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Investigations of extracellular matrix proteases, apoptotic and anti-apoptotic factors in the bovine corpus luteum

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LIST OF ABBREVIATIONS

List of abbreviations

ADP	adenosine diphosphate
acc. no.	access number
Apaf-1	apoptotic protease activating factor
Apo-1	Fas antigen
bFGF	basic fibroblast growth factor
BIR	baculoviral IAP repeat
CAD	caspase activated Dnase
CCR	c-chemokine receptor
CD95	Fas antigen
CHO	chinese hamster ovary
CL	corpus luteum
CP	crossing point
DCNP	2,6-dichloro-4-nitrophenol
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
dsDNA	double stranded desoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylendiamintetraacetat
EIA	enzyme immuno assay
FADD	Fas-associated protein with death domain
Fas	Fas antigen
FasL	Fas Ligand
FSH	follicle-stimulating hormon
GAPDH	Glyceraldehyd-3-phosphat-Dehydrogenase
GM-CSF	granulocyte-macrophage-colony-stimulating factor
GnRH	gonadotropin releasing hormone
HKG	housekeeping gene
3β-HSD	3β-hydroxysteroid dehydrogenase
IAP	inhibitor of apoptosis
ICAD	inhibitor of caspase activated DNase
INFγ	interferon γ

IGF	insulin like growth factor
IL	interleukin
LH	luteinizing hormone
MCP-1	monocyte chemoatractant protein-1
MDM2	Murine double minute 2
MMP	matrix metalloprotease
MT-MMP	membran-type matrix metalloprotease
NADPH	nicotinamid-adenosine-dinucleotid-phosphat-hydrogen
NFκB	nuclear factor kappa B
OP	onapristone
PA	plasminogen activator
PAI	plasminogen activator inhibitor
PARP	poly ADP-ribose polymerase
PBS	phosphate buffered solution
PCD	programmed cell death
PCR	polymerase chain reaction
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
P4	progesterone
qRT/PCR	quantitative reverse transcription polymerase chain reaction
RIN	RNA integrity number
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
RIA	radioimmunoassay
RING	really interesting new gene
ROS	reactive oxygen species
RT	reverse transcription
Smac	second mitochondrial derived activator of caspases
TAE	tris base / acetic acid / EDTA buffer
TIMP	tissue inhibitor of matrix metalloprotease
TNFα	tumor necrosis factor alpha
TNFR	tumor necrosis factor alpha receptor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand

TRAF2	tumor necrosis factor receptor-associated factor-2
tPA	tissue plasminogen activator
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
UV	ultraviolet
VEGF	vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis

1 Introduction and aim of the study

The corpus luteum (CL) is a transient endocrine gland, which plays an essential role in the reproductive process of the bovine species. Its main function is to produce progesterone, a steroidal hormone, which is necessary for the establishment and maintenance of pregnancy. The development of the CL begins with the ovulation of a Graafian follicle. This happens normally every 21 days in the cow if no implantation of an embryo occurs. After ovulation an active endocrine gland is formed in best time to produce progesterone, which can reach more than 10 ng/ml in the blood plasma (Döcke 1994). During formation of the CL, 50% of the cells are mitotic, primary to form new blood vessels (angiogenesis), which are necessary to assure an optimal support of the steroidal cells. Some researchers compare the angiogenesis in the corpus luteum with angiogenesis in a malignant tumor, but even in a highly malignant and fast growing Glioblastoma multiforme only 9% of all cells are mitotic. In malignant mammary gland tumors or prostata carcinomas even only 2%. During development the CL grows from 200 mg tissue to a several grams swaying endocrine gland with a diameter of 1-3 cm (Augustin and Kuhn 1999). The adult CL is highly vasculated und consists of 53% of endothelial cells. The steroidal cells represent with 19% (small luteal cells) and 4% (large luteal cells) only a small contingent of the cellular composition. The CL consists more than 17% of fibroblast and 7% of plasma cells, lymphocytes and leukocytes (McCracken *et al*. 1999). If no embryo gives a signal to maintain pregnancy the luteolytic PGF2α released from the uterus reaches the CL and luteolysis occurs. This happens through a counter-current mechanism from the vena uteri to the ateria ovarica, which supports the ovary. Within 24 hours after PGF2α induced luteolysis the blood plasma level of progesterone drops to basal values of under 1 ng/ml (Schallenberger *et al.* 1984). This decline of progesterone is characteristic for the so called functional luteolysis. After 2-3 days the corpus luteum has only half of its original volume and after a few weeks only a small, avascular scar remains (Augustin and Kuhn 1999). These degeneration of the tissue is called structural luteolysis.

The CL with its high rate of angiogenesis and its normally smooth degradation is an unique formation in the adult organism. The smooth process of CL development and degradation needs a fine balanced interaction between numerous endocrine and locally acting factors. At the endocrine level it is thought that luteolysis in the cow occurs as follows: Progesterone inhibits the PGF2α synthesis in the endometrium of the uterus. Through the decline of its progesterone sensitivity and the stimulation through oxytocin occurs an increased release of PGF2α in the endometrium. This leads to secretion of luteal oxytocin and PGF2α, which increases in turn the endometrial production of PGF2α

inducing a positive feedback between CL and endometrium. If the concentration of PGF2α reaches a certain level, luteolysis occurs (Schams and Berisha 2002; Döcke 1994). The role and interaction of the locally acting factors are not totally known today.

The maintenance of the CL function is dependent on the presence of an embryo that prevents the signal cascade released from the endometrium to induce luteolysis. To ensure the survival of the conceptus not only the secretion of progesterone from the CL is necessary, but although provision of an optimal environment and nutrition in the uterus. Up to 40% of the embryonic losses are estimated to occur between day 8 and day 17 of pregnancy (Thatcher *et al.* 1994). To improve embryo survival during this critical period before implantation (Rüsse and Sinowatz 1994c) a better knowledge of the uterus-conceptus interaction is required.

Aim of the study

Control of the oestrous cycle in dairy cows plays a central role in herd management of dairy farms. A high gravidity rate and short calving intervals assure the profitability of these farms. The exact knowledge of the physiological processes during oestrous cycle and gravidity is required to understand and treat pathological events correctly. The investigation of factors, which are necessary during luteolysis, can furthermore lead to more knowledge about fundamental processes during wound healing and tumor regression.

The aim of the first part of this study was to develop a method for the investigation of different factors in the uterus milk during preimplantion in cattle. Therefore uterus fluid was gained from pregnant heifers using an *in vivo* uterus flushing technic at specific time points. The samples were analysed with enzyme immunoassay (EIA) and radioimmunoassay (RIA).

The aim of the second part was to investigate if extracellular matrix (ECM) degradation and apoptosis occurs in the CL during oestrous cycle and induced luteolysis. Therefore the mRNA expression of different ECM proteases were evluated in follicles 20 hours after GnRH (Gonadotropin realising hormone) injection (shortly before ovulation) for comparable reasons and in the CL during oestrous cycle and induced luteolysis. Furthermore the mRNA expression of apoptotic and anti-apoptotic factors were evaluated in the CL during oestrous cycle and induced luteolysis.

2 Literature

2.1 Regulation of the oestrous cycle in cattle

2.1.1 Folliculogenesis

In female mammalia the complete number of oocytes is already present at the time of birth in the ovaries. 'Follicle' describes the structural and functional unit of oocyte and follicle-epithel, less than 1% of them will ever reach ovulation. All other follicles go through atresia and degeneration at all stages of development. Until its ovulation, the oocyte is arrested in the prophase of its first meiotic division. Depending on the size of the oocyte and the development of the follicle-ephitel, different stages of the follicle are distinguished: In the primordial follicle, the oocyte with a diameter of approximately 30µm is embedded in a single layer of flat epithelial cells (Epithelium folliculare). Before puberty, the cells of some follicles generate small primary follicles. Here, the oocyte is surrounded by a single layer of cubic epithelial cells and the zona pellucida. This glycoprotein barrier allows only a single, artspecific sperm to fertilise the oocyte. The secondary follicle shows an enlarged volume with a diameter of approximately 50-100µm. Within this stage, the follicle ephitel grows and forms several layers, furthermore, stroma cells differentiate into the theka follicularis. A tertiary follicle is characterised through the development of the antrum folliculare, a liquid filled cavity. In this stage, the oocyte reaches its maximum diameter of approximately 150µm. The follicle epithel differentiates into the granulosa cell layer and the cumulus oophorus, both have nourishment- and control functions. Final stage of the follicular development is reached with the Graafian follicle: in this mature, praeovulatory follicle with a diameter up to 2cm, the oocyte loses its conection to the follicle layer and moves freely in the liquor folliculi. The oocyte finishes the first meiotic division, in which the genetic material is equally distributed among two nuclei. However the cytoplasma is not divided equally, after a second meiosis only one oocyte is able to get fertilised (Rüsse and Sinowatz 1994a).

