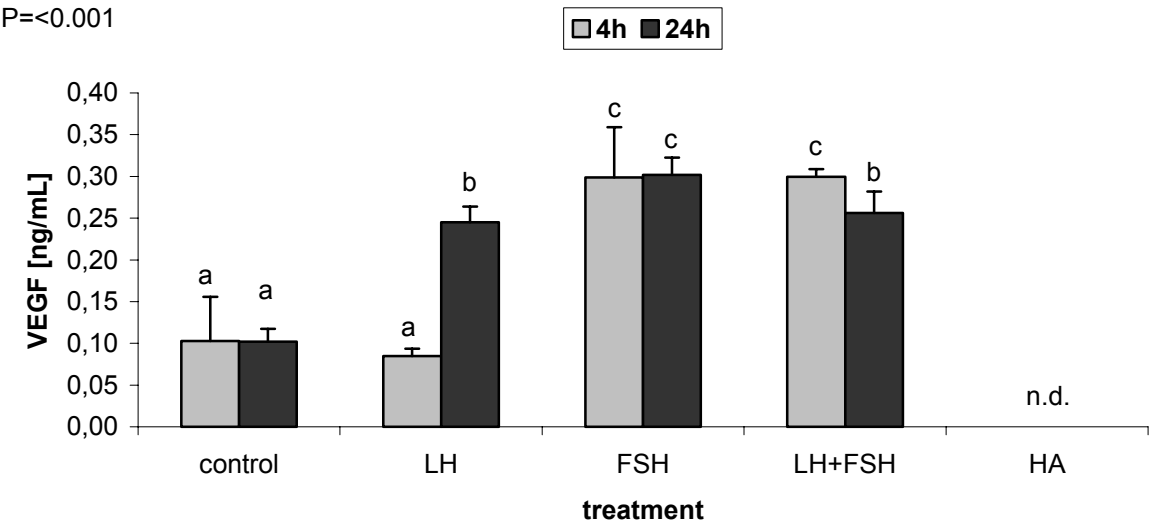
7.8.1. VEGF secretion of bovine GC under gonadotropins and hyaluronan

Recent studies indicate that VEGF undergoes specific modulations during the estrous cycle in the oviduct [186] and GC, respectively [26].

In order to evaluate VEGF protein synthesis in vitro, cultured bovine GC were exposed to gonadotropins (Fig. 23). According to the potential influence of HA on cellular behavior, VEGF secretion was also measured in presence of 1 mg/mL HA (Fig. 23).

To compare the in vivo data of follicular VEGF concentrations, RIA-examination of VEGF in the supernatant of cultured GC were performed and elucidated no time-dependant effects on the occurrence of VEGF in non stimulated culture. Whereby P4 levels are known to increase during luteinization (see Fig. 7a).

Figure 23: VEGF secretion of cultured bovine granulosa cells treated with gonadotropins (LH, FSH, LH+FSH) and hyaluronan (1 mg/mL). Culture media were measured for VEGF by RIA (detection limit <0.06 ng/mL, n.d. = not detectable). Statistical analysis were done by one way ANOVA following Student-Newman-Keuls method. Data are depicted as means±SD. Significant differences ($p<0.05$) between the groups are indicated by letters.



The most abundant increase of VEGF were examined for the FSH-treated GCs (Fig. 23), showing a rapid rise immediately after its administration. In contrast, LH caused no significant increases in VEGF secretion until 24 hours of culture. In spite of stimulatory effects on VEGF protein secretion of both gonadotropins, neither an additive nor an inhibitory effect could be observed after 4 hours of co-stimulation. Just a slight drop was examined after 24 hours compared to FSH stimuli. Further results in other species will support the observed gonadotropin dependant effect on VEGF. In human luteinized GC cultures rhFSH as well as hCG stimulate VEGF mRNA in a dose dependent manner. Additionally, these effects were also time dependent, because the highest concentrations were measured at day two of cultivation. After the third day the mRNA levels of VEGF declined [298]. As in human, Christenson and Stouffer examined in the rhesus monkey an increase of both VEGF and P4, caused by r-hFSH, r-hLH as well as hCG [299]. The stimulatory effect last up to six days for VEGF and up to three days for P4 respectively.

Results of recent studies indicated that high molecular HA inhibits angiogenesis during follicular development [300] as well as during tumor growth [301], whereas its products of degradation by hyaluronidase are angiogenic [302]. Tempel and coworkers postulate an anti-angiogenic shield of HA in preovulatory rat follicles, preventing the penetration of the follicular wall by new-build blood vessels [303]. This inhibitory effect is ascribed to the COC as well, which showed reduced migration of endothelial cells in co-culture. But when DON (6-diazo-5-oxo-1-norleucine) or hyaluronidase were added in vivo as well as in vitro this observed reduced in migration partly

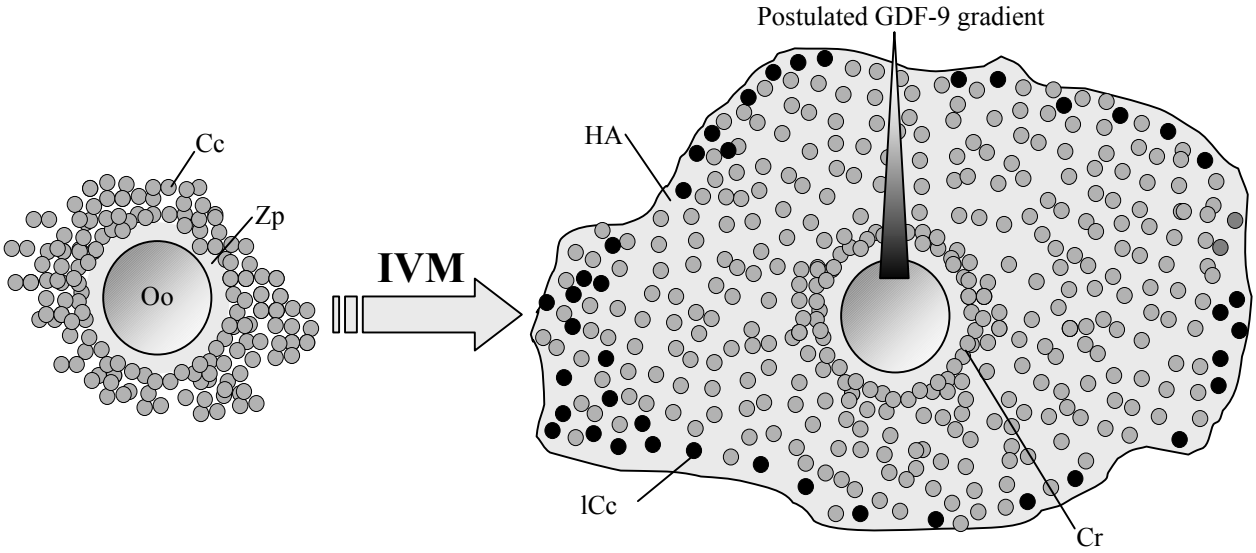
disappeared. Furthermore, in vivo administration of DON led to a precocious infiltration of endothelial cells through the basement membrane and into the GC layer [303]. Additionally, the ovulation was inhibited in mouse experiments [304]. It still remains unclear which mechanism is involved in this strict inhibition of VEGF demonstrated by the present results of HA supplementation to GCs (Fig. 23). Therefore further examinations have to be done to reveal this remarkable process.

In summary, the data demonstrate that FSH-like as well as LH-like gonadotropins directly stimulate VEGF synthesis by granulosa cells. It could assumed that the rise in VEGF is possibly not dependent on P4, which was found increased in luteinizing GCs. Collectively, these data suggest an important role for the gonadotropin surge in mammalian species to stimulate an increase in VEGF production, which may be important for follicle rupture and/or luteinization. Additionally, the high FSH-responsiveness of the GCs to VEGF secretion could possibly indicate the signal of vascularization in the developing follicle and CL respectively. The drop in VEGF protein inside the oocyte may represent the end of oocyte maturation. Therefore, a progression of vascularization is no longer necessary for oocyte development. In parallel, the reduction in mRNA expression could be a result of RNA polymerase inhibition through the MPF system, inhibiting the transcription when GMVB occurs.

8. Conclusions

On the basis of an established in vitro maturation (IVM) protocol it was possible to demonstrate that the bovine cumulus oocyte complexes (COC) were able to modulate their environment concerning the selected steroid hormones, showing almost the same patterns as known in vivo. A predominant increase could be ascribed to estradiol-17β (E2), which was additionally mirrored by the aromatase P450 mRNA expression. In contrast, progesterone slightly increased during IVM. This slow increase could be a consequence of the oocyte derived growth and differentiation factor-9 (GDF-9), repressing LH receptors in cumulus cells and consequently the luteinization could not or retarded occur in these cells. During IVM the cumulus cells dispersed from the oocyte and a possible GDF-9 gradient arised across the expanded cumulus (Fig. 24) leading to an induction of LH receptors in the outer cumulus cell layers. This assumption was confirmed by the rise of LH receptor mRNA above detection limit after 24 hours of IVM.

Figure 24: Hypothetical model of a GDF-9 gradient possibly occurring across the expanded cumulus. Corona radiata (Cr), cumulus cells (Cc), luteinazing cumulus cells (lCc), hyaluronan matrix (HA), Oocyte (0o), Zona pellucida (Zp),



This local steroidogenic system represents also a possible target for endocrine disruptors often acting in very low doses, as shown by the treatment of granulosa cell (GC) cultures with tributyltin. Therefore, the GC culture as well as the specific IVM system, initiated by isolation out of the intact follicle, could serve as a suitable system to study endocrine disrupting substances and its potential effects on the resting as well as the growing pool of oocytes.

Beneath the steroidogenic signal transduction, a system of direct connection of neighboring cells by gap junctions was also detectable during IVM persumably interacts with the meiotic maturation of the oocyte by modulation of cAMP levels. Connexin 43 (Cx43), the predominant gap junction protein in various tissues, was present during all stages of IVM. Its declining levels paralleled with the maturation time assuming a possible influence on meiotic maturation. Additionally, Cx32 was existent in the present system but in a lower excess, whereas Cx26 was not detectable.

Alternatively, cell to cell interactions can occur via the small molecule nitric oxide (NO), produced by diverse NO synthases (NOS). In this study it was possible to show that two of these synthases were present in both GC and COC systems, but showed different expression patterns. In GC only the inducible NOS (iNOS) was detectable, with direct dependence on FSH as well as LH treatment. Messenger RNA levels of iNOS significantly arose after 4 hours of incubation but declined to control levels within 24 hours. It was not possible to detect the endothelial NOS (eNOS) in the GC, probably based on low expression, supported by literature data. In COC, eNOS increased between 4 and 12 hours of IVM, whereby iNOS continuously decreased until the end of IVM. Therefore, a potential function of NO in maturation or ovulation can be postulated, because these results are in temporal context to the meiotic resumption and the extrusion of the first polar body.

As shown for other species, the two oocyte derived factors GDF-9 and bone morphogenetic protein-15 (BMP-15) were present throughout the whole IVM indicating almost slight alterations in mRNA levels. It can be assumed that the mRNA is potentially stored in the oocyte until its usage, because mRNA polymerase is inhibited during the meiotic process through the maturation promoting factor system. The existence of their potential BMP receptors BMPR-1A, BMPR-1B and BMPR-2 in cultured and isolated GCs as well as theca interna cells could assign a functional system. To improve this assumption concerning a functional BMP/GDF-system, a recombinant protein of the native GDF-9 was generated. First results of recGDF-9 treatment indicated, that its functionality possibly requires the production of the preform of GDF-9, following cleavage by paired basic amino acid cleavage enzyme and protein folding in eukaryotic cells allowing right disulfide linkage as well as glycosylations.

Furthermore, in the present study it could be shown, that the hyaluronan synthase 2 (HAS2) may be the predominant enzyme producing the hyaluronan (HA) rich extra cellular matrix surrounding the oocyte. Its mRNA expression was significantly influenced by gonadotropins in GC culture. Although HAS3 is less abundant a possible function could be assigned in IVM. The HA receptor CD44 increased time delayed to HAS2 mRNA and was also under direct influence

of gonadotropins in GC. The mRNA of the receptor for HA mediated motility (RHAMM) declined during IVM but appears independent of any gonadotropin treatment in GC. The treatment of GC with HA resulted in slight reduction of HAS2 and HAS3 respectively, whereas RHAMM mRNA was increased. Therefore, a negative feedback system between the enzymes and its catalytic product can be assumed. Astonishingly, a complete abolishment of the vascular endothelial growth factor (VEGF) protein was found in response to HA administration.

VEGF might be responsible for the vascularization of the follicle and the CL, respectively. In vivo, the VEGF protein concentration continuously increased in follicular fluid, peaking at ovulation. In vitro VEGF mRNA as well as its protein declined during IVM in the ooplasm. In consequence it was shown that VEGF improved cleavage rates of zygotes when supplemented to the maturation medium.

As shown in the present study, a complkex inter- and intracellular communication is of fundamental importance in regulation and timing of bovine oocyte maturation. These results represent a step forward towards understanding the complex interactions of this fascinating and interesting biological events of oocyte maturation in bovine system.

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Appendix

App. 1. Internationally reviewed publications of the author

App. 1.1. Schoenfelder M, Schams D, Einspanier R.: Steroidogenesis during in vitro maturation of bovine cumulus oocyte complexes and possible effects of tri-butyltin on granulosa cells. J Steroid Biochem Mol Biol. 2003 Feb; 84(2-3): 291-300.

Work solely done by the author of this dissertation

The following work for the paper was done solely by the author: including all cell handlings, molecular biology examinations, statistical analysis, and at least the basic writing of the paper. Additional data were presented by a poster presentation.

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Steroidogenesis during in vitro maturation of bovine cumulus oocyte complexes and possible effects of tri-butyltin on granulosa cells☆

M. Schoenfelder, D. Schams, R. Einspanier*

Research Center for Milk and Food Weihenstephan, Institute of Physiology, Technische Universität München, Weihenstephaner Berg 3, D-85350 Freising-Weihenstephan, Germany

Abstract

Steroids are known as important factors on the route of oocytes development and cumulus oocyte complexes (COC) as well as follicular granulosa cells (GC) are suggested to be themselves involved in steroidogenesis. The aim of this study was to characterize such a local sex steroidogenic system during in vitro maturation (IVM) of bovine COCs according to the production of estradiol (E), testosterone (T) and progesterone (P). The expression of two steroid-converting key-enzymes was measured in parallel by quantitative RT-PCR. Furthermore, possible effects of the environmental pollutant tri-butyltin (TBT) were elucidated for the first time on bovine COC and GC in vitro concerning that steroidogenic system.

During IVM of bovine COCs concentrations of P increased continuously, corresponding with steady-state levels of 3-beta-hydroxy-steroid-dehydrogenase (HSD) transcripts. In contrast, E together with P450 aromatase mRNA (ARO) increased in the first hours of IVM but declining thereafter, whereas T reached almost balanced levels. However, TBT showed only slight effects during IVM of COC. In cultured GC, LH caused highest P- and E-production within 24 h and treatment with 50 pM TBT induced a significant decrease of E in contrast to 100 pM TBT and the control. These results indicate, that (1) COCs were able to modulate their steroidogenic environment in vitro and that (2) TBT may possibly influence or disturb steroidogenesis in the cows reproductive tract shown here for GC.

Keywords: Steroidogenesis; Cumulus oocyte complexes; Granulosa cells; Tri-butyltin

1. Introduction

In the last decades important basic research focused on the developmental events during the maturation of mammalian oocytes investigating primordial up to antral and Graafian follicles. Taking advantage of some knowledge of these events in vitro maturation (IVM) of bovine cumulus oocyte complexes (COC) has become a usual technique for commercial production of bovine embryos [1]. It has been recognized that sex steroids fundamentally influence the maturation process in different ways: estradiol (E) may promote alterations in the reactivity of the calcium liberation system during cytoplasmic oocyte maturation [2] influencing the typical calcium oscillation during fertilization [3]. Additionally, the presence of both aromatisable and non-aromatisable androgens during bovine IVM increases oocyte cleavage rates without affecting embryo development. In contrast, progesterone (P) inhibited bovine oocyte maturation leading to reduced embryo development after cleavage [4].

Steroidogenesis within the follicle occurs depending on its developmental stage and gonadotropin status. In preantral follicles, P is produced by cells of the theca interna, indicated by high expression rates of 3-beta-hydroxy-steroid- dehydrogenase (HSD) and cholesterol side chain cleavage enzyme (P450scc), whereby in antral follicles androgens are synthesized and converted by granulosal aromatase P450 (ARO) into 17β-estradiol (reviewed in [5]). Biosynthesis of steroids in GC and cumulus has been demonstrated by several authors for different species: in the cow [6,7], in humans [8], in rats [9] and pigs [10]. Therefore, steroidogenesis should be expected in cultured bovine COCs and GCs as well.