2.1.2 Regulation of the oestrous cycle

Each bovine oestrous cycle (with a duration of about 21 days, fig.1) shows mostly three follicular waves (Evans and Fortune 1997). Each wave contains the recruitement of a follicle cohort and the selection of one dominant follicle which continues growing whereas all others go through atresia (Stock and Fortune 1993). Dominant follicles control the

development of other follicles through hormones (like oestradiol, inhibin), which act locally as well as systemic (Savio *et al.* 1993). Follicle growth is promoted by two types of gonadotropins, which are secreted from the anterior pituitary as a reaction to pulsatile GnRH release from the hypothalamus (Roche 1996). These two types are the follicle stimulating hormon (FSH) and the luteinizing hormon (LH), both of them sequestered within the whole oestrous cycle. Naturally only the dominant follicle of the last wave comes to ovulation (Ginther *et al.* 1989). Ovulation of the Graafian follicle is the release of the oocyte. In this inflammation like process, the follicle liquid rinses the oocyte as well as the zona pellucida and the cumulus oophorus into the oviduct. Compulsory for this impact is the LH peak, which arises through an increase of frequency and amplitude of the LH pulses (Piquette *et al.* 1991) triggerd by the pulsatile release of oestradiol-17β.

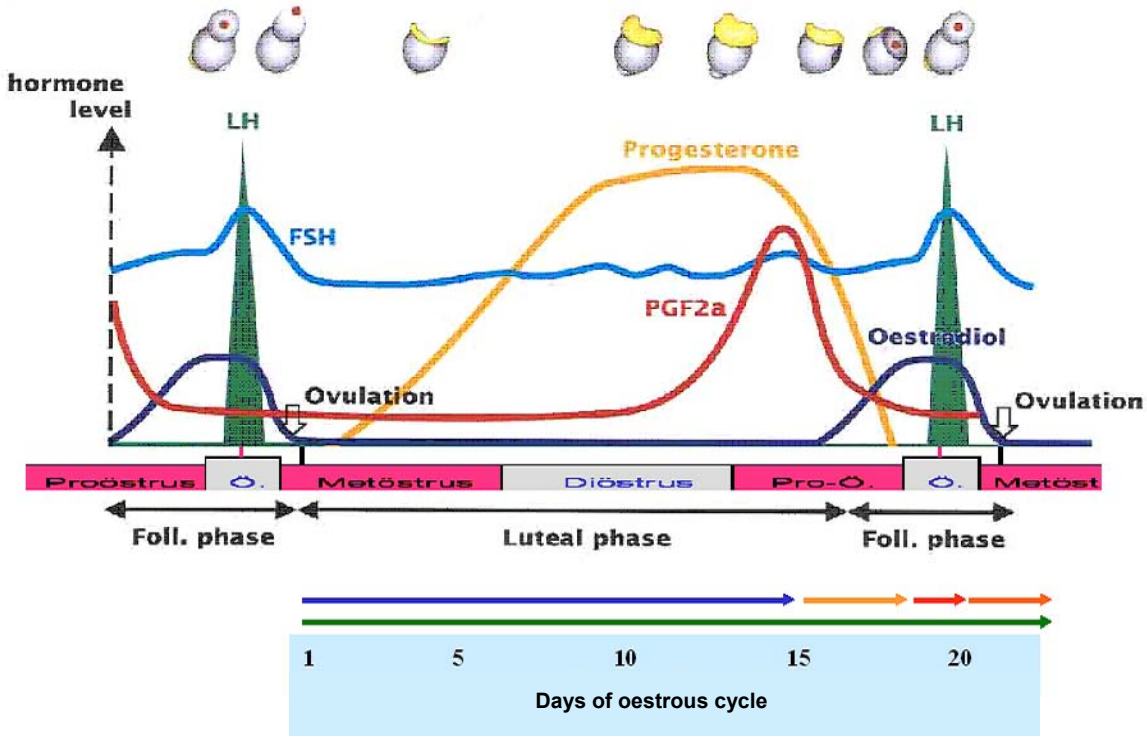


Figure 1: Hormon profiles, development and regression of the corpus luteum during the oestrous cycle of cattle (Intervet modified).

2.1.3 Corpus luteum (CL) formation, function and regression (luteolysis)

The above mentioned LH peak is also responsible for the formation of a temporary endocrine gland, the CL, which is built from the remaining granulosa- and theka interna cells after ovulation. This formation starts with the folding of the granulosa cell layer, its infiltration by theka cells and its vascularisation. During this process, also a variety of

other cell types, such as pericytes, fibroblasts and macrophages enter the granulosa. The rapid angiogenesis in the early CL development provides a tense capillary network. Parallel occurs the luteinisation of the theka and granulosa cells, which is marked through the inclusion of lipochroms and an increase in volume and synthesis rate. Cells of the follicle granulosa tissue develop into large luteal cells, while former theca cells become small luteal cells. This cubic cells synthesize steroid hormons, mostly progesterone. Progesterone is categorised into the group of gestagens, its main formation takes place in the CL. The preparation of the uterus mucosa for the implantation of the embryo as well as the maintenance of gravidity are the main functions of progesterone (Hesch 1989). Progesterone has a gonadotropin inhibiting effect, more precisely it decreases the pulse frequency. Thus, its inhibiting function aims at the LH peak (Döcke 1994), which means that ovulation only can take place in the absence of a CL and progesterone secretion. Small luteal cells produce less progesterone, but are present in greater numbers than large luteal cells.

If no fertilisation takes place, it is essential for the introduction of a new oestrous cycle, that the CL regresses. Thus, the uterus releases prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) around day 16. $PGF_{2\alpha}$ was discovered as a physiological luteolysin nearly three decades ago, cellular events associated with luteolysis however remained poorly characterised. $PGF_{2\alpha}$ interacts with its G protein-coupled receptor, present predominantly on large luteal cells, but also present on small luteal and endothelial cells of the CL (McCracken *et al.* 1999) and activates G_q/phospholipase C/protein kinase C pathway (McGuire *et al.* 1994) resulting in decreased steroidgenesis. The intracellular signalling events that lead to structural regression of luteal tissue are poorly characterised. However, proteases (Curry and Osteen 2003; Liu *et al.* 1997) and programmed cell death seem to play central roles in development and regression of the CL in several species (Bacci *et al.* 1996; Yadav *et al.* 2002).

2.2 Early pregnancy in cattle

After fertilisation of an oozyte the development of an embryo begins. The first three divisions of the zygote take place in the oviduct. The conceptus reaches the 8-cell stage after 65 to 72 hours and can be found in the apex of the ipsilateral uterus horn after 4-5 days as a morula. It is transported from the apex to the place of implantation, which is mostly found in the middle of the ipsilateral horn. A migration to the contralateral horn is seldom (Rüsse and Sinowatz 1994b). On day 8 the early blastocyst stage is reached and

the embryo is divided into an “inner cell mass”, also known as embryoblast, and the trophoblast. After nine days the blastocyst emerges the zona pellucida and the differentiation of the ecto- and endoderm follows. The beginning of the elongation stages is reached at day 13. From this time on, the conceptus elongates till it reaches the contralateral uterus horn at day 18-19, which is filled out at day 22. The conceptus has no contact to the endometrium during the preimplantation period before day 18-19. It floats freely in the uterus lumen being nourished only by the so called uterus milk, which is secreted by the endometrium (Guillomot 1995). This uterus milk is found abundantly in the uterus lumen from the early blastocyst stage till day 24 of gravidity (Chang 1952). Bartol et al. (1981) were able to detect 18 different proteins in intrauterine fluid at day 19 of gravidity, four of them could not be found in cycling cows during different cycling stages. Some of these proteins have been already characterised like retinol-binding-protein (MacKenzie *et al.* 1997), granulocyte-macrophage-colony-stimulating factor (Emond *et al.* 2000), bovine granulocyte-chemotactic-protein-2 (Teixeira *et al.* 1997), bovine Ubiquitin cross-reacting protein (Austin *et al.* 1996), insulin-like growth factor-binding proteins (Keller *et al.* 1998) and PGF (Bartol *et al.* 1981). It seems possible, that the maternal environment given through the uterine secretion plays an essential role in the survival rate of blastocysts and early concepti till day 16 of gravidity (Keller *et al.* 1998).

2.3 Endocrine regulatory factors

2.3.1 Progesterone

Progesterone is mainly produced through the CL in cattle and functions as a signal for the uterine mucosa to be prepared for the implantation of an embryo. It is also necessary for the maintenance of gravidity (Hesch 1989). Progesterone inhibits the action of gonadotropins through a down-regulation of the pulse frequence. In this kind of way (negative feedback) a blocking of the LH peak occurs. A Graafian follicle can only ovulate, when there is no progersterone secreting CL present (Döcke 1994).

2.3.2 Oestradiol-17β

The most important oestrogen, oestradiol-17β is mainly produced in follicles of the ovaries. Its synthesis is stimulated by FSH, which is released from the adenohypophysis. Oestradiol-17β itself acts in a negative feedback mechanism on the secretion of FSH. Granulosa cells of the follicles receive androgens, which are produced in theca interna

cells, and transform them into oestrogens with the help of aromatases. These enzymes produce the biological effective forms of oestrogens together with cytochrom P450, NADPH (nicotinamid-adenosine-dinucleotid-phosphat-hydrogen) and oxygen. The high preovulatory synthesis of oestrogens by the ovulatory follicle triggers the LH peak. Oestrogens cause also a proliferation of the uterine mucosa (Döcke 1994). Oestradiol-17β is not only produced in follicles, but also in 13 days old bovine embryos (Chenault 1980).

2.3.3 Prostaglandin F2α (PGF2α)

The regression of the CL, if no fertilisation occured, is essential for the beginning of a new oestrous cycle. The luteolytic agent in cattle is PGF2α, which is secreted in a pulsatile manner by the endometrium. Progesterone and oestradiol-17β are responsible for the initiation of the PGF2α release, whereas progesterone acts as inhibitor and oestradiol as promotor (Meyer *et al.* 1988). PGF2α is inhibited during the luteal phase. At the start of luteolysis the secreted oestradiol promotes a rapid distribution of PGF2α (Döcke 1994). Also oxytocin stimulates its secretion. The endometrial PGF2α and the luteal oxytocin are connected by a positive feedback mechanism. The receptor of oxytocin is in turn affected by progesterone and oestradiol (Schams *et al.* 1985). Endothelin-1 seems also important, because it shows a suppression of the oxytocin effect and progesterone production in microdialysis experiments (Miyamoto *et al.* 1997). PGF2α is not only secreted by the endometrium at time of luteolysis, but also by bovine blastocyst from day 13 of gravidity on. The prostaglandin production rises each day of gravidity, whereas the blastocysts *in vitro* secrete much more PGF2α then PGE2 (Shemesh *et al.* 1979). Co-incubation with tissue from the endometrium changes the secretion pattern to more PGE2 than PGF2α (Lewis and Waterman 1983). PGE2 is able to act as a luteotropic agent during early pregnancy and can enhance the progesterone production in luteal tissue (Bazer *et al.* 1991; Godkin *et al.* 1977).

2.4 Locally produced factors

2.4.1 Vascular endothelial growth factors (VEGFs)

VEGFs are glycoproteins which induce angiogenesis. They are a potent mitogens for endothelial cells (Ferrara and Henzel 1989) and enhance the vascular permeability (Senger *et al.* 1986). VEGF-A is a homodimeric heparin binding glycoprotein of 46-48

kDa. Several additional members of the VEGF gene familiy have been identified, including VEGF-B, VEGF-C, Placenta growth factor and VEGF-D (Garrido *et al.* 1993, Ferrara 2001). The human VEGF gene has a length of approximately 12 kb and is composed of eight exons, separated by seven introns. Alternative exon splicing of a single VEGF gene results in the generation of four different molecular species, having respectively 121, 165, 189 and 206 amino acids after signal sequence cleavage ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$). The bovine VEGF is one amino acid shorter then the human factor. $VEGF_{121}$ is a soluble secreted form, $VEGF_{165}$ is the most common molecular form produced by a variety of normal and transformed cells. In contrast, $VEGF_{206}$ is a very rare form (Houck *et al.* 1991). The different isoforms of VEGF have different properties *in vitro* and this may apply also to their *in vivo* functions. VEGF binds on two tyrosinkinase receptors: VEGF-R1 (= flt-1, fms-like-tyrosine kinase-1) and VEGF-R2 (= flk-1, fetal liver kinase-1), whereas the affinity of VEGF-R2 to VEGF is a bit lower than of VEGF-R1. Both receptors are localised on endothelial cells (Jakeman *et al.* 1992). The major regulator of VEGF is the oxygen content of the tissue. Hypoxia induces the expression of VEGF and its receptors. Further stimulating factors are: LH, IGF-1 (insulin-like growth factor-1), EGF (epidermal growth factor), TGF-β (transforming growth factor β), TNF-α (tumor necrosis factor α), IL-1 (interleukin-1) and IL-6 (interleukin-6) (Ferrara and Davis-Smyth 1997; Schams *et al.* 2001). High permeability, uterine oedema and angiogenesis occurs during implantation of rabbit embryos. VEGF seems to be critical for a successful implantation in rodents, because it was prevented by inhibition of VEGF (Rabbani and Rogers 2001). Although VEGF and its receptors have been implicated as key players in vascular remodeling and placentation in women (Ahmed *et al.* 1995), sheep (Bogic *et al.* 2001), pigs (Winther *et al.* 1999) and marmosets (Wulff *et al.* 2002).

2.4.2 Housekeeping Genes

Housekeeping genes (HKG) are genes, which are permanently expressed on the same level to keep cell functions sustainable and to assure the survival of the cell. It is assumed that mRNA from HKG is expressed in excess, so that the cells are able to compensate for ineffective posttranscriptional, translational or posttranslational processes (Warrington *et al.* 2000). Quantitative gene expression is typically referenced to an internal control for compensation of fluctuations in the used mRNA amount and quality, which can occur because of discrepancies in the RNA extraction efficiency or through extraction of RNA from different individuals. Interpretation failures should be avoided by elimination of unspecific variations. Ideally the experimental conditions have no influence on the

expression of HKG (Bustin 2000). There is a general consensus, that one HKG as control gene is enough for the normalisation of expression data. GAPDH (Glyceraldehyd-3-phophat-Dehydrogenase), β-Actin, 18S rRNA and 28S rRNA are most often used as HKG (Suzuki *et al.* 2000). But there are physiological situations existing, where even the expression of HKG show strong variations. Therefore not only one HKG, but the mean value of serveral carefully selected HKG should be used for normalisation (Vandesompele *et al.* 2002). There are numerous references existing, that also HKG can be regulated (Bustin 2000; Suzuki *et al.* 2000, Thellin *et al.* 1999). The mRNA expression of GAPDH, an enzyme of the glycolysis and gluconeogenesis, is massivley regulated by the following effects: different cell cycle stages, gravidity, dexamethason, glucose, insulin, growth hormons, oxidativ stress, hypoxia, apoptosis etc. β-Actin is a structural protein of the cytoskeleton and its mRNA expression can be influenced by hypoxia, ionising radiation, different growth factors, adrenocorticotropin or gonadotropin (Bustin 2000; Suzuki *et al.* 2000). Ubiquitin is used for the degradation of proteins and is 2-fold increased during DCNP (2,6-dichloro-4-nitrophenol) induced apoptosis in human Chang liver cells (Qi and Sit 2000a). An up-regulation of Ubiquitin is also seen in PGF2α induced luteolysis in primates, when signs of apoptosis are recognisable (Young *et al.* 1998). Histones are abundant, basic proteins, that bind to the DNA to form nucleosomes (Zong 2004). They compromise the protein portion of the nucleosoms, which function to compact the DNA into chromatin and to help regulate its activities (Sedelnikova *et al.* 2003). Several Histone variants have been found to regulate gene expression and even acting as apoptotic factors (Pusarla and Bhargava 2005).

2.4.3 Extracellular matrix proteases

2.4.3.1 General aspects

The life cycle of the CL involves several strictly regulated proteolytic processes that take place within the same CL during a relatively short period of time, including angiogenesis, tissue remodeling (Smith *et al.* 1994b) and tissue degradation (Kiya *et al.* 1999). The CL therefore provides a useful model to study the regulation and functional roles of matrix remodeling proteases *in vivo.* In other species like human (Duncan *et al.* 1998), rat (Nothnick *et al.* 1996), mouse (Liu *et al.* 2003), porcine (Pitzel *et al.* 2000), ovine (Ricke *et al.* 2002a; Towle *et al.* 2002) and in the bovine species (Zhang *et al.* 2002; Smith *et al.* 1996; Goldberg *et al.* 1996; Juengel *et al.* 1994) the matrix metalloproteases (MMPs), their tissue inhibitors (TIMPs) and the plasminogen activator system seem to play a critical

role in development and regression of the CL. Our request was therefore to investigate these factors at well defined time points in follicles during 20 hours after GnRH application and in the CL during oestrous cycle and induced luteolysis.