This fine-tuned steroidogenic system, involved in normal sexual differentiation and reproduction, could be dramatically influenced by endocrine disruptors, interfering

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* Corresponding author. Tel.: +49-8161-713510; fax: +49-8161-714204.
E-mail address: einspanierl@wzw.tum.de (R. Einspanier).

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with steroid hormone receptors [11] or interacting with steroidogenic enzymes [12]. Often such disruptors are man-made synthetic chemicals used as pesticides, especially trialkyl organotin compounds. A member of these substances is the environmental pollutant tri-butyltin (TBT), which has been used since the 1950s as a biocide for wood preservation, marine antifouling paints, antifungal action in textiles and industrial cooling systems, paper mills, and breweries [13]. The effects are widespread and depend on the concentration and exposure time. In spite of restrictive usage, the ecological effects of TBT are still present, e.g. impose syndromes (pseudohermaphroditism) in snails after exposure to TBT at concentration lower than 5 pg/ml [14]. Additionally, TBT shows immunotoxicity to human natural killer cells leading to a decreased binding capacity to tumor cells [15], causes apoptosis of rodent thymocytes [16] and induces irritations of the skin as well as lung epithelia [17–19]. Informations about unwanted effects on mammalian reproduction are somehow unavailable.

The aim of the present study was (1) to detect a functional steroidogenic system in bovine COCs during IVM and in granulosa cells (GC) in vitro in response to LH, FSH and FSH + LH in combination. Secondly possible effects of industrial pollutants like TBT on reproductive endocrine systems should be elucidated in that model by recording production of P, T and E in parallel to transcript changes of the steroid-converting enzymes HSD and ARO.

2. Materials and methods

2.1. Cell sampling and culture conditions

Non-cystic bovine ovaries were collected, irrespective to stage of the estrus cycle, at a local abattoir as described by Einspanier et al. [20]. Oocytes were received by aspiration and underwent a common in vitro maturation IVM [21]. COCs were cultured in 400 μl IVM-medium MPM199 containing TCM199 (M-2520, Sigma, GER) supplemented with 10% (w/w) FCS (Seromed, Biochrom KG, GER), 50 μg/ml gentamycin (Selectevet, GER), 0.23 mg/ml pyruvate (15220, Serva, GER), 50 μg/ml ascorbic acid (A-4034, Sigma, GER), 0.55 mg/ml calcium lactate (Merck, GER) and 0.01 U/ml of bovine FSH (NIH B1 activity) as well as LH (NIH LH B10) (Sioux Biochemicals, USA).

GCs were recovered by rinsing antral follicles with PBS (0.24 g/l KH_2PO_4; 8.0 g/l NaCl; 0.2 g/l KCl; 1,44 g/l $Na_2HPO\,2H_2O$; adust to pH7.4 with NaOH) and a 20G needle (Sterican, Braun, GER), washed three times in TCM199 containing 50 μg/ml gentamycin. After final centrifugation at 800 × *g* for 5 min, washed cells were resuspended in culture medium and seeded into 12-well uncoated tissue culture plates (4×10^5 cells per well) by using 2 ml of IVM-medium. To eliminate the potential influence of the oocyte all cultures were essentially COC-free. Gonadotropin stimulation (onset) was done 1.5 d after seeding when all GCs were attached to the bottom and well proliferating.

2.2. RNA extraction and RT-PCR

Cultured COCs and granulosa cells were washed twice in sterile PBS and introduced to RNA extraction performed by spin columns (NucleoSpin RNA II, Macherey-Nagel, GER) including a DNase 1 digestion to reduce contamination by genomic DNA. RNA was quantified spectroscopically by OD260/OD280 nm absorption (Biophotometer, Eppendorff, GER). Total RNA (COC: 200 ng, GC: 500 ng) was reverse transcribed with 200 U M-MLV-reverse transcriptase (Promega, GER) using 2.5 μM random hexamer primers (Gibco BRL, USA) and 0.5 mM dNTP's (Roche Diagnostics, GER) as described earlier [20].

Primers for target genes, designed according known sequences and generated by MWG Biotech (Ebersberg, Germany), were checked according their optimal annealing temperatures (AT) in a gradient cycler (Eppendorff, GER) using pooled bovine cDNA. Verifying specific gene amplification, PCR-products were isolated based on spin column technique (NucleoSpin Extract, Macherey-Nagel, GER) and sequenced (TopLab, Martinsried, GER). Following primer sets were newly selected according to data base search (EMBL accession) and trimmed for LightCycler PCR according the quantification temperatures (QT) of the PCR-products: ubiquitin (UBI) forward 5′-AGA TCC AGG ATA AGG AAG GCA T-3′, reverse 5′-GCT CCA CCT CCA GGG TGA T-3' (EMBL: Z18245; 198, 426 and 654 bp; AT = 60 °C; QT = 85 °C), aromatase P450 (ARO) forward 5′-CAT CAT GCT GGA CAC CTC TAA C-3′, reverse 5′-ATG TCT CTT TCA CCA ACA ACA GTC-3′ (EMBL: U18447; 457 bp; AT = 62 °C; QT = 80 °C) and 3-beta-hydroxy-steroid-dehydrogenase (HSD) forward 5′-TAC CCA GCT GCT GTT GGA G-3′, reverse 5′-ATG CCG TTG TTA TTC AAG GC-3′ (EMBL: X17614; 322 bp; AT = 60 °C; QT = 84 °C).

2.3. LigthCycler real-time PCR

For LightCycler reaction a 10 μl mastermix (Roche Diagnostics, GER) of the following reaction components was prepared containing the indicated end-concentrations: LightCycler mix (1×), $MgCl_2$ (4 mM), each primer (0.4 mM) and sample cDNA (COC: 0.33 ng/μl, GC: 0.83 ng/μl). QT was set 4.0 °C below melting temperature of specific PCR-product. For specific gene amplification the following LightCycler protocol was used:

(a) *Denaturation*: 95 °C for 10 min.
(b) *Amplification and quantification*:
denaturation at 95 °C for 3 s,
AT (indicated above) for 10 s,
extension at 72 °C for 10 s,
single fluorescent measurement at QT (indicated above) for 3 s.

(c) *Melting curve* (60–99 °C):
with a heating rate of 0.1 °C/s, a continuous fluorescent measurement and finally a cooling step at 40 °C for 60 s.

Relative quantification requires the determination of the crossing points (CP) for each transcripts. They are defined as the points at which the fluorescence signals rise appreciably above the background fluorescence [22]. CPs were analyzed by the LightCycler software 3.5 (Roche Diagnostics, GER).

2.4. PCR-Efficiency and relative quantification

2.4.1. PCR efficiency

The PCR efficiency (E) of each primer set was determined in a distinct detection range (2.7 pg up to 8.33 ng of cDNA). Each PCR efficiency was calculated according to the Eq. (1) [22]:

$$E = 10^{-1/s} \quad (1)$$

A standard curve ($n = 3$) served as basis to create the slope (s) and the regression (r). The following PCR efficiencies were detected: 2.00 for UBI ($s = -3.320$; $r = -0.994$), 2.09 for ARO($s = -3.121$; $r = -0.99$), and 1.95 for HSD ($s = -3.435$; $r = -1.00$).

2.4.2. Relative quantification

A mathematical model adopted from LigthCycler software package [23] served to calculate the relative differences between groups. Relative expression ratios were shown by Eq. (2):

$$\text{ratio} = (\text{efficiency of targetgene})^{\text{CP}_{\text{control}} - \text{CP}_{\text{sample}}} \quad (2)$$

where $\text{CP}_{\text{control}}$ is defined as crossing point of the untreated group at the onset of the experiment and $\text{CP}_{\text{sample}}$ represents the treatment groups.

2.4.3. Intra- and inter-assay variation of LightCycler data

To confirm reproducibility and accuracy of real-time PCR, intra-assay precision was performed three times within one LightCycler run as well as inter-assay variation at three different days by using different batches of LightCycler premixes and standard curve (indicated above). Variation tests were carried out with the ubiquitin primer set and resulting standard curve. Reproducibility ($n = 3$) within one run were calculated by using CP mean variation of 0.35% (absolutely 6.05%). Concerning the inter-assay variation a mean variation of 0.48% (absolutely 8.40%) could be detected. The calculation of test precision and variability is based on the standard deviation of CP mean values additionally converted by $(E^{\text{standard deviation}} - 1) \times 100\%$ into absolute data.

2.4.4. Expression of reference genes during gonadotropin and or TBT stimulation

To evaluate a constant efficiency of the RT-reaction it is requisite to compare all samples due to an endogenous standard. Therefore, mainly non-regulated reference genes or housekeeping genes were applicable [24]. For basic cell functions in nucleated cells the presence of these housekeeping gene is important. While screening for a suitable reference gene, we detected a significant upregulation of 18S rRNA ($P = 0.001$) through the LH application compared to the control group in GC cell culture. In contrast, ubiquitin expression seems not to be significantly influenced by the treatments ($P = 0.092$). Therefore, arithmetical normalization of the expression ratios was not carried out.

2.5. Steroid hormone measurements (EIA)

All corresponding supernatants of cell cultures were measured for hormone levels of P, T and E: therefore a simple direct enzyme immuno assay (EIA) on microtiter plates using the second antibody coating technique and horseradish peroxidase (HRP) as the enzyme label (EIA–HRP) were used for quantification [25,26]. The detection limits of the different EIAs ranged form 0.05 up to 25 ng/ml for P, between 2 and 1000 pg/ml for E, and 10 up to 5000 pg/ml for T. If hormone levels were below standard, the concentrations were set to the half of the lowest standard to enable a correct statistical evaluation.

According to the charcoal treatment of the FCS, the hormone levels of P, T as well as of E were eliminated and situated under the specific detection limits of the EIA (data not shown).

2.6. Statistical analysis

One way ANOVA was used to test main effects of LH, FSH, FSH + LH and TBT treatments. If normality test were passed, differences between groups ($n = 6$) of mRNA-levels as well as steroid hormone levels were identified by pair wise multiple comparison procedure (Student–Newman–Keuls method), when normality was failed, differences were analyzed by Kruskal–Wallis one way ANOVA on ranks (Dunn's method). Analyses were done on raw data and performed with SigmaStat 2.0 (Access Softek Inc.). All hormone data are presented as means ± standard deviation (S.D.). Additional, results of mRNA expression were depicted by the calculated ratios according to the control group as explained before by using REST© software tool [27].

3. Results

Initial transcript experiments using RT-PCR showed remarkable amounts of both ARO and HSD mRNA in bovine COC (Fig. 1). During a 24 h IVM distinct expression changes were found and therefore transcript quantifications, done by real-time RT-PCR (LightCycler), were subsequently initiated.

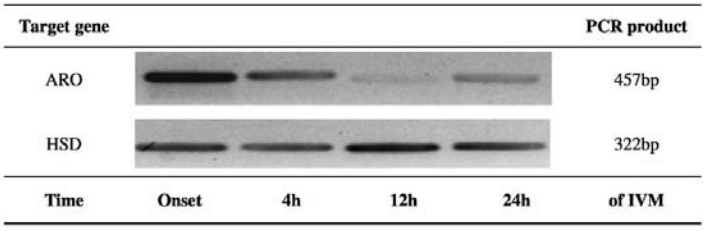

Fig. 1. PCR-products of COC cDNA during IVM, generated by conventional block PCR and separated by 1.5% agarose gel electrophoresis (inversed picture). Target genes: aromatase P450 (ARO), 3-beta-hydroxy-steroid-dehydrogenase (HSD).

3.1. Steroid production of COC during IVM and after TBT treatment

Cultured bovine COCs produced P in a time dependent manner showing highest concentrations after 24 h (Fig. 2a). TBT-treated COCs secreted slightly more P. Corresponding HSD) transcripts significantly declined rapidly and stayed on a lower level until the end of IVM (Table 1) with lowest concentrations after TBT. Concentrations of T in COC supernatants (Fig. 2b) showed a significant in-

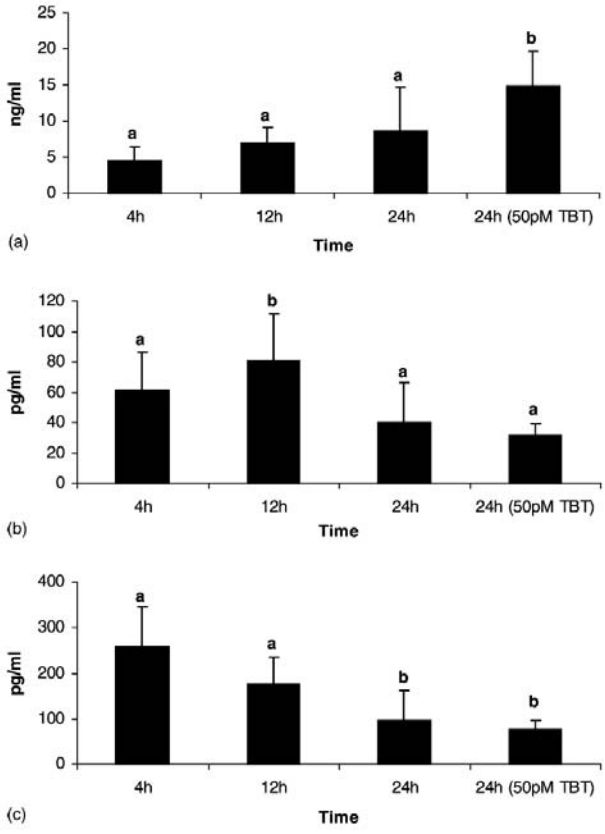

Fig. 2. Steroid hormone concentrations in culture media of COCs during IVM: (a) progesterone, (b) testosterone and (c) estradiol. Data ($n = 6$) are presented as means ± S.D. $P < 0.05$ (significant difference indicated by letters).

Table 1
Ratio of aromatase P450 (ARO) and 3-beta-hydroxy-steroid- dehydrogenase (HSD) mRNA

Target gene	Time (h)				
	0	4	12	24	24 (TBT 50 pM)
Expression ratios (%) to onset (0 h) during COC IVM					
HSD	100 a	42.7 b	36.3 b	49.1 b	32.1 b
ARO	100 a	71.8 a	1.9 b	0.3 c	0.5 c

$P < 0.05$ (significant difference indicated by letters), $n = 6$.

crease after 12 h, but appeared unchanged until 24 h of IVM irrespectively of TBT supplementation. E was found highest within the first hours (Fig. 2c), but declined significantly during 24 h of IVM with comparable low levels after TBT. This continuous decrease of E was accompanied by the significant decrease of ARO expression (Fig. 1 and Table 1).

3.2. Effects of gonadotropins on steroidogenesis granulosa cells culture

Immediately after replacement of the old culture media the GC began to produce P reaching significant increased levels after 4 h under gonadotropins (Fig. 3a). FSH showed the highest stimulus concerning P-secretion followed by LH. After 24 h P reached highest levels when treated with LH compared to the control and FSH. In contrast, co-stimulation with FSH + LH showed a decrease of P. Such P-concentrations were supported by the constant mRNA expression of HSD during the whole experimental period (Table 2).

In the same experiment testosterone (T)-levels (Fig. 3b) did not show significant alterations between the groups after 4 h. Only after 24 h the T-concentration of the FSH stimulated GC cultures was elevated. Obvious differences of E-secretion (Fig. 3c) could be detected when

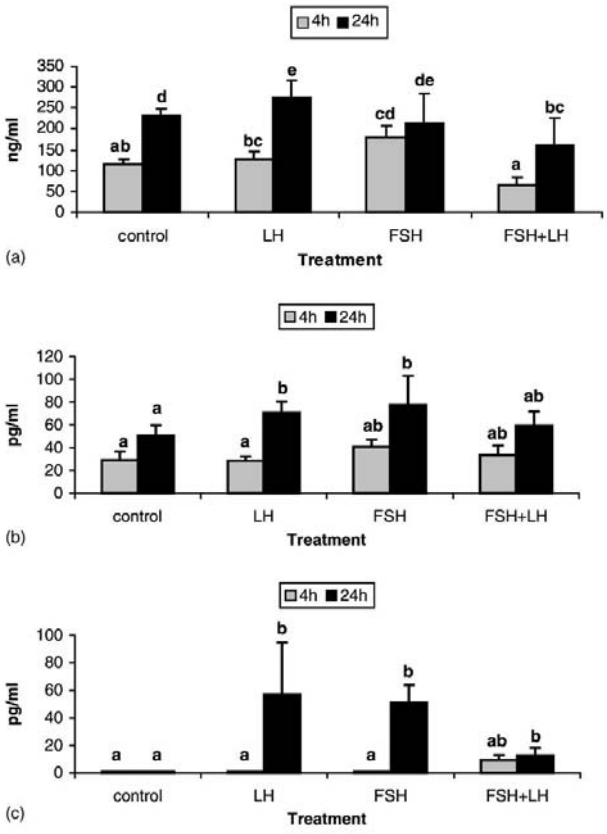

Fig. 3. Steroid hormone concentration in culture media of GC during gonadotropin stimulation: (a) progesterone, (b) testosterone and (c) estradiol. Data ($n = 6$) are presented as means ± S.D. $P < 0.05$ (significant difference indicated by letters).