2.4.3.2 Matrix metalloproteases (MMPs)

Degradation of specific extracellular matrix (ECM) components occurs by the action of MMPs, which belong to a large family with at least 25 related proteolytic enzymes (Woessner 2002). They are classified on their substrate specificity as collagenases, stromelysins, gelatinases, membran-type MMPs (MT-MMPs) and other MMPs (Kuzuya and Iguchi 2003). All MMPs share certain common features like being zinc- and calcium-dependent endopeptidases (Visse and Nagase 2003), which are synthesised as preproenzymes and secreted in an inactive form into the extracellular space (Nagase 1997). Except of MMP-11 and MMP-14 (MT1-MMP), which are activated prior to secretion by furin-like proteases (Kuzuya and Iguchi 2003), MMPs are activated *in vivo* by serine proteases like plasminogen activator (Nagase 1997), mast cell proteases (Suzuki *et al.* 1995), and other MMPs (Sato *et al.* 1994). Also non-proteolytic agents as SH – reactive agents, reactive oxygen and denaturants are activators of MMPs. For activation the prodomain of the MMPs has to be removed (Kuzuya and Iguchi 2003).

A different kind of activation occurs to MMP-2. It takes place on the cell surface by mediation of the membrane-type MMPs like MMP-14, which functions as receptor for MMP-2, and by the interaction with TIMP-2, which belongs to the family of tissue inhibitors of metalloproteases (Strongin *et al.* 1995; Butler *et al.* 1998; Wang *et al.* 2000). By binding to the hemopexin region of proMMP-2 TIMP-2 forms a tight complex and connects to MMP-14 on the cell surface. The now surface - bound - proMMP-2 is then activated by a MMP-14, which is free of TIMP-2. An absence or even a high concentration of TIMP-2 can decrease MMP-2 activation (Strongin *et al.* 1995). Degradation of the ECM by MMPs is required in endothelial cell migration, organization and, hence, angiogenesis (McCawley and Matrisian 2000). All these processes occur during development of the CL, while during luteolysis degradation of the different collagen components is necessary to enable the rapid regression of the CL.

Subdivision	MMP	ECM Substrate
Collagenases	MMP-1	Type I, II, III, VII, X collagen, gelatin
	MMP-8	Fibrillar Collagen
	MMP-13	Fibrillar Collagen
	MMP-18	Fibrillar Collagen
Gelantinases	MMP-2	Type I, IV, V u. fibrillar collagen; gelatin
	MMP-9	Type IV, V collagen, gelatin
Stromelysins	MMP-3	Laminin, fibronectin, non-fibrillar collagens
	MMP-10	Laminin, fibronectin, non-fibrillar collagens
	MMP-11	Laminin, fibronectin, non-fibrillar collagens
Membran-type MMPS	MMP-14	Gelatinase A, fibrillar collagens, Proteoglycans
	MMP-15	Gelatinase A
	MMP-16	Gelatinase A
	MMP-17	
	MMP-24	
	MMP-25	
Other MMPs	MMP-7	Laminin, fibronectin, non-fibrillar collagens
	MMP-12	Collagen type I, IV, elastin, fibronectin, gelatin, laminin
	MMP-19	Aggrecan, type VI collagen, laminin, fibronectin
	MMP-20	
	MMP-26	Fibronectin, gelatin

Table 1: Members of the matrix metalloprotease family

2.4.3.3 Tissue inhibitors of metalloproteases (TIMPs)

Activity of MMPs is strictly regulated in the extracellular environment by the family of tissue inhibitors of metalloproteases (TIMPs). These family of small proteins (21-30 kDa) contains four members, TIMP-1, TIMP-2, TIMP-3 and TIMP-4. For inhibiting active MMPs they form non-covalent complexes by binding to the active site of the catalytic domain of the MMPs. These happens in a 1:1 stechiometric ratio. TIMPs act selectively on different MMPs. For example, TIMP-2 has a high affinity for MMP-2, whereas TIMP-1 preferentially binds to MMP-9. It is reported that TIMPs not only regulate the action of MMPs, but also promote embryo growth (Satoh *et al.* 1994), act as antiangiogenic agents (Murphy *et al.* 1993), stimulate cell growth (Hayakawa *et al.* 1994), influence apoptosis (Talhouk *et al.* 1992) and recruit quiescent cells into the cell cycle (Wick *et al.* 1994).

2.4.3.4 Plasminogen activator (PA) system

Also an important role in degradation of the ovarian ECM plays the plasminogen activator (PA) system (Smith *et al.* 1999). It consists of plasminogen, the inactive form of plasmin, tissue and urokinase plasminogen activator (tPA, uPA), the uPA-receptor (uPAR) and two plasminogen activator inhibitors (PAI-1, PAI-2). tPA and uPA transform plasminogen into its active form plasmin in the extracellular space (Luck 1994). uPA is able to bind on its cell surface receptor (uPAR) for building a stable complex. Its purpose is thought to focus the plasmin activity at the cell surface. Plasmin is not only able to degenerate specific ECM components like types III and IV collagen, fibronectin, laminin and proteoglycans (Roldan *et al.* 1990; Barnathan *et al.* 1990), but also activates proenzyme forms of MMP-1, MMP-2 (Monea *et al.* 2002) MMP-3 and MMP-9 (DeClerck and Laug 1996; Lijnen *et al.* 1998; Makowski and Ramsby 1998; Murphy *et al.* 1999). Working hand in hand together the MMPs and the plasminogen activator system are able to degrade all ECM components in the ovary (Dow *et al.* 2002).

2.4.3.5 Monocyte chemoattractant protein-1 (MCP-1)

MCP-1 is a member of the intecrine β family of cytokines. The cytokines in this group are thought to be involved in inflammation and tissue repair (Oppenheim *et al.* 1991). MCP-1 has a potent chemoattractant effect on monocytes and $CD4^+$ and $CD8^+$ T lymphocytes. MCP-1 is produced by a variety of cells types including endothelial cells, fibroblasts, monocytes and T lymphocytes. MCP-1 binds to two receptors, CCR-2 and CCR-4, sharing these receptors with other cytokines of its family (Penny 2000). Results of recent studies indicate that MCP-1 is produced in the CL during luteal regression and might aid in its destruction (Penny *et al.* 1998; Townson *et al.* 1996; Townson *et al.* 2002; Bowen *et al.* 1996; Tsai *et al.* 1997). MCP-1 is of particular interest because, once expressed within blood vessels, this chemokine facilitates the attachment and migration of immune cells, especially monocytes, macrophages and T lymphocytes, from the blood stream into sites of inflammation (Mukaida *et al.* 1992). In this regard, macrophages and T lymphocytes have been demonstrated to accumulate in regressing CL of many species (Penny *et al.* 1999; Townson *et al.* 2002; Townson *et al.* 1996; Hehnke *et al.* 1994) and have been implicated in phagocytosis of luteal cells (Paavola 1979), degradation of ECM (Endo *et al.* 1993) and secretion of proinflammatory mediators that influence luteal steroidogenesis (Benyo and Pate 1992; Rueda *et al.* 2000b).

2.4.4 Apoptosis

2.4.4.1 General aspects

Apoptosis or programmed cell death is a evolutionary highly conserved mechanism, that allows the organism to tightly control cell numbers, tissue size and protect itself from dangerous cells that threaten homeostasis. It is regulated by proteins of the organism that form an intrinsic and extrinsic signaling cascade. The intrinsic apoptotic signaling cascade is generally thought to be activated by apoptotic stimuli that are caused within a cell in response to certain drugs, radiation or growth factor withdrawal and primarily lead to changes in mitochondrial permeability through alterations in the ratio of pro-apoptotic to anti-apoptotic Bcl-2 family members (Adams and Cory 1998). The extrinsic apoptotic signaling cascade on the other hand is activated by extracellular signals like TNFα or Fas-Ligand that interact with cell surface receptors to start cell death (Nagata 1997). All these signals activate a cascade of intracellular proteases known as caspases (fig. 2). Once activated, caspases cleave various cellular substrates including actin, poly(ADP-ribose) polymerase (PARP), DFF45/inhibitor of caspase-activated DNase (ICAD), fodrin, and lamin which contribute to the morphological changes typically for apoptotic cells (Martin and Green 1995). The final cellular event during apoptosis is reached with the fragmentation of DNA. This is mediated by a huge variety of endonucleases such as DFF40/caspase-activated DNase (CAD), Nuc70 etc (Yadav *et al*. 2005).

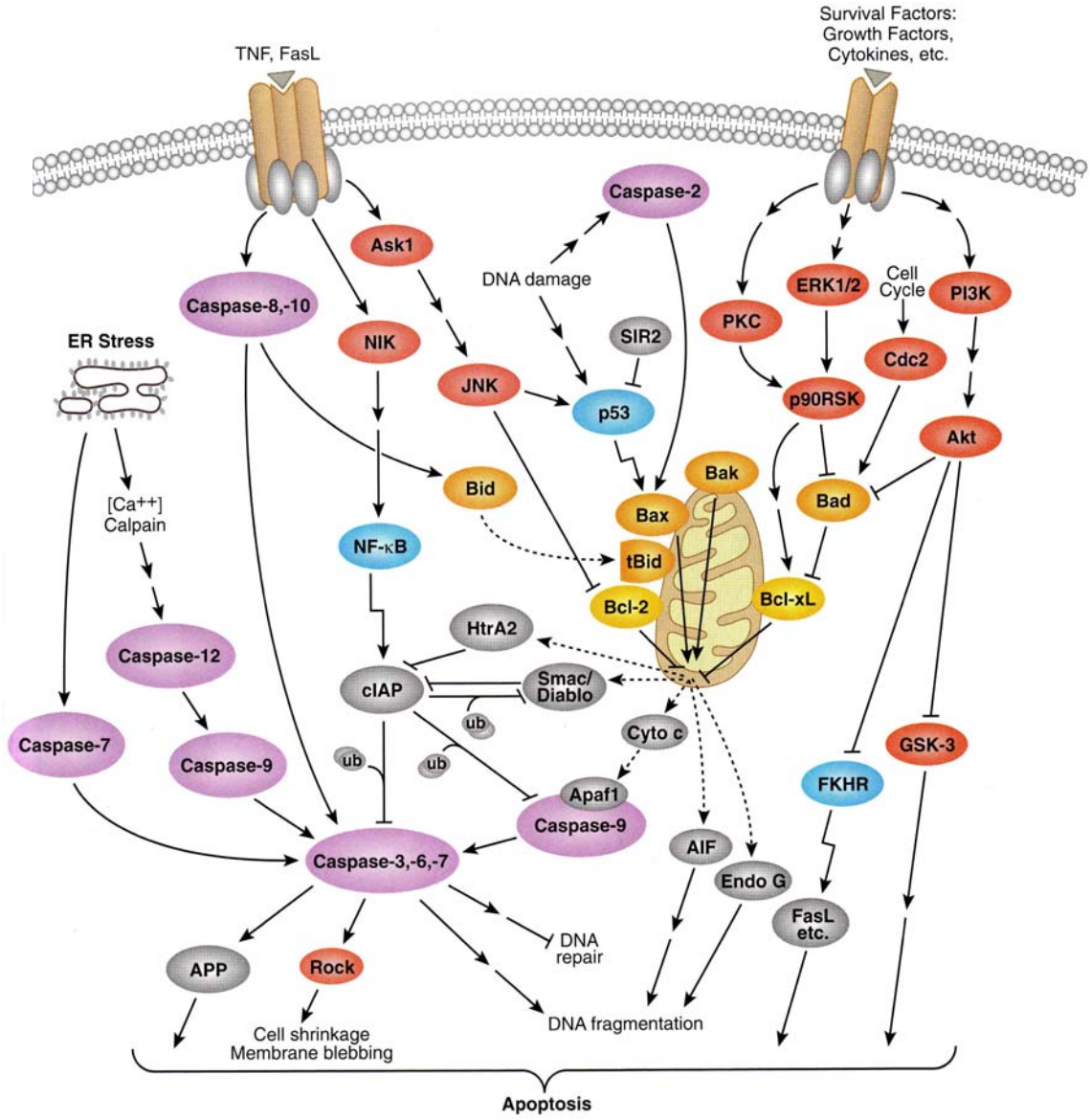


Figure 2: Apoptotic pathways (Cell Signaling Technologies 2005/2006)

2.4.4.2 Extrinsic pathway

2.4.4.2.1 Tumor necrosis factor family (TNFα, TNFR1, TNFR2)

TNFα is a nonglycosylated protein with 17kDa of molecular mass, which belongs to the family of cytokines. TNFα has first been described as a tumoricidal factor, which is produced by activated macrophages. Nowadays it is known, that besides macrophages also granulosa cells (Zolti *et al.* 1990) and luteal cells (Hehnke-Vagnoni *et al.* 1995) are candidates which express TNFα within the ovary. In rats (Brannstrom *et al.* 1994a) and cows (Skarzynski *et al.* 2000) it is shown, that macrophages are attracted to and infiltrate the CL during luteolysis. This accumulation of macrophages in the regressing CL is

believed to be in response to a chemotactic factor. A likely candidate for this is monocyte chemoattractant protein-1 (MCP-1), its expression has been demonstrated to be regulated by local growth factors and cytokines such as TNFα (Kayisli *et al.* 2002). Various researches verify that TNFα stimulates prostaglandin synthesis by luteal cells, and that it furthermore inhibits gonadotropin supported progesterone production in bovine luteal cells (Benyo and Pate 1992). This is supported through further studies proving that locally expressed cytokines such as TNFα induce follicular atresia and CL apoptosis (Friedman *et al.* 2000). TNFα is able to bind to two different TNF-receptors (TNFR1, TNFR2), which are expressed on microvascular endothelial cells in the CL (Okuda *et al.* 1999). TNFR1 mRNA is expressed on bovine luteal and endothelial cells and is responsible for TNFα induced apoptosis during structural luteolysis via the extrinsic pathway mediated by TNFR1 (fig. 2), which mainly effects the endothelial cells (Friedman *et al.* 2000). There is a controverse discussion about the action of TNFR2 in concern to trigger or inhibit apoptosis. Some groups report that TNFα is not only able to induce apoptotic cell death, but can also induce cell proliferation by binding to TNFR2. This receptor can induce gene transcription for cell survival, growth and differentiation, and therefore TNFR2 is involved in an anti-apoptotic effect of TNFα (Inoue *et al.* 2000; Wajant and Scheurich 2001; Stanger *et al.* 1995; Shu *et al.* 1996). On the other hand TNFR2 is able to bind the TNF receptor-associated factor 2 (TRAF2), which is a necessary factor for the activation of the anti-apoptotic NFκB pathway by TNFR1. Depending if TNFR1 is costimulated with TNFR2 or not, apoptosis occurs or not (Fotin-Mleczek *et al.* 2002) (fig. 3).

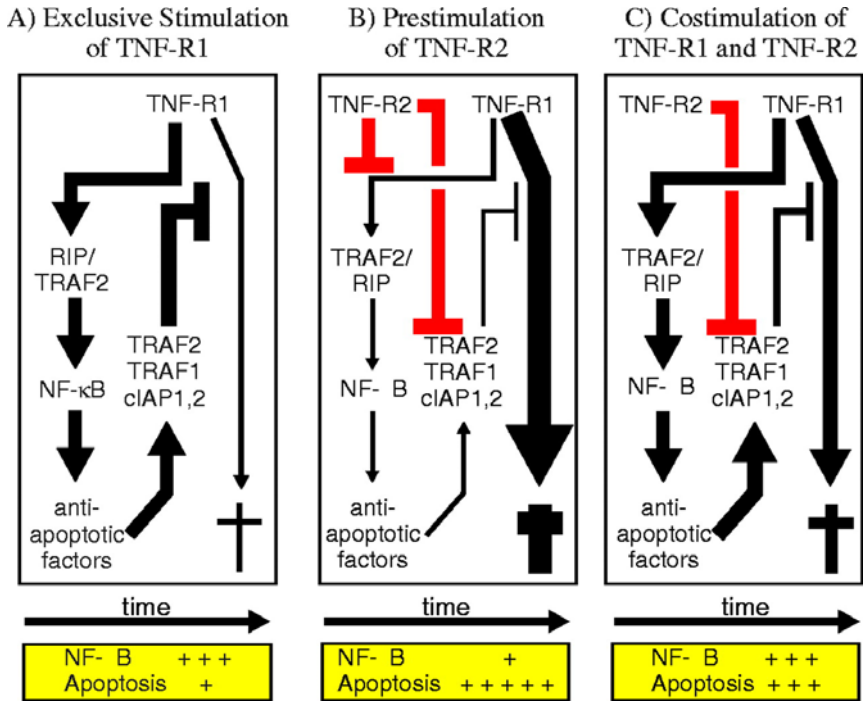


Figure 3: Model of the apoptotic TNFR1 / TNFR2 crosstalk. A) Exclusive stimulation of TNFR1. B) Prestimulation of TNFR2. C) Costimulation of both TNF receptors. (Fotin-Mleczek *et al*. 2002)