Table 2
Ratio of aromatase P450 (ARO) and 3-beta-hydroxy-steroid- dehydrogenase (HSD) mRNA according to experimental onset (0 h, 100%)

	Target gene			
	HSD (time (h))		ARO (time (h))	
	4	24	4	24
Expression ratios (%) to onset during cultivation of GC (FSH and/or LH)				
Control	64.6	75.0	134.4	254.0
LH	78.8	73.0	255.3	186.3
FSH	81.9	85.4	317.3	289.8
FSH + LH	51.7	74.6	115.3	395.9

$P < 0.05$ (no significant difference), $n = 6$.

GCs were incubated with all gonadotropins. Whereby the E-concentrations in the controls were always below the detection limit (2 pg/ml), after 24 h E was clearly increased in response to LH and FSH but remarkably lower after a combined treatment. Compared to the aromatase mRNA (ARO) dynamics slightly but not significantly increased transcript levels could be elucidated (Table 2).

3.3. Toxicity of TBT to granulosa cell culture

In order to recover cytotoxic effects of TBT, GC were initially incubated with different TBT-concentration over a wide concentration range (50 pM, 50 nM and 50 μM) and visually characterized. At 50 μM it was obviously that the cultured cells were damaged and detached from the bottom of the dishes (data not shown). Low cellular total RNA concentrations under 50 μM TBT accompanied visual observations. No difference neither in appearance nor total mRNA concentration could be detected in all other GC cultures treated with concentration at or below 50 nM TBT.

3.4. Effects of tri-butyltin on steroidogenesis granulosa cells culture

In the following experiments the possible influence of selected TBT supplementations (50 and 100 pM) on steroidogenesis in cultured GCs should be elucidated.

In contrast to all other tested steroids, P increased significantly after 24 h under 50 pM TBT.(Fig. 4a). However, higher TBT-levels lead to a minor increase of P compared to both gonadotropins applied individually. Accompanying P-levels a slight increase of HSD mRNA (Table 3) could be seen only after 4 h. At the end of the incubation period HSD transcripts in controls were more prominent than TBT-treated samples. As shown previously for gonadotropin stimulations, as well TBT alone had no effect on the T-concentrations (Fig. 4b). Effects of TBT on E-secretion were found contrary to the P measurements: high E contents were significantly reduced under low TBT-concentrations (Fig. 4c). In contrast to low TBT-concentration, 100 pM TBT induced a slight increase in E, but the concentration

Table 3
Ratio of aromatase P450 (ARO) and 3-beta-hydroxy-steroid- dehydrogenase (HSD) mRNA according to experimental onset (0 h, 100%)

	Target gene			
	HSD (time (h))		ARO (time (h))	
	4	24	4	24
Expression ratios (%) to onset during cultivation of GC (TBT)				
Control	51.8	253.5	44.8	321.5
TBT 50 pM	102.2	133.5	787.3	112.5
TBT 100 pM	33.9	69.0	107.5	303.1

$P < 0.05$ (no significant difference), $n = 6$.

Table 4
Ratio of aromatase P450 (ARO) and 3-beta-hydroxy-steroid- dehydrogenase (HSD) mRNA according to experimental onset (0 h, 100%)

	Target gene			
	HSD (time (h))		ARO (time (h))	
	4	24	4	24
Expression ratios in % to onset during cultivation of GC (TBT + FSH)				
Control (FSH)	181.9	331.7	130.1	5.0
TBT 15 pM	680.7	376.6	58.6	1.7
TBT 50 pM	603.4	47.0	21.4	96.3
TBT 50 nM	233.4	226.6	41.0	607.1

$P < 0.05$ (no significant difference), $n = 6$.

remained below the controls. Although E-levels were decreased, ARO expression (Table 3) was increased after 4 h in GC when treated with 50 pM TBT, whereby 100 pM TBT did not cause any significant effects concerning the control.

In order to detect possible synergistic effects between gonadotropins and TBT on GC, additional investigations with FSH and different concentrations of TBT (15 pM, 50 pM and 50 nM were introduced. All three steroids were significantly increased after 24 h of culture under 50 nM of TBT in combination with FSH (Fig. 5). All other TBT treatment groups reacted similarly without significant differences between these each other. Obviously increased ARO mRNA-levels during this experimental setup (Table 4) at 50 nM TBT support directly the measured high E-concentrations in the supernatants.

4. Discussion

Follicular maturation and development are complex processes influenced both by intra- and extra-ovarian events leading to successful ovulation. The LH-surge initially stimulates the secretion of both androgens and estrogens [28], but E-concentrations decline several hours after the LH-surge while P-concentrations are increasing [29]. Furthermore, intra-follicular events such as cumulus expansion may terminate gap junctionial communication between cumulus cells and the oocyte resuming meiosis followed by the germinal vesicle break down. Early experiments demonstrated that

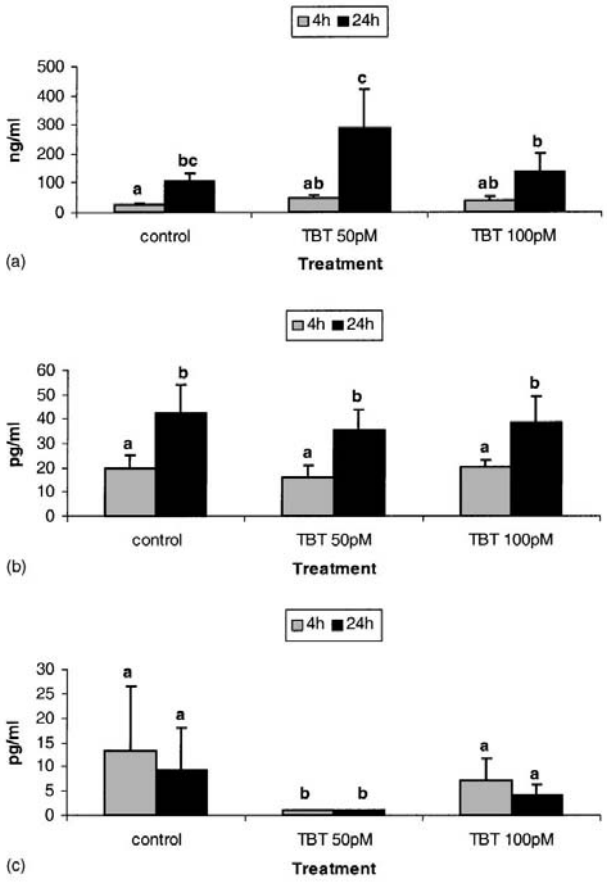

Fig. 4. Steroid hormone concentration in culture media of GC during TBT treatment at different concentrations: (a) progesterone, (b) testosterone and (c) estradiol. Data ($n = 6$) are presented as means ± S.D. $P < 0.05$ (significant difference indicated by letters).

oocytes, removed from the follicle, were also sometimes able to undergo spontaneously resumption of meiosis even without the gonadotropin stimulus [30], a independent local steroid system may contribute to that development.

4.1. Steroidogenesis during IVM of COC

The present results indicate that bovine COCs are able to secrete several steroid hormones during IVM without support of follicular granulosa or theca cells and possess a selection of important steroidogenic enzymes. The observed increase of progesterone is not as high as described for intact follicles after the LH-surge [29]. Such progesterone concentrations in COC culture remained below in vivo data of follicle fluid concentrations, but were comparable to values found in peripheral serum during the estrus cycle [31]. In contrast to progesterone, the secretion dynamics of estradiol were found reversed continuously decreasing. Similar effects have been described for in vivo conditions after the LH-surge [32]. The reduction of estradiol could be seen as a consequence of decreased mRNA expression of FSH receptor (unpublished own data) because FSH receptors are known to activate aromatase or increase cAMP accumulation in granulosa cells [33]. Therefore, cumulus cells, which derived from granulosa cells could still resemble similar reactivity.

4.2. Effects of TBT during IVM of COC

An aberrant composition of local steroids during IVM is known to influence oocyte maturation [7,34] and finally development of the embryo [4,35]. After 24 h of COC

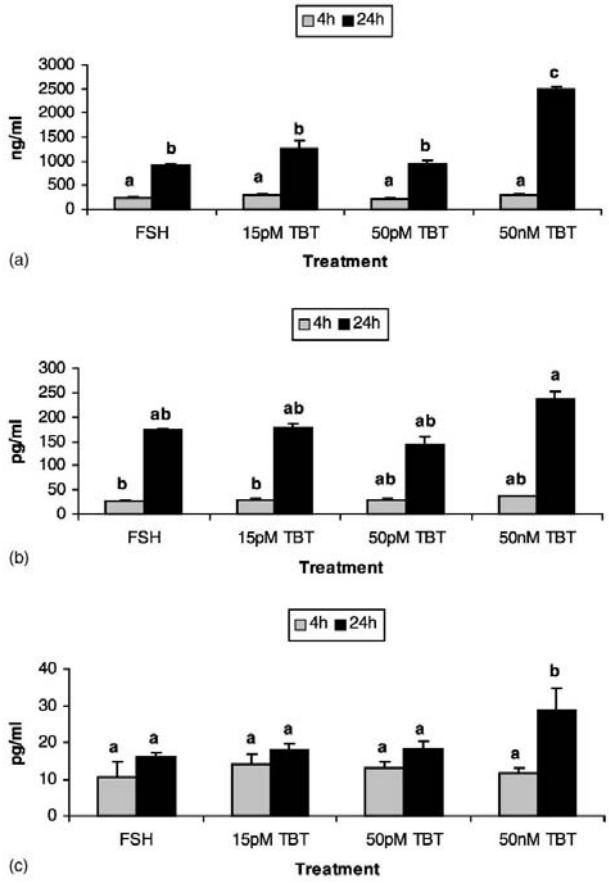

Fig. 5. Steroid hormone concentration in culture media of GC FSH stimulation and simultaneous TBT treatment at different concentrations: (a) progesterone, (b) testosterone and (c) estradiol. Data ($n = 3$) are presented as means ± S.D. $P < 0.05$ (significant difference indicated by letters).

maturation TBT induced either a significant increase in progesterone and a slight reduction in T and E. The decrease of E could be caused either by the reduced mRNA-levels of aromatase or though TBT-induced ARO enzyme-inhibition as previously described for human [36,37]. Furthermore, Whalen and Loganathan [38] described a rapid decrease of intracellular cAMP-levels in human natural killer cells after exposure to TBT. A proposed similar cAMP breakdown could as well happen in the bovine cumulus or granulosa cells, which may lead to a pre-term resumption of meiosis in the oocyte.

4.3. Steroidogenesis during GC culture in response to FSH and LH

A granulosa cell culture was used to further elucidate possible effects of (1) gonadotropins, (2) TBT and (3) gonadotropins and TBT in combination. Although the bovine GCs may undergo luteinisation after removing from the follicle, they showed typical effects after FSH and LH supplementation. Without gonadotropins, the GCs produced P but less estradiol as expected for luteal cells [39]. Although rapid morphological and steroidogenic changes are described for cultured GC [40], our GCs responded well to LH and FSH by increased E beside almost unchanged T- and P-secretion. Supporting such unaffected P-levels, HSD mRNA expression showed no significant changes during gonadotropin treatment.

In contrast, initial ARO mRNA increase caused by FSH and LH led to delayed secretion of E whereby co-treatment induced just slight increased E-levels. These results possible indicate a estrus cycle dependent reactivity of the GCs to different gonadotropin ratios, which were existent in distinct but changing ratios in vivo.

4.4. Steroidogenesis during GC culture in response to TBT

Recent studies would expected that TBT inhibits the conversion of androgens to estrogens [36,37] and therefore T-secretion should increase. However, in our system no significant alteration of the T-levels are induced by TBT possibly indicating that T is not the predominant substrate for the estrogen synthesis. It is known that ARO could also converts androstendione to estrone. Therefore, additional experiments have to elucidate possible effects of TBT on the androstendione pathway.

The most interesting result was that 50 pM TBT blocked completely the production of E although ARO mRNA could be detected at higher concentration as compared to the control GC culture. In contrast, P-concentration were increased by 50 pM TBT despite of lowered HSD mRNA transcripts. These data support the results of Heidrich et al. [37], postulating that TBT blocks ARO through only moderate affecting HSD enzyme activity. However, higher concentrations of TBT (100 pM) led to an increase of E when compared to 50 pM indicating a strict dose-dependant effect of this pollutant in that system.

4.5. Combined effects of TBT and FSH during GC culture

To further enlighten possible interactions between endogenous hormones and the pollutant, a co-treatment was introduced. It was made use of the known stimulatory effect of gonadotropins on the E-production in GC (see this communication and [39]) to simulate an in vivo-like scenery. The main reason for choosing especially FSH as co-stimulator was, that FSH receptor mRNA was much more abundant in GC than the LH receptor (personal data).

To our surprise the highest TBT treatment led to an increase of all steroids in GC cultures additionally provided with FSH, but the previously measured effects of low TBT were erased by simultaneous application of FSH. Distinct dose-dependent and synergistic effects of TBT on bovine GC in cultures have to be taken into account. A possible influence of TBT on the FSH receptor expression may be observed in this content.

Although TBT alone caused lowered E-levels at 50 pM in co-stimulation with TBT such effects were not detectable. Higher concentrations of TBT (50 nM) were necessary to evoke alterations in steroid ratios. Therefore, our results indicate that in vivo effects of TBT could be much more complex as shown in the isolated experiments.

In conclusion, cumulus oocyte complexes were able to produce their own micro-environment according to the three steroids progesterone, testosterone and estradiol, although they are separated from their natural milieu. In addition, it seems that mRNA expressions of aromatase P450 and 3-beta-hydroxy steroid dehydrogensase in parallel reflect the measured steroid products. Our first results concerning COCs under TBT exposure indicate that this potential biocide could influence steroidogenesis of the maturing oocyte in mammals especially through increasing progesterone. In the future cAMP-levels after TBT treatment, potentially modulating meiotic arrest of the oocyte, should be tested. Experiments on bovine granulosa cells supported that gonadotropins clearly elevated levels of estradiol whereby aromatase mRNA data may indicate immediate modulation of transcription. As well as in the cumulus oocyte complexes TBT showed a possible effect onto steroid hormone composition in the culture media. Besides testosterone also changes in androstendione—likewise a substrate of aromatase P450—should be examined as well as short term effects on mRNA expression. There is strong evidence that TBT acts in time—as well as concentration-dependent manner on the steroid hormone producing reproductive cells furthermore interacting with circulating gonadotropins. More efforts are necessary to illuminate the complex action of potent pollutant as TBT. Possible disturbing influences of TBT on the mammalian steroid system within the ovary cannot be excluded.

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App. 1.2. Schoenfelder M, Einspanier R: Expression of hyaluronan synthases and corresponding hyaluronan receptors is differentially regulated during oocyte maturation in cattle. Biol Reprod. 2003 Jul;69(1): 269-77.

Work solely done by the author of this dissertation

The whole work for the following paper was done by the author: including all cell handlings, molecular biology examinations, histological and statistical analysis, and at least the basic writing of the paper.