2.4.4.2.2 Fas and Fas-Ligand (FasL)

The Fas antigen (also known as APO-1 or CD95) is a transmembrane glycoprotein of 48 kDa and encodes a cysteine-rich transmembrane protein of 335 amino acids. It is a member of the TNF family of cell surface receptors (Nagata and Golstein 1995), which also includes other receptors like TNFR1 and TNFR2, death receptor-3, two receptors for the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and many more. Fas is expressed on the cell surface in various tissues like heart and kidney, furthermore on thymocytes and activated T cells (Nagata, 1999). Fas-Ligand (FasL) is a cytokine that belongs to the TNF family (Suda *et al.* 1993). FasL is predominantly expressed in activated T lymphocytes and natural killer cells. It is a type II membrane protein with an extracellular region of about 150 amino acids and an cytoplasmic region of 77 amino acids. Induced expression of the FasL gene occurs through the binding of NFκB to the 5' flanking region of the FasL gene, stresses as UV radiation or gamma-irradiation also show the same effect. Itoh et al. (1991) showed that binding of FasL to Fas induces apoptosis in Fas bearing cells. TNF, the prototype of the TNF family has two different receptors TNFR1 and TNFR2, especially the interaction of TNFR1 and TNF mediates apoptosis. Fas and TNFR1 share a homologous domain in their cytoplasmic domain and

mutational analysis indicated that this domain is necessary to transduce the apoptotic signal. Thus, this domain has been designated a death domain (Nagata, 1999). Fas and TNFR1 must be oligomerized to be activated, and since x-ray diffraction analysis showed that TNF induces trimerization of its receptor, the similarity to the structure of FasL suggests that FasL also induces trimerization of Fas. The trimerized cytoplasmic region then has the ability to transduce the apoptotic signal via the recrutation of FADD (FAS-associated protein with death domain) and the following binding of caspase8. The activation of caspase8 and the further extrinsic pathways is already described above.

2.4.4.3 Intrinsic pathway

2.4.4.3.1 Tumor suppressor p53

The tumor suppressor p53 is often described as a “guardian of the genome”, because it is a critical component of cellular mechanisms that respond to genotoxic stresses like DNA damage, hypoxia etc. to maintain the genomic integrity in part by arresting the cell cycle progression or by inducing apoptosis. p53 is a short-lived protein with a half life of about 20 minutes that is maintained at very low or undetectable levels under normal circumstances. Under exposure of stressful stimuli, p53 is activated through post-translational modifications that increase its stability and activity (Levine 1997). The human p53 protein contains 393 amino acids and has been divided structurally and functionally into four domains: an acidic amino-terminal domain, which is required for transcriptional activation; a central core sequence-specific DNA-binding domain; a tetramerization domain and a C-terminal regulatory domain (Somasundaram 2000). p53 can alter the transcriptional activity of genes associated with cell death. It was found that p53 increases the expression of Bax, an apoptotic factor of the internal pathway, and by the other hand decreases the Bcl-2 expression, which is a anti-apoptotic factor that inhibits the action of Bax (Miyashita *et al.* 1994; Miyashita and Reed 1995). It is also reported that p53 is able to activate Fas, which belongs to the extrinsic pathway (Kobayashi *et al.* 1998). p53 expression occurs in apoptotic granulosa cells of atretic rat follicles (Tilly *et al.* 1995) and is also induced by reactive oxygen species (ROS) in bovine luteal cells (Nakamura and Sakamoto 2001).

2.4.4.3.2 Bax and Bcl-X_L

The intrinsic pathway is used extensively in response to internal insults such as DNA damage (Rich *et al.* 2000). The diverse response pathways affect the mitochondria through the activation of a set of apoptotic regulators, the Bcl-2 family. This family is divided into three groups, based on structural similarities and functional criteria. Some molecules like Bcl-2 and Bcl-X_L are attached to the mitochondria-membrane, others can shuttle between the cytosol and organelles (Gross *et al.* 1999). Members of group I (like Bcl-2, Bcl-X_L) posses anti-apoptotic activity, whereas group II (Bax, Bak) and group III (Bid, Bik) members promote cell death. The Bax protein was originally identified via its ability to interact with Bcl-2 in cells. Bcl-X is a unique gene among the other members of this family as it produces both positive ("short" isoform, death inducer) and negative ("long" isoform, death suppressor) regulators of the cell death pathway (Goodman *et al.* 1998). Bcl-2 members control cell death through their ability to form heterodimers from pro- and anti-apoptotic molecules. Thus, heterodimerization can be thought as resulting in neutralization of these proteins. This leads to the point, that overall levels of pro-and anti-apoptotic factors have to be compared in order to estimate whether cells are sensitive to death or resistant (Hengartner 2000). How the increase in the expression levels of Bcl-2 members is achieved has not been fully investigated yet, but it is obvious, that p53 plays a significant role (Miyashita and Reed 1995). The key function of Bcl-2 family however is to regulate the release of pro-apoptotic factors from the mitochondrial intermembrane compartment into the cytosol (Adams and Cory 1998). The most prominent factor among these is cytochrome c, an electron carrier. This protein is one of the components required for the activation of caspase9 in the cytosol. Unlike other caspases, proteolytic processing of procaspase9 has only a minor effect on its activity. The urgent requirement for the activation of caspase9 is its association with the adaptor protein Apaf-1 (Li *et al.* 1997). Apaf-1 thus is nowadays believed as an essential regulatory subunit of a caspase9 holoenzyme, the so called apoptosome. Heat shock proteins act as multiple steps in the pathway to regulate apoptosis (Xanthoudakis and Nicholson 2000). The extrinsic and intrinsic pathway then converge at the level of caspase3 activation (fig. 2).

2.4.4.3.3 Second mitochondria derived activator of caspases (Smac)

In the intrinsic pathway, the key event leading to the activation of caspases is the release of several pro-apoptotic proteins from the intermembrane space of mitochondria into the cytosol (Wang 2001). One such protein is the second mitochondria derived activator (Smac), which is also called Diablo (Verhagen *et al.* 2000). The newly synthesised Smac protein contains of 239 amino acids. Its N-terminal 55 residues encode the mitochondrial targeting sequence and are proteolytically removed in the mature Smac protein (Du *et al.* 2000). This cleavage results in the exposure of four hydrophobic amino acids at the N-terminus of the mature Smac. This tetrapeptide represents the founding member of a family of inhibitors of apoptosis proteins (IAPs) binding motifs in mammals (Shi 2002a). The binding of Smac to IAPs prevent their interactions with caspase9 and the inhibition of the intrinsic pathway (Srinivasula *et al.* 2001).

2.4.4.3.4 Survivin

Survivin belongs to the family of inhibitors of apoptosis proteins (IAPs). These proteins prefent aberrant activation of caspases, which can be detrimental to cells. To protect against inadvertent damages and death, cells have evolved a system of checks and balances. At the heart of the system is the evolutionarily conserved IAP family. Membership of this family requires a 80 amino acid zink-binding module, which is referred to as a baculoviral IAP repeat (BIR) domain. At least eight distinct IAPs have been identified in the mammalian genome, each of which contains 1-3 copies of the BIR domain. Except of survivin, all other IAPs contain other functional domains such as RING (really interesting new gene) (Deveraux and Reed 1999; Salvesen and Duckett 2002). Survivin contains only one BIR domain and does not inhibit caspase activity *in vitro* (Shi 2002b). In contrast to the relatively stable expression levels of other IAPs, expression of survivin oscillates with cell cycle and peaks at the G2/M phase (Li *et al.* 1998). Survivin is able to inhibit Smac and prevent cells from apoptosis (Song *et al.* 2003). Survivin is expressed in human endothelial cells of newly formed blood vessels (Tran *et al.* 1999).

2.4.4.4 Caspase3, -6, -7

Caspases share homology with the Caenorhabditis elegans gene, Ced-3, its product is required for mediating apoptosis during development. Caspase3 is the vertebrate-caspase most similar to Ced-3 concerning amino acid sequence homology as well as substrate specificity. The active mammalian form, consisting of p17 and p12 subunits, is processed from an inactive 32-kDa proenzyme after auto- or heterocatalysis, preferentially cleaving substrates containing a DEVD motif (Nicholson and Thornberry 1997). The active form of mammalian caspase6 consists of p18 and p11 subunits (processed from a 34-kDa proenzyme). caspase7 shares its signature cleaving site with caspase3 and thus has a great similarity in subtrat specifity. Studies have demonstrated the existence of at least 11 additional homologous mammalian proteases, caspase1 through -14, that together compose the caspase family of cysteine proteases. All identified mammalian caspases are synthesised as inactive proenzymes and activated after cleavage at a specific N-terminal domain aspartate cleavage sites. Within this family of enzymes, caspase7 and caspase9 are considered to be most homologous in sequence to caspase3 and -6, while the amino acid sequences of caspase8 and caspase10 are the next most related (Miller 1997; Nicholson and Thornberry 1997). Although the mechanism of caspase action is highly conserved, differences among primary sequences account for different substrate specificity. As a group, caspase members generally form a cascade of enzymatic activity that results in the initiation and amplification of early apoptotic events (e.g. caspase8, -9, so called initiator-caspases) as well as mediating the later executioner steps of apoptosis (e.g. caspase3, -6, -7, the effector-caspases) (Nicholson and Thornberry 1997). Both apoptotic pathways result in the activation of this cascades, independend wether it is done directly or via activation of the mitochondrial death program. On one hand, the extrinsic pathway starts with ligand-binding to death-receptors such as CD95 (Apo-1/Fas). As an effect, those receptors form membrane-bound signalling complexes, which recruit through adapter proteins several molecules of caspase8. The induced proximity model (Muzio *et al.* 1998) posits that under these conditions the molecules cleave and thus activate each other. As described above, this activated caspase now activates further caspases of the cascade like -3, -6, -7, which leads to cleavage of specifc death substrates. The intrinsic pathway on the other hand also activates caspase3 and following caspases, however it starts out in quite a different way.

3 Material

3.1 Development of a method to gain uterus milk *in vivo*

3.1.1 Equipment

blood withdrawal system	Vacuette 20G, 450.077, Vacuette K3E EDTA K3, 455.036, Greiner Labortechnik, (Kremsmünster, Austria)
buckler	Stopfen Sarstedt 65.806 (Nürnbrecht)
catheter	disposable embryo flushing catheter steril incl. steel mandrin, CH 12, Minitüb (Tiefenbach)
cellulose tissue	Ärztekrepp, Heiland (Hamburg)
centrifuge	Sigma 3-16K (Osterode)
clipping machine	Typ GT 104, Aesculap AG & Co.KG (Tuttlingen)
microscope	Zeiss Axiovert 25, Carl Zeiss AG, (Hallbergmoos)
microtiter plates	Nunc-Immuno-Platte Maxisorb F 96, Nunc Gmbh (Wiesbaden)
microtiter plate dispensor	Manifold-8-Changonel, SLT Labinstruments (Crailsheim)
microtiter plate washer	SLT 96.PW, SLT Labinstruments (Crailsheim)
needles sterile	Microlane 3 18G x 1½, BD (Drogheda, Ireland)
pipettes	Abimed, Gilson (Frankreich)
	Eppendorf (Hamburg)
	Pipetus akku, Hirschmann Laborgeräte (Eberstadt)
pipettetips	Diamond D10 tips, Gilson (Hamburg)
	Standardtips Eppendorf (Hamburg)
	Sarstedt (Hamburg)
reaction tubes	2ml, Eppendorf (Hamburg)
	5ml Sarsted-Röhrchen 55.525 (Nürnbrecht)
	15ml Cell Star 188.271, Greiner (Frickenhausen)
syringes disposable	Terumo 5ml, Heiland (Hamburg)
	Basik 20ml, Heiland (Hamburg)
tissue culture dish	Corning, Sigma-Aldirch (München)
vaginal flushing system	Irrigator 2L, Heiland Vet (Hamburg)
	flexible hose, Heiland Vet (Hamburg)

3.1.2 Medicaments

Buserelinacetat	Receptal, Intervet Deutschland GmbH

	(Unterschleißheim)
Cloprostenol	Estrumate, Essex Tierarznei (München)
Gentamicinsulfat	Genta-Sleecol 5, A. Albrecht GmbH + Co. KG (Aulendorf)
0,9% NaCl	Fresenius Kabi Deutschland Gmbh (Bad Homburg)
Procainhydrochlorid	Procasel 2%, Selectavet Dr. Otto Fischer GmbH (Weyarn-Holzolling)

3.1.3 Biochemicals

99% acetylsalicylic acid	Merck (Darmstadt)
BSA	Fraktion V, pH 5,2; Standard Grade, Serva, 11930 (Heidelberg)
citric acid x 1 H_2O	Merck, 244 (Darmstadt)
ethanol (100%)	Merck (Darmstadt)
H_2O_2-urea	Merck, 818356 (Darmstadt)
H_2SO_4	Merck, 100731 (Darmstadt)
Kathon	pH 5.0, Rohm & Haas Ltd., 62368 (U.K.)
NaCl	Merck (Darmstadt)
Na_2CO_3 x 10 H_2O	Merck, 6391(Darmstadt)
$NaHCO_3$	Merck, 6329 (Darmstadt)
Na_2HPO_4 x 2 H_2O	Merck, 6580 (Darmstadt)
sperm deep frozen	FV Weber IN 184593 27.07.01, D-KBR015 EWG (München - Grub)
TMB	Boehringer, 784974 (Mannheim)
0,05% Tween-80	Sigma, P 1754 (Taufkirchen)

3.1.4 Reagents

assay-buffer	7.12 g/l Na_2HPO_4 x 2 H_2O
	8.5 g/l NaCl pH 7.2
	1.0 g/l BSA (0.1%)
coating buffer	4.29 g/l Na2CO3 x 10 H_2O
	2.93 g/l $NaHCO_3$ pH 9.6
substrate solution A	1 g/l H_2O_2-urea
	18 g/l Na_2HPO_4 x 2 H_2O
	10.3 g citric acid x 1 H_2O
	0.1 ml Kathon
	pH 5.0 photosensitiv
substrate solution B	300 mg TMB (Tetramethylbenzidin)

	40 ml DMSO (Dimethylsulfoxid)
	960 ml H_2O
	10.3 g citric acid x 1 H_2O
	pH 2.4 photosensitiv
peroxidase-labeled oestradiol-17β	self-production 17β/2pool1 26.07.95
unlabeled oestradiol-17β	Serva 31100 (Heidelberg)
oestradiol-17β antibody (rabbit)	self-production E2/2pool1 23.11.77
peroxidase-labeled PGF2α	self-production 29.10.97
unlabeled PGF2α	Sigma, P0424 (Taufkirchen)
PGF2α antibody (rabbit)	R. Claus, AS S5, Ka III, E. 20.09.87, (Hohenheim)
peroxidase-labeled progesterone	Sigma, P3659 (Taufkirchen)
unlabeled progesterone	Sigma, P 0130 (Taufkirchen)
progesterone anti-body (rabbit)	K. Okuda, OK-1 03.12.97, (Japan)
prostaglandin stabilisator	111.7g 0.3 m EDTA
	10g 1% acetylsalicylic acid
	pH 7.4 photosensitiv
VEGF-165 antiserum	self-production H21 30.9.93
VEGF-165 protein, bovine, recombinant	D. Gospodarowicz, 28.10.97 (Chiron, Oakland, USA)

3.2 Expression of housekeeping genes, proteases, apoptotic and anti-apoptotic factors in the CL during oestrous cycle and induced luteolysis

3.2.1 Tissue collection

3.2.1.1 Follicles 20 hours after GnRH application

For comperable reasons follicles (n=5) were taken 20h after GnRH application from the experiment published by Berisha et al. (2006). German Fleckvieh cows were superovulated using FSH (Ovagen; Immunochemical Products Ltd, Auckland, New Zealand) injections (in total seven), which were given i.m. at 12h intervals in gradually decreasing doses for 3.5 days, starting between days 8 and 11 of the oestrous cycle after previous oestrus observation. After the sixth FSH injection, a luteolytic dose of 500 µg PGF_2 analogue (cloprostenol, Estrumate; BERNA Veterinärprodukte AG, Bern, Switzerland) was injected i.m., and then 40h after $PGF_{2\alpha}$ injection, 100 µg gonadotrophin-releasing hormone (GnRH) (Receptal; BERNA Veterinärprodukte AG, Bern, Switzerland) were injected to induce the LH surge. For confirmation of LH surge, blood samples were collected from the jugular vein at –24, –12, –1 and 0h before and 3 and 12h after GnRH application. Lack of an endogenous LH surge prior to GnRH was confirmed by LH determination in blood plasma (range 0.8–1.0 ng/ml); 4h after GnRH the mean LH level (induced LH surge) was 11.50 ng/ml (range 8.5–14.1 ng/ml) and at 12h 0.73 ng/ml (range 0.2–1.0 ng/ml). The ovaries were collected by transvaginal ovaryectomy (*n*=5 cows/group) 20h after GnRH application.