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Expression of Hyaluronan Synthases and Corresponding Hyaluronan Receptors Is Differentially Regulated During Oocyte Maturation in Cattle[1]

Martin Schoenfelder and Ralf Einspanier[2]

Institute of Physiology, Technical University of Munich, D-85354 Freising, Germany

ABSTRACT

In response to the gonadotropin surge, the compact cumulus-oocyte complex (COC) undergoes expansion by synthesis of the mucopolysaccharide hyaluronan (HA) accompanying oocyte maturation. The objective of the present study was to quantify mRNA transcripts of the HA synthase (HAS) 1, HAS2, and HAS3 and the HA-receptors CD44 and RHAMM (receptor for HA-mediated motility). Additionally, we determined the histological localization of HA and its receptor, CD44, in maturing bovine COCs and cultured granulosa cells (GCs). Full-length transcript of bovine HAS2 and a part of the bovine RHAMM sequence has been made available. Real-time reverse transcriptase-polymerase chain reaction was used for individual mRNA expressions of bovine COCs in comparison to follicular GC gonadotropin treatment. Localization of CD44 and HA were done by immunohistochemistry and biotinylated HA-binding protein, respectively. Gonadotropins caused a rapid, 120-fold increase of HAS2 mRNA, whereas a delayed, 2-fold up-regulation of HAS3 mRNA was observed. The HAS1 transcripts were barely detected. Expression of CD44 mRNA greatly increased during in vitro maturation of COCs, indicating an important role when compared to an unchanged, steady-state RHAMM expression. As a consequence, HA was locally enriched after COC expansion, but only limited change was observed in the GCs. In cultured GCs, HAS2 expression was stimulated through FSH application, followed by the effective treatments of FSH+LH and LH. Treatment with LH induced the highest increase of the CD44 receptor, followed by FSH and FSH+LH treatments. These results suggest that HAS2 is mainly responsible for rapid HA synthesis in bovine COCs and GCs. In bovine COCs, the transcriptional up-regulation of both HAS2 and the receptor CD44 appear to be important prerequisites for initiating HA-mediated effects during final oocyte development and sperm-egg interaction.

cumulus cells, gene regulation, granulosa cells, oocyte development

INTRODUCTION

During the development of ovarian follicles, the granulosa cells (GCs) form a multilayered epithelium surrounding the maturing oocyte. At the end of follicle growth, the GCs differentiate into two subpopulations: the oocyte-attached cumulus cells, and the mural GCs. Several hours after the endogenous LH surge, the cumulus-oocyte complex (COC) undergoes expansion in cattle [1] and other species [2, 3]. This process is thought to be stimulated by FSH or epidermal growth factor [4–7]. One of the principal components of the expanded COC is the glycosaminoglycan (GAG) hyaluronan (HA), which is composed of repetitive disaccharides of D-glucuronate and *N*-acetylglucosamine. This large, linear mucopolysaccharide (2000–25 000 disaccharide units) contributes to tissue homeostasis [8] and biomechanics [9]. To support these complex interactions, different soluble proteins appear to be involved [10]. Some of these HA-binding molecules are of cumulus cell origin [11, 12] and have proposed effects on COC functionality. The resulting spongiform COC matrix may facilitate the extrusion of the oocyte at ovulation and prevent the dispersion of the surrounding cells. Furthermore, HA is supposed to play a key role during fertilization and sperm capacitation [13]. In contrast to the other intracellular-produced GAGs, HA is synthesized at the cell membrane by HA synthases (HAS) [14], which extrude the growing polymer into the extracellular space. In mammals, the HAS family consists of three known isoenzymes (HAS1, HAS2, and HAS3) [15, 16].

During COC expansion in mammalian species, HAS2 [3, 17] was suggested to synthesize the huge amount of HA found in ovarian follicles [18]. Recently, receptors for HA have been identified and include the transmembrane receptors CD44 (named by clusters of differentiation) and RHAMM (receptor for HA-mediated motility). The glycoprotein CD44, probably the most common HA receptor, is characterized by a variety of isoforms caused by alternative splicing and posttranslational modifications [19]. This receptor is responsible for cell-to-cell and cell-to-extracellular matrix (ECM) interactions [20], inhibition of apoptosis [21], endocytosis of HA [22], augmentation of tumor cell motility and metastasis [23], and stimulation of lymphocytes [24]. In contrast, little information is available concerning the regulation of CD44 gene expression, though recent studies indicate that CD44 may influence fertility and quality of human oocytes [25].

The other known HA receptor, RHAMM, possesses two potential HA-binding sites [26]. Interactions between HA and RHAMM could activate protein kinases that modulate cellular behavior. Known to promote cell movement, RHAMM is located both in the cell as well as at the cell surface [27]. Furthermore, the HA receptor may be involved in wound healing [28], migration of smooth muscle cells [29], and modulation of ciliary beating of the airway epithelial cells [30].

The aim of the present study was to characterize the expression profiles of HAS1, HAS2, and HAS3 as well as the corresponding receptors, CD44 and RHAMM, during in vitro maturation (IVM) of bovine COCs. By use of real-time reverse transcriptase-polymerase chain reaction (RT-PCR), the mRNA transcripts of HAS1, HAS2, HAS3, CD44, RHAMM, ubiquitin, and 18S rRNA were relatively

[1]Supported by the DFG.
[2]Correspondence: Ralf Einspanier, Institute of Physiology, Weihenstephaner Berg 3, D-85354 Freising, Germany. FAX: 49 8161 71 4204; e-mail: einspanier@wzw.tum.de

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quantified. Additionally, immunohistochemistry of CD44 and localization of HA by biotinylated HA-binding protein (bHABP) were used to describe the HA system in ovarian cumulus and GCs when under the influence of gonadotropin.

MATERIALS AND METHODS

Cell Sampling and Culture Conditions

Noncystic bovine ovaries were collected, irrespective of stage of the estrous cycle, at a local abattoir and transported at 37°C in sterile Ringer solution to the laboratory within 1 h. Immediately, COCs were recovered by aspiration and sorted out for grade 1 COCs with a compact and multilayered cumulus oophorus as well as homogeneous ooplasm [31]. To reduce estrous cycle-dependent effects, groups of 10 COCs underwent IVM [32, 33]. The COCs were cultured in 400 μl of bicarbonate-buffered modified Parker medium (MPM, pH 7.2) supplemented with 10% (w/w) fetal calf serum (Seromed; Biochrom KG, Berlin, Germany). The MPM was composed of 15.0 g/L of Medium 199 (M-2520; Sigma, Taufkirchen, Germany), 2.2 mg/ml of sodium bicarbonate (S-5761; Sigma), 50 μg/ml of gentamicin (Selectevet; Germany), 0.23 mg/ml of pyruvate (15220; Serva, Heidelberg, Germany), 50 μg/ml of ascorbic acid (A-4034; Sigma), 0.55 mg/ml of calcium lactate (Merck, Darmstadt, Germany), and 0.01 IU/ml of bovine FSH (NIH B1 activity) as well as 0.01 IU/ml of LH (NIH LH B10; Sioux Biochemicals, Sioux Center, IA).

Granulosa cells were recovered by rinsing antral follicles with PBS (0.24 g/L of KH_2PO_4, 8.0 g/L of NaCl, 0.2 g/L of KCl, and 1.44 g/L of $Na_2HPO{\cdot}2H_2O$; pH 7.4) using a 10-ml, single-use syringe (Braun, Melsungen, Germany) connected to a 20-gauge needle (Sterican; Braun); washing three times in culture medium; and seeding into 12-well uncoated tissue-culture plates (4×10^5 cells per 2-ml well). To reduce contamination of atretic GCs, only small follicles up to 8 mm were punctured. Potential influence of simultaneously extracted COCs was prevented by sorting out the GC cultures for oocytes. Gonadotropin stimulation with FSH, LH, or both in combination started 1.5 days after seeding (onset), when all GCs were attached to the bottom and proliferating. The FSH and LH were each used at final concentrations of 0.01 IU, as indicated above. Sampling was carried out at the onset, after 4 h, and after 24 h by removing culture medium and cell lysis for total RNA extraction directly in culture dishes.

RNA Extraction and RT-PCR

For total RNA extraction, all culture media were removed. Before cell lysis, all cultured COCs and GCs were washed twice in sterile PBS and put through RNA extraction performed by spin columns (NucleoSpin RNA II; Macherey & Nagel, Düren, Germany) including DNase1 digestion (Macherey & Nagel) to reduce contaminating DNA. The RNA integrity was verified by optical density (OD) 260/OD280-nm absorption (Biophotometer; Eppendorff, Hamburg, Germany). Total RNA (COC, 200 ng; GC, 500 ng) was reverse transcribed with 200 IU of MMLV-Reverse Transcriptase (Promega, Madison, WI) using 2.5 μM random hexamer primers (Gibco BRL, Carlsbad, CA) and 0.5 mM dNTPs (Roche Diagnostics, Heidelberg, Germany) as described previously [33].

The following primer sets were selected based on published data as indicated. Optimal annealing temperatures (AT) were established in a gradient cycler (Mastercycler; Eppendorff) and tested for quantification temperature (QT) by a melting-point analysis in the LightCycler (Roche): 18S rRNA, forward 5′-AAG TCT TTG GGT TCC GGG-3′, reverse 5′-GGA CAT CTA AGG GCA TCA CA-3′ (EMBL, AF176811; product, 365 base pairs (bp); AT, 60°C; QT, 82°C); ubiquitin, forward 5′-AGA TCC AGG ATA AGG AAG GCA T-3′, reverse 5prime;-GCT CCA CCT CCA GGG TGA T-3′ (EMBL, Z18245; product, 198 bp; AT, 60°C; QT, 85°C), HAS1, forward 5′-GGT ACA ACC AGA AGT TCC TGG G-3′, reverse 5′-CGG AAG TAC GAC TTG GAC CAG-3′ (EMBL, AB017803; product, 184 bp; AT, 55°C; QT, 79°C); HAS2, forward 5′-GGM TGT GTC CAG TGC ATT AGC GGA C-3′, reverse 5′-CAG CAC TCG GTT CGT TAG RTG CCT G-3′ (EMBL, U54804; product, 144 bp; AT, 68°C; QT, 80°C); HAS3, forward 5′-ACA GGT TTC TTC CCC TTC TTC C-3′, reverse 5′-GCG ACA TGA AGA TCA TCT CTG C-3′ (EMBL, AJ293889; product, 166 bp; AT, 60°C; QT, 84°C); HA-receptor CD44, forward 5′-TAT AAC CTG CCG ATA TGC AGG-3′, reverse 5′-CAG CAC AGA TGG AAT TGG G-3′ (EMBL, X62881; product, 221 bp; AT, 64°C; QT, 83°C); and HA-receptor RHAMM, forward 5′-TGT TGA ATG AAC ATG GTG CAG CTC-3′, reverse 5′-CCT TAG AAG GGT CAA AGT GTT TGA T-3′ (EMBL, AF310973; product, 249 bp; AT, 61°C; QT, 77°C). To verify specificity of each gene amplification, PCR products were isolated and sequenced (TopLab, Martinsried, Germany).

Quantitative Real-Time PCR

For each sample, a standard 10-μl real-time PCR reaction mix (Roche) was prepared containing the following components: 1 μl of LightCycler mix, 4 mM $MgCl_2$, 0.4 mM primers, and sample cDNA (COC, 0.33 ng/μl; GC, 0.83 ng/μl). The QTs were set below the individual melting peak of each PCR product. For specific gene amplification, a standard protocol of 50 cycles was used in the LightCycler: After initial polymerase activation at 95°C for 10 min, primer-specific amplification and quantification cycles were run at AT and QT as indicated above. To evaluate specific amplification, a final melting curve was created (60–99°C) under continuous fluorescent measurement.

Relative quantification was determined using sample crossing points analyzed with LightCycler software 3.5 (Roche) by the second derivative maximum method.

PCR Efficiency and Relative Quantification

PCR efficiency. The PCR efficiency (E) of each primer set was determined within distinct detection ranges (2.7 pg to 8.33 ng of cDNA) and calculated according to the following equation [34]:

$$E = 10^{-1/\text{slope}} \quad (1)$$

From resulting standard curves (n = 3), PCR efficiency, slope (s), and regression (r) were determined as follows: for 18S rRNA, $E = 1.95$, $s = -3.436$, and $r = -0.997$; for ubiquitin, $E = 2.00$, $s = -3.320$, and $r = -0.994$; for HAS1, $E = 1.81$, $s = -3.856$, and $r = -0.991$; for HAS2, $E = 1.81$, $s = -3.874$, and $r = -0.993$; for HAS3, $E = 1.82$, $s = -3.841$, and $r = -0.995$; for CD44, $E = 2.00$, $s = -3.323$, and $r = -0.996$; and for RHAMM, $E = 1.98$, $s = -3.453$, and $r = -0.995$.

Relative quantification. A mathematical model adopted from the LightCycler software package [35] served to calculate the relative differences between groups. Relative expression ratios were calculated by the following equation:

$$\text{Ratio} = (\text{efficiency of target gene})^{CP_{\text{control}} - CP_{\text{sample}}} \quad (2)$$

where CP_{control} is the crossing point of the untreated group at the onset of the experiment and CP_{sample} is the crossing point of the treatment group.

To evaluate the efficiencies of the preceding RT reaction, unregulated reference genes are suggested as control [36]. Therefore, all cDNA samples were compared to endogenous standards. Because of its highly balanced expression ($P = 0.092$), ubiquitin and 18S rRNA served as the main housekeeping genes in our system. To ensure reproducibility and accuracy of real-time PCR, assay precision was confirmed using ubiquitin PCR within one LightCycler run (n = 3) or at three different days using different batches of LightCycler premixes showing an intraassay variation of 6.05% and an interassay variation of 8.40%.

Full-Length cDNA Sequencing

Full-length cDNA sequencing of the bovine HAS2 was performed using the SMART-RACE cDNA-Kit (Clontech, Palo Alto, CA) introducing total RNA of bovine COCs. One microgram of RNA and 200 IU of MMLV-Reverse Transcriptase were used for first-strand synthesis in the 5′- as well as 3′-direction. Resulting cDNA libraries were subsequently used for amplification of specific PCR products by introducing the described HAS2 primers in a thermal cycler (Master Gradient Cycler; Eppendorff) performing 25 cycles of denaturation at 94°C for 5 sec, annealing at 68°C for 10 sec, and elongation at 72°C for 2 min and 30 sec. The PCR products were sequenced commercially (TopLab).

Statistical Analysis

One-way ANOVA was used to test the effects of LH, FSH, and FSH+LH application. Differences between groups (n = 6) were identified by least significant difference Student t-test ($P < 0.05$). Analyses were performed with SAS release 8.01 (SAS, Inc., Cary, NC). Raw data are presented as the crossing point (mean ± SD) and the variation quotient (in %). Results were depicted by the calculated expression ratios according to the control group.

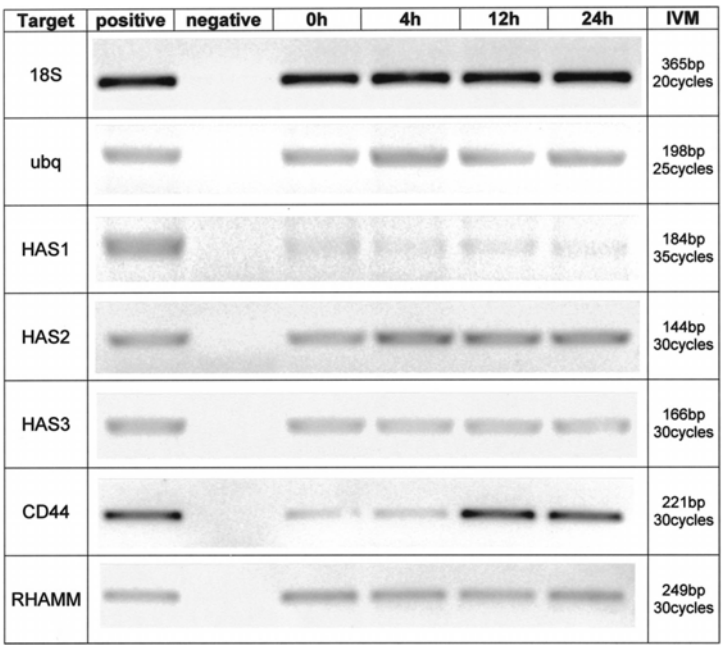


FIG. 1. Messenger RNA expression (one of six experiments) of selected genes during 24-h IVM of bovine COC detected by a conventional RT-PCR. Housekeeping genes: 18S rRNA (18S) and ubiquitin (ubq); target genes: HAS1, HAS2, HAS3, and HA receptors (CD44 and RHAMM). Bovine follicular cell RNA served as the positive control. Specific amplicons were separated by gel electrophoresis.

Histological Localization of HA and Immunodetection of CD44

Cultured bovine COCs and ovary were embedded in TissueTek (Miles, Inc., Torrance, CA) and shock-frozen in liquid nitrogen. Serial cross-sections (COC: thickness, 14 μm; ovary: thickness, 7 μm) were performed using a cryotome (HM 505E; Microm, Walldorf, Germany) and mounted on preheated (37°C) HistoBound glass slides (Marienfeld). The GCs were grown and treated directly on sterilized glass slides provided with flexiPERM chambers (Vivascience AG, Germany). Before specific staining, all cryosections as well as cultured GCs were air-dried for 30 min at 37°C and fixed with ethanol for 5 min at room temperature at −30°C for HA localization or with 3.7% formaldehyde in PBS for 15 min at room temperature for CD44 immunodetection.

For HA localization, all tissues were incubated for 30 min with 5 ng/ml of bHABP (catalog no. 385911; Calbiochem, San Diego, CA) diluted in PBS. Unbound bHABP was removed by washing twice in PBS. To detect bound bHABP, slides were treated for 30 min with a streptavidin fluorescein conjugate (SAF; 1 μg/ml, catalog no. 189734; Calbiochem), washed twice in PBS, counterstained for 5 min with propidium iodide in PBS (2.5 μg/ml), and mounted in antifading solution Citiflour AF1 (Agar Scientific, Essex, U.K.). Negative controls were performed either without bHABP or without SAF.

Immunolocalization of bovine HA-receptor CD44 was accomplished by using rat-anti-porcine CD44 immunoglobulin (Ig) G (1:100 dilution; SM488, lot 130900; DPC Biermann, Bad Nauheim, Germany) as first antibody and horseradish peroxidase (HRP)-labeled goat-anti-rat IgG (1:200 dilution; R1378HRP, lot 6563; DPC Biermann) for visualization by diaminobenzidine (DAB; D-5905; Sigma). After fixation in formaldehyde, samples were washed twice in PBS and incubated for 30 min in 1% H_2O_2 to block endogenous peroxidases. Background was reduced by treatment with 10% normal goat serum for 30 min. First-antibody incubation was done overnight, followed by three washing steps in PBS and incubation with HRP-labeled goat-anti-rat IgG for 1 h. Specific staining of CD44 were visualized by 0.05% DAB in 0.01% H_2O_2. Additionally, samples were counterstained by Mayers Haemalaune, dehydrated with graded alcohols, and embedded in Eukitt (Kindler, Freiburg, Germany). Specificity of the immunodetection was proved by several negative controls: 1) replacement of the first antibody, 2) replacement of secondary antibody, and 3) incubation with DAB alone to show efficiency of blocking endogenous peroxidases. All samples were visualized with a Axioscope microscope (Zeiss, Jena, Germany) and documented by digital imaging (Zeiss AxioCam MR with AxioVision software) or color slides (Kodak Elite 100; Eastman Kodak, Rochester, NY).

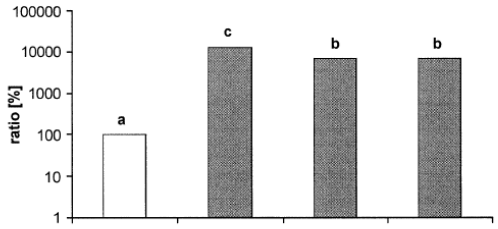
RESULTS

The PCR-based amplifications of the bovine COC mRNA, obtained during IVM, detected discrete components of the HA system, including enzymes and receptors (Fig. 1). Different signal intensities were identified for HAS2, HAS3, CD44, and RHAMM. Figure 1 indicates that HAS1 mRNA was hardly detected when amplified for 35 PCR cycles. The four more-abundant members of the HA system were subsequently detected and quantified by real-time RT-PCR.

Sequencing of the bovine HAS2 mRNA by use of rapid amplification of cDNA ends technology lead to an mRNA of 2946 bases possessing the 5′ untranslated area and the entire 3′ untranslated region containing a putative polyadenylation signal (AATAAA) at base 2888, leading further into the poly-A-tail. An open reading frame was identified between bases 545 and 2201 encoding a 552-amino acid protein (63 kDa). This HAS2 cDNA sequence has been submitted to a nucleic acid database to permit public access (EMBL, BTA004951). To our knowledge, it is the first description of the complete bovine HAS2 mRNA sequence derived from bovine COC RNA, enabling further transcript characterization in cattle. The deduced open reading frame showed high homologies to known HAS2 cDNA as well as to deduced protein sequences of other species (cDNA homology, 93.6% human, 90.7% rat, and 88.9% mouse; protein homology, 97%–98%).

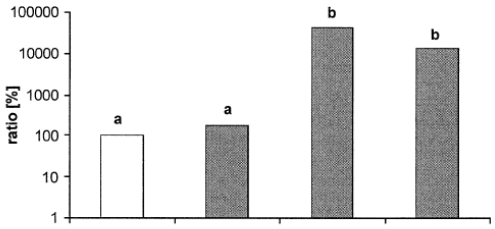
Furthermore, a functional PCR setup amplifying bovine RHAMM transcripts was introduced (Fig. 1). Sequencing

Fig. 2A: HAS2 mRNA in COC



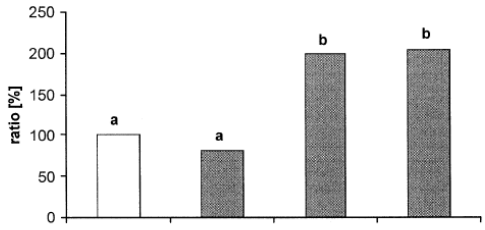
Time [h]	0	4	12	24
CP mean	29.88	21.71	22.72	22.76
SD [CP]	±0.56	±0.39	±0.15	±0.54
VQ [%]	1.88	1.79	0.66	2.38

Fig. 2C: cd44 mRNA in COC



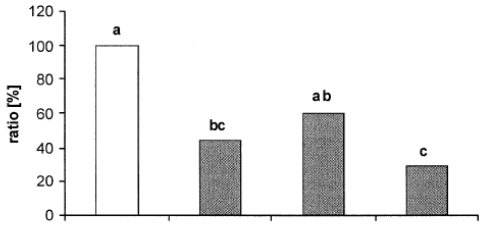
Time [h]	0	4	12	24
CP mean	37.73	36.89	28.98	30.67
SD [CP]	±0.35	±1.58	±0.82	±0.37
VQ [%]	0.92	4.29	2.82	1.21

Fig. 2B: HAS3 mRNA in COC



Time [h]	0	4	12	24
CP mean	31.06	31.37	29.91	29.87
SD [CP]	±0.18	±0.25	±0.05	±0.01
VQ [%]	0.57	0.80	0.18	0.03

Fig. 2D: RHAMM mRNA in COC



Time [h]	0	4	12	24
CP mean	29.02	30.19	29.74	30.78
SD [CP]	±0.24	±0.14	±0.08	±1.01
VQ [%]	0.82	0.45	0.27	3.27

FIG. 2. Expression ratio during 24-h IVM detected for HAS2 (**A**), HAS3 (**B**), CD44 (**C**), and RHAMM (**D**) in bovine COC. Statistically significant differences are indicated by letters ($P < 0.05$). Means of crossing points (CP mean), SD of crossing points (SD), and variation quotient (VQ) are depicted in the bottom charts.

of this cDNA provided a new partial sequence of this bovine HA-receptor type, including two potential HA-binding sites (EMBL, AJ439694) with high homologies to other RHAMM sequences (cDNA homology, 98% sheep, 89% human, and 82% rat).

HA Synthases

In both cumulus as well as GCs, HAS2 showed the most prominent increase in mRNA expression after gonadotropin treatment (FSH+LH in COCs and LH in GCs). In COCs, HAS2 transcripts reached the highest levels (120-fold) within 4 h (Fig. 2A) and decreased thereafter, reaching a plateau at 60-fold higher when compared to the experimental onset ($P < 0.001$). In GCs, the HAS2 elevation (Fig. 3A) was highest under FSH (5.5-fold, $P = 0.04$), followed by FSH+LH in combination (3.5-fold, $P = 0.003$) and then LH (2.6-fold, $P = 0.05$). After 24 h, HAS2 transcripts returned to or dropped below the baseline level.

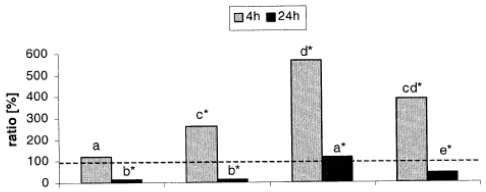
Although HAS3 increased, if not as much as HAS2, significant alteration of HAS3 transcripts was detected after 12- and 24-h IVM in COCs (Fig. 2B). The relative concentration at the onset of IVM and 4 h later (indicated by low crossing points) as well as the increase after 12 h in HAS3-specific mRNA (2-fold, $P = 0.003$) was always below the height found for HAS2. In contrast, cultured GCs always showed a significant decline of HAS3 transcripts within the first 4 h compared to the onset (Fig. 3B). Regardless of gonadotropin treatment, the HAS3 expression was adjusted to the lowest levels at the end of each experiment after 24 h.

In bovine COCs as well as in GCs, HAS1 mRNA was not detectable with the real-time RT-PCR system (2 pg of cDNA). The validity of these primers was demonstrated using whole ovarian follicle mRNA (Fig. 1).

HA-Receptors CD44 and RHAMM

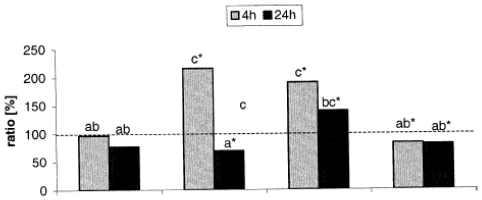
Within the first 4 h of COC maturation, very low mRNA quantities of the HA-receptor CD44 were present (Fig. 2C). After 12 h of IVM, CD44 expression increased more than 100-fold ($P = 0.001$) and stayed high until the end of mat-

Fig. 3A



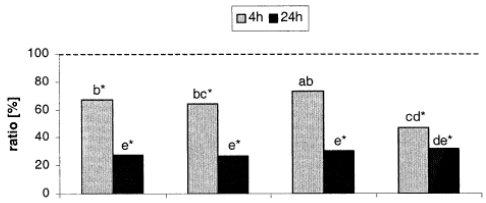
Treatment	control		LH		FSH		FSH+LH	
CP mean	27.37	30.77	26.06	31.21	24.75	29.33	25.39	29.02
SD [CP]	±1.35	±0.15	±0.77	±1.86	±0.27	±0.39	±1.92	±0.08
VQ [%]	4.93	0.49	2.94	5.95	1.09	1.32	7.57	0.28

Fig. 3C



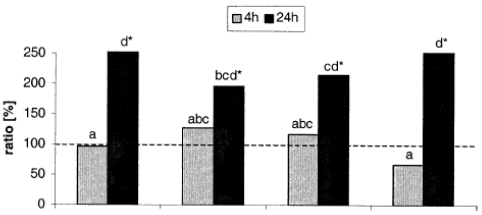
Treatment	control		LH		FSH		FSH+LH	
CP mean	24.78	25.11	23.62	25.28	23.60	24.25	25.01	25.06
SD [CP]	±0.34	±0.19	±0.57	±0.36	±0.12	±1.38	±2.37	±0.09
VQ [%]	1.39	0.77	2.39	1.42	0.52	5.68	9.49	0.37

Fig. 3B



Treatment	control		LH		FSH		FSH+LH	
CP mean	29.11	30.58	29.17	30.65	28.96	30.40	29.69	30.32
SD [CP]	±0.34	±0.01	±0.26	±0.88	±0.07	±0.24	±0.78	±0.15
VQ [%]	1.16	0.29	0.88	2.88	0.26	0.79	2.62	0.48

Fig. 3D



Treatment	control		LH		FSH		FSH+LH	
CP mean	29.08	27.64	28.69	28.06	28.80	27.93	29.62	27.49
SD [CP]	±0.78	±0.20	±0.56	±2.21	±0.24	±0.66	±1.90	±0.04
VQ [%]	2.69	0.71	1.96	7.88	0.82	2.35	6.41	0.15

FIG. 3. Expression ratios during 24-h culture of bovine GCs in relation to the experimental onset for HAS2 (**A**), HAS3 (**B**), CD44 (**C**), and RHAMM (**D**). Treatments: control (MPM199 [MPM] + fetal calf serum [FCS]), LH (MPM+FCS+0.01 IU/ml of LH), FSH (MPM+FCS+0.01 IU/ml of FSH), and FSH+LH (MPM+FCS+0.01 IU/ml each of FSH and LH). Dashed line represent expression level (100%) at the onset (0 h). Statistical analysis were done by one-way ANOVA (least significant difference test), whereby differences between groups ($P < 0.05$) are indicated by letters and between onset and groups by an asterisk. Mean of crossing point (CP mean), SD of crossing point (SD), and variation quotient (VQ) are depicted in the bottom charts.

uration (24 h, $P = 0.047$). In GCs, response to both gonadotropins was detected, but at a lower level (Fig. 3C). In these cells, LH induced the highest increase (2.2-fold, $P = 0.019$) within the first hours, followed by FSH (1.9-fold, $P = 0.047$). In addition, FSH showed a more prolonged stimulus on CD44 expression after 24 h ($P = 0.028$). However, no effect was observed when both gonadotropins were supplemented simultaneously (4 h, $P = 0.546$; 24 h, $P = 0.496$).

The PCR results for RHAMM indicated that its mRNA is detectable at all stages of IVM in COCs (Fig. 2D) as well as during GC culture (Fig. 3D). The initial expression of RHAMM decreased until the end of COC maturation and reached significant differences compared to the control after 4 and 24 h of IVM. In contrast, during the first hours, the RHAMM mRNA levels in GCs stayed unchanged, but it reached significantly higher levels in all groups after 24 h. Neither FSH, LH, or their combination provoked significant alterations compared to the untreated control.

Localization of HA-Receptor CD44 in the Ovary, COCs, and Cultured GCs

Serial cross-sections of the whole bovine ovary showed a distinct staining for immunoreactive CD44 in small antral follicles (Fig. 4A). Specific staining could be detected for the theca interna as well as for the theca externa. However, no specific localization for CD44 was observed in mural GCs or in COCs of immature follicles (Fig. 4A).

In sections obtained from matured bovine COCs with an expanded cumulus, specific staining for CD44 was observed only for several cumulus cells (Fig. 4B). In contrast, cells of both corpora lutea (Fig. 4C) and cultured mural GCs (Fig. 4D) were positive for the HA-receptor CD44. Furthermore, in GC cultures, an irregular staining was observed (Fig. 4D, arrows).

Localization of HA in COCs and GCs

In histological cross-sections of COCs, it could be shown that the cumulus forms a compact and multilayered

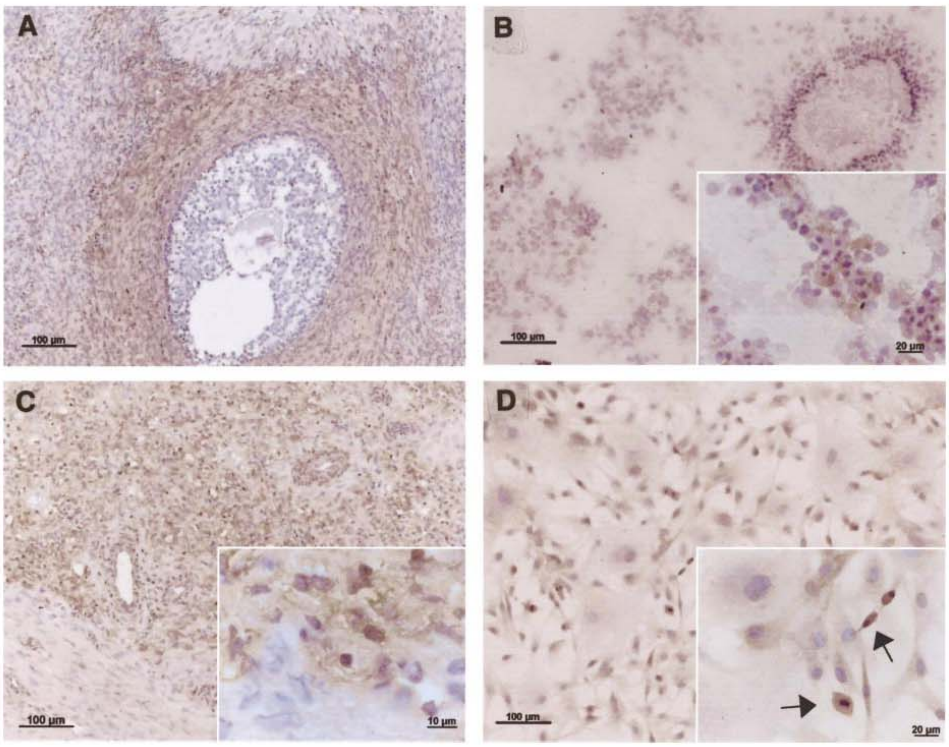


FIG. 4. Histological detection of CD44 by specific antibody. Specific localizations were detected by a brown DAB-chromophore. Nuclei were counterstained with Mayers Haemalaune. A) Cryosection of an early antral follicle. B) Cryosection of COC 24 h after IVM. C) Cryosection of corpus luteum. D) Cultured GCs (arrows depict CD44-specific immunoreaction).

envelope around the oocyte (Fig. 5A). At the onset of IVM, all cumulus cells were tightly attached to each other, and only a faint HA-specific staining could be detected in the extracellular space. After 24 h of IVM (Fig. 5B), an increase of HA, indicated by the green fluorescence, could be observed. Most of the cumulus cells were dispersed from the oocyte but were anchored in a large coat of HA. Only a single-cell layer, the corona, was still attached to the oocyte. All the controls remained free of unspecific staining (data not shown). In cultured GCs, HA was hardly detectable at the beginning of the experiment (Fig. 5C), but after 4 h, sporadic signals specific for HA became visible (Fig. 5D). In GC cultures grown in medium supplemented with both gonadotropins, increased and cloud-like, HA-specific fluorescence was observed (Fig. 5E). However, 24 h after gonadotropin stimulation, all cultured GCs appeared to be nearly depleted of cell-bound HA molecules (Fig. 5F).

DISCUSSION

In the present study, a functional HA-synthesizing and -binding system was detected during IVM of bovine oocytes. Of the three identified mammalian HA-producing enzymes, only two, HAS2 and HAS3, were observed. Their obvious regulation throughout oocyte maturation may be mediated by gonadotropins. After determining the missing gene information for the bovine HAS2 and RHAMM, first transcript quantifications could detect a distinct and immediate amplification of HAS2, HAS3, and CD44 within the first hours of IVM by use of real-time RT-PCR.

These findings were supported by the excessive local accumulation of HA, shown using histological techniques, that matched the increased enzyme transcription. These rapid changes indicate extensive alterations of the microenvironment during bovine IVM, leading to cumulus expansion, probably under peripheral hormonal control. Ubiquitous HA is known to influence major cellular events, such as cell migration and proliferation [37], and might be an important polymer during final oocyte development and fertilization [13]. Compared to HAS3, HAS2 is the most abundantly expressed HA-synthesizing enzyme during bovine COC expansion, as has been reported in mouse cumulus cells as well [38]. However, in contrast to the rapid mRNA expression of HAS2 in the cow, recent reports by Kimura et al. [3] concerning porcine IVM showed a significant increase of HAS2, but not until 24 h. Additionally, HAS3 mRNA was located exclusively in porcine oocytes and was lowered by substitution with gonadotropins. These lower amounts of porcine HAS3 expression are comparable to the present bovine system when LH and FSH were added to the maturation medium. However, the lower transcript concentration does not exclude HAS3 from also being important for HA production during cumulus expansion [16]

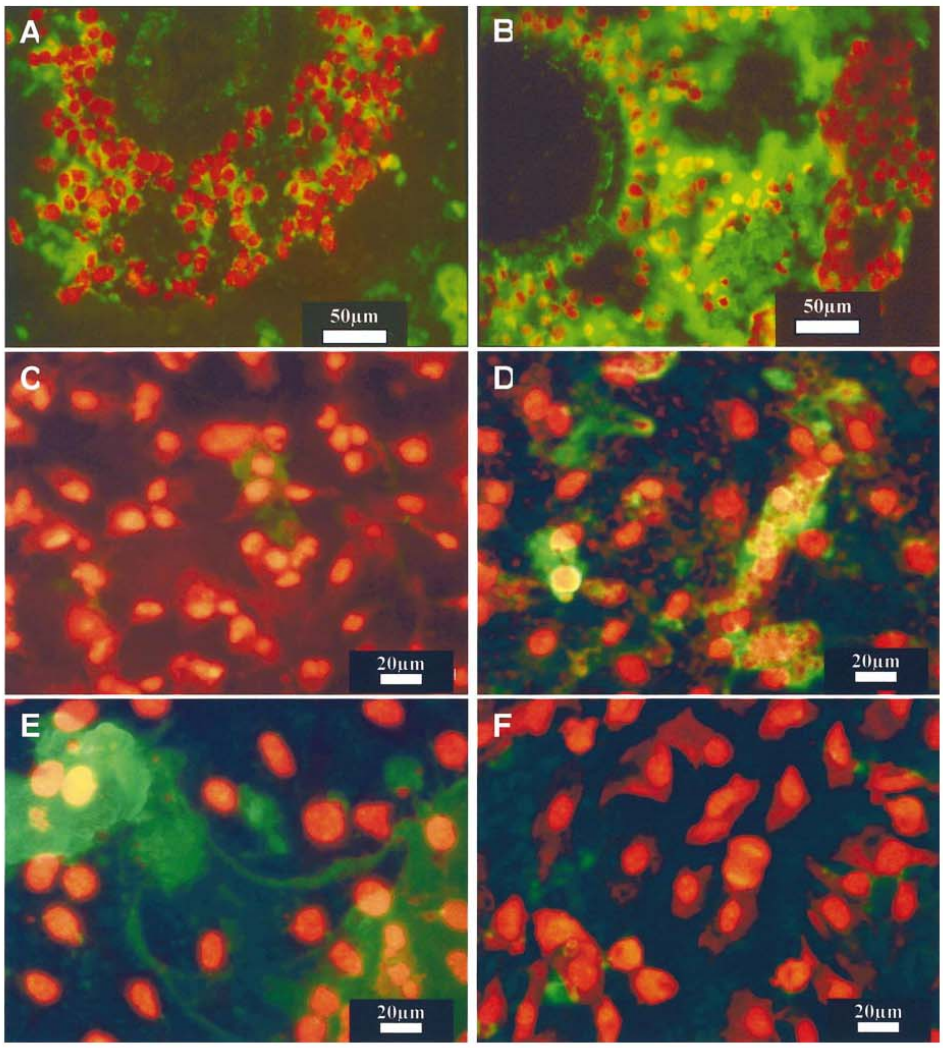


FIG. 5. Histochemical localization of HA by bHABP conjugated with SAF and counterstained with propidium iodide. **A** and **B**) Serial cross-sections of COC at the beginning of IVM (**A**) and after 24 h of cultivation (**B**). **C–F**) GCs grown on glass slides. Untreated control culture is shown at the onset (**C**) and after 4 h without gonadotropins (**D**). Also shown are GCs treated with FSH+LH after 4 h (**E**) and after 24 h (**F**).

when considering the diverse processing characteristics of these enzymes, as shown earlier [39]. The HAS3 possesses intrinsically higher catalytic activity than other HAS enzymes, and the chain length of HA molecules synthesized by HAS3 is shorter when compared to HAS2-generated polymers. Low-molecular-weight HA will directly influence the expression of cytokines and cell shape in eosinophils [40], but the proportion as well as the effect of the different HA length classes in the bovine cumulus is unknown. The immediate response of HAS2 to gonadotropins indicates its local importance, but the influence of the later, much-less-expressed HAS3 has to be established in the bovine COC relative to different functions of the resulting polymer. Independent of biomechanical effects during ovulation, HA could transmit signals through binding to receptor proteins and, potentially, influence cell functions and properties in a paracrine manner [41].

Although little is known about regulation of the HA-receptors CD44 and RHAMM in reproductive tissue, the few experiments that have been conducted suggest that CD44 plays an important role during human oocyte maturation [25] and prevents apoptosis in human GCs [21]. The CD44 protein has been found by immohistochemistry in a variety of mammalian epithelial cells, including oviduct, uterus, and vagina [42]. Such observations suggest a local communication between the cumulus-derived HA and the

HA receptors on the reproductive epithelium (e.g., the oviduct). Cycle-dependent regulations of CD44 in the human tissues have been described with increased levels during the uterine secretory phase, indicating a possible steroid influence [43]. Conversely, our results suggest an endocrine influence on the mRNA expression of the HA-receptor CD44. Although GCs produced steroid hormones under FSH or LH application, the production of progesterone and estradiol lagged behind the initial increase of CD44 mRNA at 4 h [44]. Therefore, the major influence on CD44 induction in GCs is likely based on gonadotropins rather than on steroid hormones. In contrast to the lack of CD44 modulation in GCs, with simultaneous treatment of LH and FSH the same conditions led to a significant increase of CD44 mRNA transcripts during COC maturation. Hence, our results suggest both an endocrine as well as a paracrine regulation of CD44 through gonadotropins and, probably, oocyte factors, respectively. A few so-called oocyte-derived factors (e.g., growth and differentiation factor 9) may be potential modulators for the essential interplay between the oocyte and the surrounding somatic cells [45]. Additionally, the altered expression pattern of HAS3 in oocyte-surrounding cumulus cells when compared to the GCs indicates especially the oocytes feasible influence. Although RHAMM was unregulated under gonadotropins, possible functions of this HA receptor in the bovine reproductive tract could be important, because basic expression levels during IVM were found to be compared to CD44 expression. The RHAMM may mediate movement and attachment of human cumulus cells [46], or it could modulate ciliary beating of oviduct epithelia, as described for the respiratory system [30]. Recent work discovered CD44 as the essential HA receptor throughout development of the preimplantation human embryo [47] and during trophoblast implantation [48]. Further investigations will be necessary to elucidate the detailed functions of both receptor types in the bovine reproductive system.

Our in vitro results indicate that a balance of FSH and LH is capable of modulating the main components of the HA system in bovine follicular cells. These data support an influence of the endogenous gonadotropin surge before ovulation and, subsequently, stimulating HAS2 expression within 24 h in native follicular GCs [49]. The common bovine IVM system appears to be comparable to normal physiological situations leading to fully mature COCs. An exogenous application of HA during bovine IVM showed beneficial effects on both IVM and in vitro production of embryos [50]. Additionally, Saito et al. [18] postulate that the concentration of HA in the follicular fluid could be used as an indicator to estimate oocyte fertilization capacity.

Previously described effects of HA surrounding the oocyte have indicated that it could serve as a protective shield as well as a reservoir for different growth factors. Glycosaminoglycans are negatively charged compounds and, therefore, bind unspecifically to many other substances, including growth factors. The interactions between GAGs and cytokines are one of the important mechanisms underlying communication processes between cells that are mediated by secreted and locally acting factors. Various studies have shown that quite a few cytokines are associated with the ECM by polysaccharide-binding motives [51]. For example, vascular endothelial growth factor protein has been found attached in increasing amounts at the bovine COC during final maturation [32], and such HA-rich oocyte microspheres may serve as a pool for such potent cytokines. Rapid remodeling of the ECM of COCs may trigger cell function through a possible cleavage of cytokines from that matrix. Still another mechanism of HA action together with bound cytokines could alter cell dynamics through intracellular uptake of HA via CD44-mediated incorporation [22]. A mucosal host defense has been proposed based on HA protecting cells from ciliary clearance in the lung [52]. This may draw future attention to interesting interactions between the cumulus and the oviduct after ovulation of the COCs.

In summary, our findings assign HAS2 and the corresponding receptor, CD44, a major role during final bovine oocyte maturation facilitating cumulus expansion. Interestingly, CD44 appeared to be differentially expressed in bovine cumulus in contrast to GCs: LH and FSH in combination induced an altered reaction in GCs. Considering such a diverse response, oocyte-derived local factors likely play a role in regulating the CD44 expression during IVM independent of endocrine signals. The results of investigations with HA components indicate a complex, local, and fine-tuned system during final oocyte maturation in the cow. These findings may further complete our understanding of the complex network of circulating hormones, local growth factors, and ECM components enabling optimal development of the oocyte that leads to reproductive success. Furthermore, the growing knowledge of such essential physiological processes supporting oocyte maturation will enable an improved understanding of assisted reproduction.

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App. 1.3 Einspanier R, Schoenfelder M, Müller K, Stojkovic M, Kossmann M, Wolf E, and Schams D: Expression of the vascular endothelial growth factor and its receptors and effects of VEGF during in vitro maturation of bovine cumulus-oocyte complexes (COC). Mol Reprod Dev 2002, 62: 29-36.

Work solely done by the author of this dissertation

The work was done in collaboration with the Moorversuchsgut in Oberschleißheim. The following work was solely done by the author: molecular biology examinations of VEGF and its receptors, immunhistological and statistical analysis, and at least partially writing of the paper.

MOLECULAR REPRODUCTION AND DEVELOPMENT 62:29–36 (2002)

Expression of the Vascular Endothelial Growth Factor and Its Receptors and Effects of VEGF During In Vitro Maturation of Bovine Cumulus-Oocyte Complexes (COC)

RALF EINSPANIER,[1]* MARTIN SCHÖNFELDER,[1] KARIN MÜLLER,[2] MIODRAG STOJKOVIC,[3] MICHAELA KOSMANN,[1] ECKHARD WOLF,[3] AND DIETER SCHAMS[1]

[1]*Institute of Physiology, Technical University of Munich, Freising, Germany*
[2]*Institute for Reproduction of Farm Animals, Schönow, Germany*
[3]*Institute of Molecular Animal Breeding and Genetics, Ludwig Maximilians University of Munich, Oberschleissheim, Germany*

ABSTRACT A complete VEGF system consisting of the ligand and two of its receptors has been detected for the first time in the bovine cumulus-oocyte-complex (COC). In the course of a 24 hr in vitro maturation procedure (IVM), expression of the smaller VEGF transcripts and their specific receptors flt and flk changed remarkably in a time-dependent manner as observed by RT-PCR. The transcript concentrations of VEGF declined within 24 hr of culture, whereas both receptor mRNAs were found enriched between 6 and 12 hr of IVM. In the follicular fluid of growing ovarian follicles, immunoreactive VEGF, measured by RIA, increased significantly, reaching highest concentrations immediately before ovulation of the oocyte. The immunohistochemical localization of VEGF in bovine COCs revealed strong signals within the cumulus cell complex clearly extending beyond the oocyte cytoplasm at the beginning of in vitro maturation. After 24 hr, IVM immunoreactive VEGF disappeared remarkably from cumulus cells and the oocyte cytoplasm. An exogenous application of VEGF at the beginning of a 24 hr IVM significantly improved cleavage rates of zygotes and their development into bovine embryos showing obvious synergistic effects in combination with FSH, when compared with untreated control embryos. In addition, the number of blastomeres in deriving blastocysts increased after VEGF supplementation. These results indicate a functional VEGF system controlling important events beside the known angiogenetic effect during in vivo and in vitro maturation of the bovine COC, possibly affecting the early embryonic viability. *Mol. Reprod. Dev. 62: 29–36, 2002.* © 2002 Wiley-Liss, Inc.

Key Words: Bos taurus; oocyte maturation; real-time RT-PCR

INTRODUCTION

One of the central prerequisites for successful fertilization is the full competence of the mature oocyte. Mammalian oocytes develop in ovarian follicles tightly surrounded by cumulus cells, a specialized sub-type of granulosa cells. The follicular micro-environment appears to mediate the fate of each oocyte through protein and steroid hormones, growth factors and other components present within the growing follicle (for a review, see: Adashi, 1994). Major processes like meiotic arrest, final gamete ripening, and the viability of the resulting zygote are suggested to be primed within the last maturation period until an endogenous gonadotropin surge initiates the ovulation of the cumulus-oocyte-complex (COC) (Hendriksen et al., 2000). Sustained by para- and autocrine actions in the Graafian-follicle (Monniaux et al., 1997) as well as in the oviduct (Einspanier et al., 1999a), a resulting viable oocyte paves the way for a high quality embryo.

In vitro fertilization (IVF) has become an expanding field in farm animal reproduction. Important biological developments led to well established techniques producing mature oocytes and viable zygotes (Fortune et al., 1999). However, such artificial systems are known to possess several limitations. Inadequate development of IVF-derived bovine embryos culminating in the large calf syndrome is supposed to be caused by physiological imbalances early in the IVF protocol (Behboodi et al., 1995; van Wagtendonk-de Leeuw et al., 1998; Young et al., 1998). In view of the still unresolved complex in vivo events, the quality assessment and in vitro maturation of oocytes (IVM) represent the bottleneck of IVF. Recently, some growth factors have been recognized to play important roles during ovarian follicle development and, furthermore, the heparan-binding growth factor systems of FGF and VEGF (vascular endothelial growth factor) have been identified as

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*Correspondence to: Dr. Ralf Einspanier, Institute of Physiology, Technical University of Munich, Weihenstephaner Berg 3, D-85350 Freising, Germany. E-mail: einspani@weihenstephan.de

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major mediators during remodeling of ovarian tissue (Campbell et al., 1995; Reynolds and Redmer, 1998). Moreover, the very potent VEGF-system possesses interesting physiological properties during ovarian and embryonic development by regulating tissue permeability and blood supply. As previously shown, the oxygen-driven activity of the VEGF can manage a fine tuning of the metabolic supply of cells and exert outstanding influence on physiological as well as pathological developments (for a review, see: Keshet, 1999). Such biological activities are mediated through the two VEGF tyrosine kinase receptors flk and flt (Ferrara and Davis-Smyth, 1997). Expression of both receptors is presumed to be largely restricted to the vascular endothelium (Jakeman et al., 1992), suggesting a main role in maintaining endothelial cell functions. However, a first evidence for the presence of these VEGF receptors has been shown for the bovine corpus luteum (Berisha et al., 2000b). A recent finding has indicated the potential role of VEGF in possibly influencing the bovine oocyte development (Einspanier et al., 1999b).

The aim of this study was to observe the possible expression of the angiogenic growth factor VEGF and its receptors during in vitro maturation of bovine oocytes. Additionally, the presence and concentration of VEGF in growing ovarian follicles was monitored. Furthermore, first effects of exogenous VEGF during in vitro maturation of bovine oocytes were elucidated.

MATERIALS AND METHODS

Tissue Collection

Ovaries from adult German Fleckvieh cows were collected at a local abattoir within 20 min post mortem. Ovarian follicles up to a diameter of 6 mm were aspirated and the cumulus oocyte complexes harvested. Only COCs with a complete dense cumulus and a dark, evenly granulated cytoplasm were selected, washed twice with 1 ml Ringer solution (Fresenius AG, Bad Homburg) and cultured in Tissue Culture Medium (TCM 199) for IVM as described (Stojkovic et al., 1995). Successively, 8 samples of each 10 COCs were taken every 6 hr during maturation and stored at −80°C until RNA analysis.

In parallel, bovine ovarian follicle fluid was collected, frozen at −20°C, and assayed later for steroid hormones and VEGF. Additionally, resulting granulosa cells were harvested and subsequently used for RNA extraction. According to the follicular size and the progesterone–estradiol ratio, single follicles were judged for their developmental stage (Ireland et al., 1980) as follows: only follicles which appeared healthy (i.e., well vascularized and having transparent follicular wall and fluid) and whose diameter was >5 mm were used. Large follicles (>14 mm) were collected only after corpus luteum regression, with signs of uterine and cervical mucus production and were assumed to be preovulatory. Follicles were classified according to the estradiol-17β content in the follicular fluid: (i) <0.5; (ii) 0.5–5; (iii) 5–20; (iv) 20–180; and (v) >180 ng/ml. The corresponding sizes of follicles were in the range of (i) 5–7 mm; (ii) 8–10 mm; (iii) 10–13 mm; (iv) 12–14 mm; and (v) >14 mm. Since healthy follicles have relatively constant progesterone levels within the follicular fluid, only follicles with progesterone below 100 ng/ml were used to exclude atretic ones.

Radioimmunoassay (RIA) and Immunohistochemistry

Concentrations of progesterone and estradiol-17β were determined according to Berisha et al. (2000a) directly in follicular fluids of each stage (n−17–89) with an enzyme immunoassay using the second antibody technique (Prakash et al., 1987; Meyer et al., 1990) introducing as enzyme solution progesterone-6β-hydroxy-hemisuccinate-HRP or oestradiol-17β-6-carboxy-methyl-oxim-HRP. Each polyclonal antibody was raised in a rabbit against progesterone-7a-carboxy-ethylthioether-BSA or oestradiol-17β-6-carboxy-methyl-oxim-BSA, respectively. The cross-reactivity of the antiserum used for the estradiol-17β assay was as follows: oestrone 100%, estradiol-17a 66%, progesterone and testosterone <0.1%. The antiserum used for the progesterone assay showed no cross-reactivity with estradiol-17β, testosterone and androstendione (<0.1%). The effective dose for 50% inhibition (ED_{50}) of the assay was 6 ng/ml for progesterone and 3.5 pg/ml for estradiol-17β. The intra- and inter-assay variations were 4–5 or 8–9% for progesterone and 6–7 or 9–10% for estradiol-17β, respectively.

VEGF concentrations were measured directly in 200 µl follicular fluid by RIA (Berisha et al., 2000b) using a rabbit antiserum raised in our laboratory against recombinant bovine $VEGF_{165}$. The antibody cross-reacts with the known four human isoforms of VEGF. The cross-reactivity to other growth factors (PDGFs, FGF1, FGF2, TGFa) was below 0.1%. Intra- and inter-assay variations were below 6 or 14%, respectively, showing an ED_{50} of 0.6 ng/ml.

For histological studies, all COCs were washed in PBS (pH 7.4), embedded in Tissue Freezing Medium (Tissue-Tek, Miles, Elkhart, IN) and shock-frozen in liquid nitrogen. Serial cross-sections (14 µm) were cut on a cryostat (CM 1850, Leica, Bensheim, Germany) and mounted on HistoBond-slides (Marienfeld, Lauda-Königshofen, Germany). After air-drying, all sections were fixed in ice-cold ethanol for 8 min and treated with 1% hydrogen peroxide to block endogeneous peroxidases. Non-specific background staining was reduced by use of goat serum (10% in PBS) for 30 min. The presence of VEGF detected by a specific anti-VEGF rabbit IgG (1:2000; 4°C overnight) and a peroxidase-labeled goat-anti-rabbit IgG (1:400, Sigma-Aldrich, Taufkirchen, Germany) for 1 hr. Immunoreactivity was visualized using 0.01% 3′,3′diaminobenzidine dihydrochloride (DAB, Sigma). Additionally, all sections were counterstained with Mayers Haemalaun (Merck, Germany), embedded in Eukitt (Kindler, Germany) and analyzed using a Zeiss microscope (Axioscope, Zeiss, Göttingen, Germany). The specificity

of immunochemical reaction was assessed by different methods:

1. Pre-absorption involving the respective antigen $VEGF_{165}$ (Berisha et al., 2000b)
2. Replacement of the specific antiserum with PBS or nonimmune rabbit serum
3. Incubation with DAB alone to exclude residual endogeneous peroxidase activity.

Isolation of RNA

Total RNA was prepared from mural granulosa cells or ten bovine COCs per sample according to the method of Chomcyznski and Sacchi (1987) using Trizol reagent (Gibco BRL, Gaithersburg, MD). Total RNA was quantified spectroscopically (OD 260 nm) or fluorometrically using Pico-green-dye and subsequently equilibrated for absolute quantity.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total granulosa or COC RNA (0.3 μg) was introduced to synthesize cDNA in a 60 μl reaction mixture using 2.5 μM random hexamers (Amersham Pharmacia, Freiburg, Germany) and Superscript II reverse transcriptase (Gibco BRL) as described in detail by Gabler et al. (1997). The following primers were used to amplify specific bovine transcripts for

VEGF	(following Garrido et al., 1993) detecting four isoforms 190, 318, 390, 441 bp forward 5′-TGT AAT GAC GAA AGT CTG CAG-3′ reverse 5′-TCA CCG CCT CGG CTT GTC ACA-3′
flk-1	(Gabler et al., 1999) 379 bp forward 5′-AGA CTG GTT CTG GCC CAA C-3′ reverse 5′-GAA GCC TTT CTG GCT GTC C-3′
flt-1	(Gabler et al., 1999) 351 bp forward 5′-CAC CAA GAG CGA CGT GTG-3′ reverse 5′-AAG AAG TCC TCG GAG AAG GC-3′
18S rRNA (488 bp)	(QuantumRNA, Ambion, Austin) forward 5′-TCAAGAACGAAAGTCG-GAGG-3′ reverse 5′-GGACATCTAAGGGCAT-CACA-3′

The predicted size of each RT-PCR product is assigned in parentheses. The PCR was performed in a thermal cycler (Biometra, Göttingen, Germany) as previously described (Gabler et al., 1997). Individual amplification programs were applied for VEGF (35 cycles at 94°C and 58°C, 1 min each), flk-1 (37 cycles at 94°C and 62°C, 1 min each), flt-1 (37 cycles at 94°C and 62°C, 1 min each) and 18S (25 cycles at 94°, 60°, and 72°C, 45 sec each). Five microliters of each reaction were subsequently subjected to agarose gel electrophoresis followed by ethidium bromide staining. Amplification conditions were optimized for each factor, ensuring that the PCR amplification did not reach a plateau verified by use of increasing cycle numbers.

Additionally, transcript concentrations were quantified introducing external cRNA standards by use of a real-time PCR cycler (LightCycler, Roche Diagnostics, Mannheim, Germany). Each cRNA standard was prepared using an in vitro transcription system (Ambion, Austin, TX) based on individually subcloned gene-specific PCR-products. After cRNA purification and size evaluation (100 bp Ladder, BioLabs, MA), the nucleic acid concentrations were determined spectroscopically. After serial dilutions of resulting cRNA standards, final sensitivity levels between 0.1 pg and 1 ng specific transcript per sample were performed during real-time PCR as follows:

Using 1 μl of cDNA the Master SYBR Green protocol was performed (Roche Diagnostics, Mannheim, Germany). Confirmation of the amplicon identity was obtained through melting curve analysis as well as by sequencing of resulting RT-PCR products (TOBLAP, Munich, Germany). Sequence determining of VEGF, flk, and flt PCR products confirmed in each a 100% homology to the published sequences. As negative controls, water instead of RNA was always used.

In Vitro Production of Bovine Embryos

Bovine embryos were produced using isolated bovine COCs as previously described in the section "Tissue Collection" and by Stojkovic et al. (1995). Briefly, follicles with a diameter of 2–8 mm were aspirated from the ovaries using a 20-gauge needle and a vacuum pressure of approximately 100 mmHg. The COCs were collected in a 50 ml centrifuge tube and washed twice with pre-incubated (39°C; 5% CO_2) TCM199 supplemented with 10% (v/v) estrous cow serum (ECS). Only COCs with homogenous oocyte cytoplasm and a complete, compacted, and multilayered cumulus were selected for IVM and further experiments. The COCs were washed in TCM199 supplemented with 10% ECS and 0.01 IU/ml porcine FSH (pFSH, Schering Pharmaceuticals, Kenilworth, NJ) and matured in this medium for 24 hr at 39°C in an atmosphere of 5% CO_2 in air and maximum humidity.

After maturation, the COCs were maintained in Tyrode's-Albumin-Lactate-Pyruvate medium containing 6 mg/ml BSA, 10 μg/ml heparan, and frozen/thawed bovine semen (10^6 spermatozoa/ml) that had been subjected to swim-up procedure. Such COCs were maintained in this medium for 18 hr under the same conditions as those used for IVM. Cumulus cells were removed from presumptive zygotes by vortexing (120 sec) and by gentle pipetting. Then, groups of 30–35 presumptive zygotes were washed three times and cultured in 400 μl Synthetic Oviduct Fluid Medium (SOF) supplemented with 0.1% MEM, 0.2% BME amino acids (both Gibco-BRL, Paisley, Scotland), and

10% estrous cow serum (ECS) at 39°C; 5% CO_2; 5% O_2; 90% N_2; and maximum humidity.

Following these methods, five experiments were performed subsequently:

1. Groups of 30–35 COCs (in three replicates with 876 COCs total) were matured in TCM199 supplemented with 0, 100, 300, and 1000 ng/ml human VEGF (BioTrend, Köln, Germany) for 24 hr. After 66, 162, and 186 hr post insemination (h.p.i.) the cleavage, the morula, and blastocyst rates were evaluated.
2. Additionally, in four replicates a total number of 910 COCs were matured with (a) 300 ng/ml VEGF, (b) 0.01 IU/ml pFSH, and (c) 300 ng/ml VEGF and 0.01 IU/ml pFSH.
3. From 559 COCs cumulus cells were removed and matured in three replicates.
4. Denuded oocytes were fertilized, cultured, and the developmental rates evaluated.
5. The COCs were matured in TCM199 plus 0.01 IU/ml pFSH with or without 100 ng/ml VEGF at 0 and 6 hr of IVM, fertilized, and the developmental rates evaluated. Different stage embryos were fixed with 2% paraformaldehyde at day 7–9 after IVF and the number of blastomeres per embryo was calculated microscopically after aceto-orcein staining.

Statistical Analysis

Polar body extrusion, cleavage, morula, and blastocyst rates were analyzed using Chi-square test. $P < 0.05$ was considered as significant. The data analyzed were normally distributed as tested by the Kolmogorow-Smirnow method.

RESULTS

By use of RT-PCR performed with total RNA, the presence of transcripts specific for the growth factor VEGF and its two receptors (flk and flt) could be verified in bovine COC (Fig. 1). Initial analysis of these transcripts indicated a continuous decline of VEGF-specific mRNA from highest concentrations at the beginning of IVM until the detection limit after a 24 hr maturation, when related to unchanged 18S rRNA levels. Two main isotypes of VEGF mRNA (190 + 318 bp) and a third less abundant one (390 bp) were expressed in the bovine COC encoding for the corresponding VEGF isoforms 120, 164, 188 amino acids, respectively. Only the largest one (188) is known to possess high affinity to heparan structures. In contrast to the transcription of VEGF, the amount of both VEGF-receptors flk and flt increased within the first half of the IVM, dropping thereafter below the detection limit of the RT-PCR-assay (Fig. 1). A subsequent absolute quantification of individual transcript concentrations supported the results above revealing a significant decrease of the detected VEGF mRNAs from 12 to 1 pg VEGF mRNA/μg total RNA ($P < 0.05$) during a 24 hr IVM. Conversely, a rise of

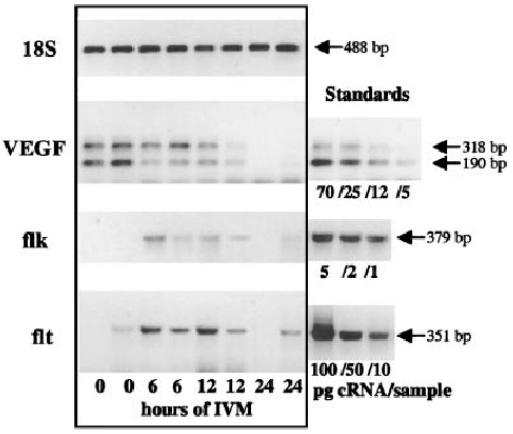

Fig. 1. RT-PCR detecting specific transcripts for VEGF, flk and flt in bovine COC during a 24 hr IVM. A pair of results is shown for each maturation point. Standardized cRNAs were used for semi-quantitative evaluation. Expression of 18S rRNA verified equal total RNA amounts per sample. Sizes of the corresponding PCR products: 18S – 488 bp; VEGF – 190, 318 bp; flk – 379 bp; flt – 351 bp. Two out of 8 independent experiments are depicted.

both receptors was measured within the first half of maturation: maximum concentrations were found between 6 and 12 hr of maturation for the relatively more abundant flt mRNA (maximum 6 pg/μg total RNA) and for the less enriched flk mRNA (below 0.1 pg/μg total RNA). Melting curve analysis revealed homogeneity of all PCR products generated by real time cycling. Consequently, all results have been normalized against absolute18S RNA contents representing the most valid housekeeping gene.

To demonstrate the presence and cellular localization of the VEGF within the bovine COCs, immunohistochemistry was applied detecting all known protein isoforms of this growth factor. Strong signals for immunoreactive VEGF could be verified in the bovine COC at the beginning of in vitro maturation: VEGF staining was present not only in the cumulus cells but also in the cytoplasm of the oocyte (Fig. 2B). After expansion of the cumulus 24 hr after gonadotropin, only the zona pellucida appeared positive with only faint signals found in the inner cumulus cell layer. No positive staining was at that time observed within the oocyte (Fig. 2D). The control sections (Fig. 2A,C) never showed an unspecific staining.

To compare these in vitro findings with the natural environment of the bovine COC, the growing follicle, VEGF concentrations were measured during follicle maturation in vivo. According to the maturation stage of each follicle, indicated through increased estradiol contents in the follicular fluid (0.5–180 ng/ml), soluble VEGF concentrations increased highly significantly until final follicle maturation (Fig. 3). This directly corresponds to obviously enhanced VEGF transcript

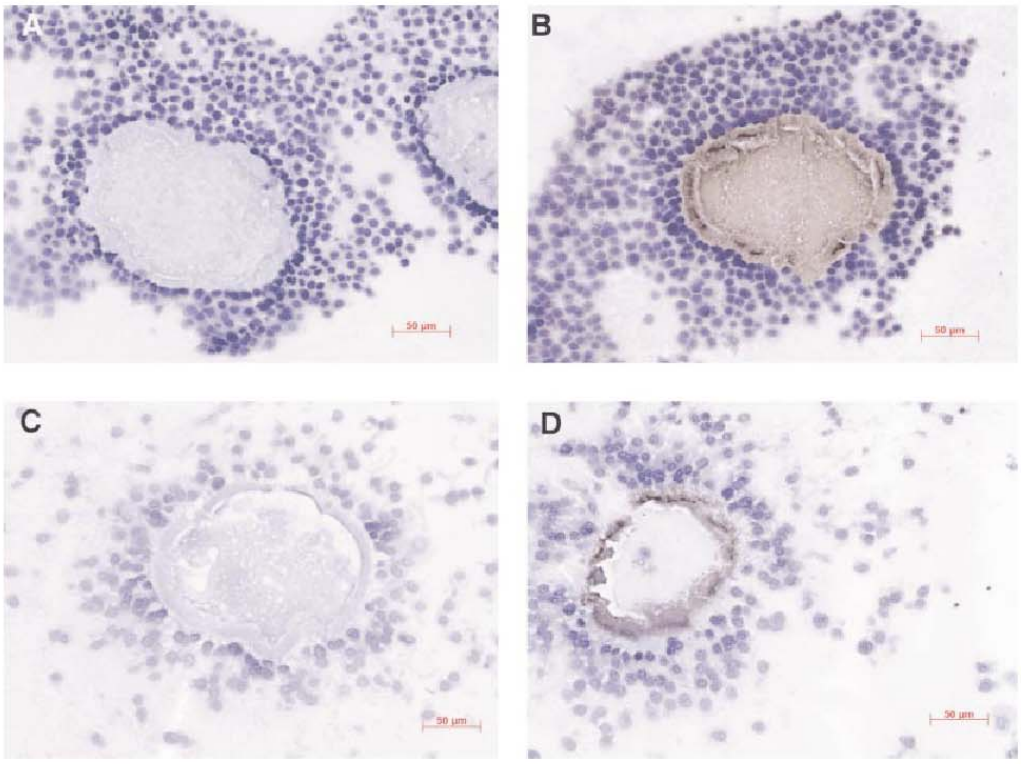

Fig. 2. Localization of immunoreactive VEGF in bovine COCs before (**A,B**) and after (**C,D**) a 24 hr maturation period. Positive immunreactions (B,D) were detected by a brown DAB–chromophor. Negative controls are indicated (A,C).

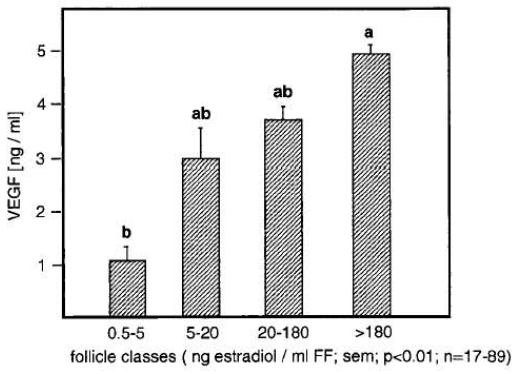

Fig. 3. Soluble VEGF concentrations in bovine ovarian follicular fluid from different developmental stages indicated by increasing estradiol concentrations. Different superscripts showing significant differences; SEM, $P < 0.01$; n – 17–89.

levels found in mural granulosa cells immediately before ovulation after the gonadotropin surge (data not shown).

When VEGF was supplemented the first time to maturation media during the in vitro maturation of bovine COCs, notable effects could be observed. VEGF increased the cleavage rate and morula, as well as blastocyst developmental rate in a dose dependent manner (Table 1). Maximal effects on each parameter were observed with 300 ng/ml VEGF. Furthermore, when combining such VEGF concentration with usual FSH levels, early cleavage and developmental rates (162 and 186 h.p.i.) were always normal or significantly increased when compared with VEGF and FSH groups (Table 2). However, polar body extrusion after 16 hr of IVM was as efficient with VEGF alone as when using normal FSH treatment (Table 3).

When lower concentrations of recVEGF (100 ng/ml) were applied twice in the course of IVM (at 0 and 6 hr), no significant difference in the outcome of in vitro fertilization was detected (n – 300 COC): division rates

TABLE 1. Effect of Chemically Defined IVM Medium Supplemented With Different Dose of VEGF on Developmental Rate of In Vitro Produced Bovine Embryos

Dose (ng/ml)	Oocyte	Cleavage 66 h.p.i. (%)	Morulae 162 h.p.i. (%)	Blastocyst 162 h.p.i. (%)	Total 162 h.p.i.(%)	Blastocyst 186 h.p.i. (%)
0	220	115 (52.3)^{a}	27 (12.3)	36 (16.4)^{a}	63 (28.6)^{a}	48 (21.8)^{a}
100	207	115 (55.6)^{a}	29 (14.0)	40 (19.3)^{a,b}	69 (33.3)^{a}	54 (26.1)^{a,b}
300	233	160 (68.7)^{b}	39 (16.7)	60 (25.7)^{b}	99 (42.5)^{b}	78 (33.4)^{b}
1,000	216	123 (56.9)^{a}	26 (12.0)	40 (18.5)^{a,b}	66 (30.5)^{a}	61 (28.2)^{a,b}

Different superscripts indicate significant differences.Three replicates; Chi square ($P < 0.05$).

TABLE 2. Effect of VEGF and FSH on Developmental Potential of In Vitro Matured Cumulus Oocyte Complexes

In vitro maturation	Oocyte	Cleavage 66 h.p.i. (%)	Morulae 162 h.p.i. (%)	Blastocyst 162 h.p.i. (%)	Total 162 h.p.i. (%)	Blastocyst 186 h.p.i. (%)
VEGF	266	177 (66.5)^{a}	42 (15.8)	64 (24.1)^{a,b}	106 (39.8)^{a}	88 (33.1)^{a}
FSH	204	139 (68.1)^{a}	35 (17.2)	50 (24.5)^{a,b}	85 (41.7)^{a,b}	68 (33.3)^{a}
FSH + VEGF	242	173 (71.5)^{a}	45 (18.6)	76 (31.4)^{b}	121 (50.0)^{b}	85 (35.1)^{a}
Without supplement	198	107 (54.0)^{b}	27 (13.6)	34 (17.2)^{a}	61 (30.8)^{c}	43 (21.7)^{b}

Different superscripts indicate significant differences. Four replicates; Chi square ($P < 0.05$).

of 63.3 ± 13.2% and blastocyst rates of 22.6 ± 13.4% under VEGF vs. 63.0 ± 10.6% and 24.7 ± 7.8% for the control. However, a single dose of low concentrated VEGF induced significant effects on the cell division of bovine blastocysts: Counting the number of blastomeres per embryo (for morulae, blastocysts, and expanded blastocysts separately), we found a partly significant increase in the cell number for blastocysts from day 7 to 9 after in vitro fertilization (Fig. 4). On day 8, blastocysts which were not yet expanded in VEGF samples had about 40% more cells ($P < 0.003$). Additionally, the percentage of blastocysts expanding already on day 7 (22 ± 5%) seems to be more stable after VEGF when compared with the control (11 ± 1%). In expanded blastocysts, the mean number of blastomers did not differ significantly between the treatments. Furthermore, a VEGF supplementation was ineffective in improving the vitality and developmental potential of denuded oocytes after in vitro fertilization (experiment 3 and 4). Using this system, no cleavage was observed (data not shown), emphasizing the necessity of the cumulus cells.

DISCUSSION

The main components of the important VEGF system were successfully monitored during the final maturation of bovine cumulus-oocyte-complexes. To our knowledge few data are known which describe the presence and action of VEGF in the female reproductive tract of mammals (Gordon et al., 1996), and only very recently was the interaction of this important growth factor with the growing oocyte investigated in more detail (Manau et al., 2000; Pellicer et al., 2000). Now, first experimental data are available on occurrence and possible importance of this growth factor during in vivo and in vitro maturation of bovine oocytes. In this study, the two truncated transcript species encoding the smaller VEGF isoforms could be identified as the main mRNAs in bovine COCs. These findings are supported by an

TABLE 3. Effect of VEGF (300 ng/ml) on Polar Body Extrusion 16 Hours After In Vitro Maturation

Treatment	Total oocyte	Pb extrusion (%)
VEGF	161	86 (53.4)^{a,b}
FSH	146	85 (58.2)^{a}
FSH + VEGF	122	65 (53.3)^{a,b}
Without supplement	130	55 (42.3)^{b}

Different superscripts indicate significant differences. Three replicates; Chi square ($P < 0.05$).

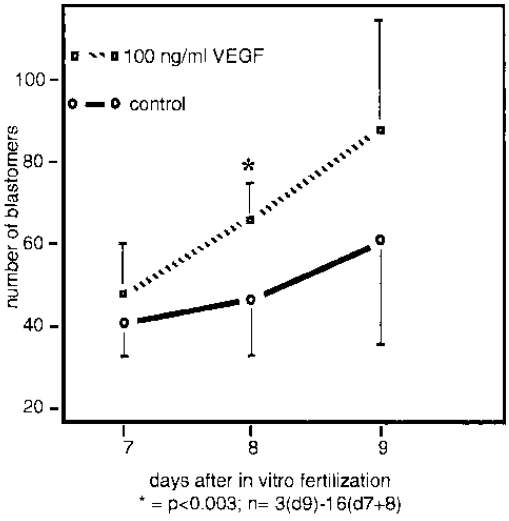

Fig. 4. Effect of IVM medium supplemented two times (0 and 6 hr of IVM) with exogenous VEGF on the number of blastomeres of in vitro produced bovine blastocysts. *indicating a significant difference; SEM; $P < 0.003$; n – 3 (d9) or 16 (d7 + 8).

earlier report (Garrido et al., 1993) identifying such shorter $VEGF_{121}$ and $VEGF_{164}$ isoforms in cultured bovine granulosa cells.

The presence of the smaller, nonheparan binding isoforms of the VEGF suggests that this factor could be potentially secreted into the supernatant (Berisha et al., 2000b). Any less abundant $VEGF_{188}$ will preferably bind onto extracellular structures like glycoproteins and receptors. According to that, immunohistology demonstrated a distinct staining pattern for VEGF, not only in the cytoplasm of the oocyte and cumulus cells but also on surface structures, confirming both cytoplasmic and membrane-bound localization. Therefore, main paracrine effects of the cumulus derived VEGF can be assumed on the oocyte and the cumulus cells themselves. Yet, the turnover of the extracellular matrix will play a major role in liberating the heparan-binding VEGF-isoform during COC maturation in vitro. In regard to the data observed in vivo for growing bovine follicles, the considerable increase of VEGF until ovulation indicates a prominent role of this angiogenic and permeability promoting system.

In women, an increased VEGF level has been recently suggested to be one candidate of interest to predict ovarian hyperstimulation during IVF (D'Ambrogio et al., 1999). In contrast, a decrease of this growth factor which we have described during in vitro culture could possibly lead to a delayed oocyte ripening. As shown here for the first time, the expression of both VEGF receptors during bovine COC in vitro maturation indicates possible local interactions of the corresponding growth factor and would support this hypothesis.

Fluctuating levels of both receptor transcripts were found during the maturation, indicating an high expression variability between each experiment. This may be due to different maturation stages of each COC being harvested from differently developed follicles and sexual cycle stages. However, the synthesis of new receptor molecules may be driven through their specifc mRNAs suggesting a distinct increase several hours after the onset of IVM.

Certainly, physiological ligand-receptor interactions could be verified using the in vitro maturation system indicating several significant influences of VEGF on the early bovine embryo development. Such effects confirm a remarkable influence of follicular fluid factors set during final oocyte maturation.

Despite an unpredictable biological activity of this introduced recombinant human VEGF, physiological effects on bovine oocyte maturations can be presumed. However, the fact that the lower effective dose (100 ng/ml) of this recombinant growth factor still showed a significant improvement of bovine embryo development may lead to a more favorable procedure in IVM which has to be proved practically.

Several main growth factor effects were found to act very early during the final maturation of bovine oocytes within the ovarian follicles (Schams et al., 1999). A complete network of such important factors is suggested to exist within the growing follicle, probably priming the oocyte development. In that context, the viability of COCs and the resulting early embryo can become strongly influenced by a better understanding of the in vitro maturation procedure. However, stimulatory effects of growth hormone (Izadyar et al., 1996), insulin-like growth factor I and epidermal growth factor (Lorenzo et al., 1994; Lonergan et al., 1996) supplementation in maturation medium on oocyte maturation were already demonstrated. In our study, chemically defined maturation medium supplemented with VEGF increased extrusion of the first polar body and the developmental potential of oocytes after IVF. In addition, a synergistic effect of FSH and VEGF resulted in the highest early cleavage and blastocyst rates. As reported recently, VEGF is suggested to play an important role in the cyclic growth of ovarian follicles being controlled through both FSH and LH/hCG receptor-activated pathways (for review, see: Geva and Jaffe, 2000). In addition to the investigated VEGF system, other growth factors and extracellular matrix proteins are expected to have important local influences on the developing oocyte as suggested early in an IVM system (Bieser et al., 1998). A future aim will be to resolve known problems of in vitro technologies (e.g., large calf syndrome), learning from cellular processes of the fine-tuned network of follicular factors. Again, the essential influence of the cumulus cells on the final oocyte maturation is remarkable. In this prospect, further optimization of established in vitro systems during assisted reproduction is expected, supported by a more precise and detailed understanding of the female reproductive tract.

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App. 2. Papers in preparation of the author

Schoenfelder M, Berisha B, and Einspanier R
The growth factors GDF-9 and BMP-15 and their receptors in maturing bovine cumulus oocyte complexes and developing follicles.
Paper is in preparation for the journal Endocrine.

Ulbrich SE, Schoenfelder M, Einspanier R
Hyaluronic acid in the bovine oviduct – regulation of synthases and receptors during the normal estrous cycle.
Paper is submitted in the journal

App. 3. Abstract publications of the author

Schoenfelder M, Schams D, and Einspanier R
Steroid secretion, expression of steroid receptors and key-enzymes of the steroid pathway during in vitro maturation of bovine cumulus-oocyte-complexes.
12/2000, Utrecht SSF Meeting

Schoenfelder M, and Einspanier R
First characterization of growth and differentiation factor-9 (GDF-9) transcripts and expression changes during in vitro maturation of bovine cumulus oocyte complexes.
5/2001, Gießen DGE Meeting

Schoenfelder M, Schams D, and Einspanier R
Possible role of Vascular Endothelial Growth Factor (VEGF) and Growth and Differentiation Factor 9 (GDF-9) during ovarian follicle development and steroidogenesis in the cow.
9/2001, Weihenstephan DGfZ/GfT-Gemeinschaftstagung

Schoenfelder M, and Einspanier R

Differential expression of hyaluronan synthase 2 and 3 as well as Hyaluronan receptor cd44 in bovine cumulus oocyte complexes and mural granulosa cells during gonadotropin and hyaluronan stimulation.

3/2002, Göttingen DGE Symposion

Schoenfelder M, Schams D, and Einspanier R

Steroidogenesis during in vitro maturation of bovine cumulus oocyte complexes and possible effects of tributyltin on granulosa cells.

5/2002, München Int. Syp. of J. Steroid Biochem. & Mol. Biol.

Schoenfelder M, Schams D, and Einspanier R

In vitro Maturierung (IVM) von bovinen Cumulus-Oocyten-Komplexen, ein mögliches System zum Screening von Einflüssen von Umweltschadstoffen auf die Reproduktion?

9/2002, Halle DGfZ/GfT-Gemeinschaftstagung

Schoenfelder M, Berisha B, and Einspanier R

Nitric oxide synthases expression during in vitro maturation of bovine cumulus oocyte complexes compared to cultured granulosa cells in response to gonadotropins.

2/2003, Wien An. Meeting Phys. And Path. of Repr.

Curriculum vitae

Particulars:

Surname	Schönfelder
First name	Martin, Fritz
Date of birth	14.10.1971
Place of birth	Ulm (Donau)
Permanent residence	Katharina-Fischer-Platz 6, D-85435 Erding

School:

1978-1982	St. Ulrich primary school Dornstadt
1982-1991	Schubart-Gynamsium, Ulm (Donau)
1991	Qualification for university entrance (Abitur)

Vocational training:

1991-1992	Military service (Military hospital in Ulm)
1992-1993	Study of medical technique (Fachhochschule Ulm)
1993-1999	Study of biology and sports (Karl-Rupprecht University Heidelberg)
1999	Graduated with diploma (1. Staatsexamen)
2000-dato	Preparation of thesis (Dr. rer. nat.)

Practical work:

1996-1999	Laboratory assistant at the Institute of Cell Biology (Karl-Rupprecht University Heidelberg)
1996	Practical training in aqua culture and hypervitaminosis (University of Bergen/Norway)
2000-2003	Employee at the Institute of Physiology (Technical University of Munich)
2003-dato	Employee at the Chair of Sports and Health Support (Technical University of Munich)