3.2.1.2 CL during oestrous cycle

The CLs (4 per group) were collected 10-20 minutes after slaughter, frozen in liquid nitrogen and kept at –80°. According to a modified method by Ireland et al. (Ireland *et al.* 1980) the CLs were divided into four groups:

- Group I + II (day 1-4):
  - Time before ovulation until development of one epithelial cell layer over the rupture site with further differenciations like:
  - Day 1-2: only a blood-clot is present, 2-6mm in diameter (group I)
  - Day 3-4: development of a small umbilicus, growing diameter (group II)

- Group III (day 5-7):
  - Day 5-7: developing stage; blood vessel formation in the periphery is seen; cutting of the CL displays a red apex; 5-12mm in diameter

- Group IV (day 8-12):
  - Day 8-12: mature stage; the apex is red, the rest of the CL yellow; the CL is clearly bigger than in group III (over 12mm of diameter) and is softly demarced

3.2.1.3 CL during induced luteolysis

CL tissue was taken from the experiment described by Neuvians et al. (2003). In this experiment 5 CLs per group were taken from cycle synchronised cows after defined time points during induced luteolysis at day 8-12 of the oestrous cycle. The time intervals were as follows:

0, 2, 4, 12, 24, 48 and 64 hours after PGF2α injection

Additionally we decided to complement the experiment with an earlier time point at 0.5 hours after luteolysis (Az: 209.1/211-2531.3-29/04). Therefore we synchonised five non lactating Brown Swiss cows with two injections of 500μg Cloprostenol (Estrumate) i.m. within eleven days. 10-12 days after the second injection luteolysis was induced by further administration of 500μg Cloprostenol. After 0.5h the ovaries were taken by transvaginal ovaryectomy. The CLs were dissected, cut in slices and either fixated in Bouin´s solution or in methanol-glacial acetic acid for immunohistochemistry, or frozen in liquid nitrogen and stored at –80°. Blood samples were taken at each PGF2α injection and daily from the second day after the second PGF2α injection on for measuring the progesterone level.

3.2.2 Equipment

blood withdrawal system	Vacuette 20G, 450.077, Vacuette K3E EDTA K3, 455.036, Greiner Labortechnik, (Kremsmünster, Austria)
buckler	Stopfen Sarstedt 65.806 (Nürnbrecht)
centrifuge	Sigma 3-16K (Osterode)
clipping machine	Typ GT 104, Aesculap AG & Co.KG (Tuttlingen)
syringes disposable	Terumo 5ml, Heiland (Hamburg)

3.2.3 Surgical instruments

cervix clamp	Hauptner (Dietlikon-Zürich, Schweiz)
effeminator	Hauptner (Dietlikon-Zürich, Schweiz)
knife by Höhne	Hauptner (Dietlikon-Zürich, Schweiz)
ligature carrier by Deschamps 45cm	Eickemeyer (Tuttlingen)
suture material	PBS 2USP, 5EP absorbable, WDT (Garbsen)
swabs sterile	Fuhrmann (Much)

3.2.4 Medicaments

Benzylpenicillin-Procain	Procillin, Alvetra (Neumünster)
Cefquinomsulfat	Cobactan 2.5%, Intervet (Unterschleißheim)
Cloprostenol	Estrumate, Essex Tierarznei (München)
Metamizol-Natrium	Vetalgin, Intervet (Unterschleißheim)
Poly(1-vinyl-2-pyrrolidon)-Jod-Komplex	Vet-Sept-Lsg., Albrecht (Aulendorf)
Procainhydrochlorid	Procasel 2%, Selectavet Dr. Otto Fischer GmbH (Weyarn-Holzolling)
Xylazinhydrochlorid	Rompun 2%, Bayer Vital (Leverkusen)

3.2.5 Laboratory equipment

beads	Matrix Green, Bio 101 Systems, MP Biomedicals (Heidelberg)
Bioanalyzer 2100	Agilent Technologies (Waldbronn)
centrifuge	Biofuge 13, Heraeus Instruments, 75003635/01 (Osterode)
	Biofuge fresco, Heraeus Instruments, 75005510, (Osterode)
	Biofuge pico, Heraeus Instruments, 75003280, (Osterode)
	Zentrifuge 5415 D, Eppendorf (Hamburg)
	Zentrifuge 5415 R, Eppendorf (Hamburg)
	Zentrifuge 5804, Eppendorf (Hamburg)
	Sigma 3K30 (Osterode)
cuvettes	Uvette, Eppendorf (Hamburg)
electrophoresis module	Standard Power Pack P25, Biometra (Göttingen)
embedding form	10mm x 20mm, Plano (Wetzlar)

embedding slide	Histosette II tissue, Simport (Beloeil, Canada)
heating plate	hotplate magnetic stirrer, Cenco (Breda, Netherlands))
homogenisator	FastPrep FP 120 Bio 101 Systems, MP Biomedicals (Heidelberg)
homogenisator tubes with cap	Lysing Matrix D, Bio 101 Systems, MP Biomedicals (Heidelberg)
gel-chamber	Agagel Mini/Midi-Wide, Biometra (Göttingen)
microtiter plates	Nunc-Immuno-Platte Maxisorb F 96, Nunc Gmbh (Wiesbaden)
microtiter plate dispensor	Manifold-8-Changonel, SLT Labinstruments (Crailsheim)
microtiter plate washer	SLT 96.PW, SLT Labinstruments (Crailsheim)
microwave-oven	Panasonic NN 6275B (UK)
photometers	Biophotometer Eppendorf (Hamburg)
	Titrek-Multiscan, Typ 310 C, Flow Laboratories with SLT-Easy-Fit, SLT Labinstruments (Crailsheim)
pipettes	Abimed, Gilson (Frankreich)
	Eppendorf (Hamburg)
pipettetips	Diamond D10 tips, Gilson (Hamburg)
	Standardtips Eppendorf (Hamburg)
	Sarstedt (Hamburg)
reaction tubes	0.2ml micro-tube, Abgene (Epson, UK)
	0.5ml Biozym 710912 (Oldendorf)
	1.5ml; 2.0ml, Eppendorf (Hamburg)
	5ml Sarsted-Röhrchen 55.525 (Nürnbrecht)
	10ml Sarstedt-Röhrchen 26.323 (Nürnbrecht)
	15ml Cell Star 188.271, Greiner (Frickenhausen)
	50ml Cell Star 227.261, Greiner (Frickenhausen)
RNA nano chips	Agilent Technologies (Waldbronn)
rotor-gene 3000	Corbett Research (Australia)
scales	Sartorius MC 210 P (Göttingen)
	Sartorius 2001 MP2 (Göttingen)
shaker	Vortex-Genie 2 Bender & Hobein (München)
thermoblock	Thermomixer 5436, Eppendorf (Hamburg)
thermocycler	Mastercycler gradient, Eppendorf (Hamburg)
video-documentationsystem	Image Master JVDS, Pharmacia
	80-6247-01 (Freiburg)
warming cupboard	Shinko MCS, Bachofer (Reutlingen)

3.2.6 Biochemicals

agarose	Amresco 710-50G (Ohio, USA)
bromphenolblue	Merck 8122 (Darmstadt)
BSA	Fraktion V, pH 5.2; Standard Grade, Serva, 11930 (Heidelberg)
butanol tert.	Roth (Karlsruhe)
buthyl methyl ether	Roth (Karlsruhe)
chloroform	Merck, 2445 (Darmstadt)
DEPC (diethylpyrocarbonat)	Aldrich 15.922-0 (Steinheim)
DMSO	Serva, 20385 (Heidelberg)
dNTP (each 25 µmol)	deoxynucleoside triphosphate set, R0182, Fermentas (St. Leon-Rot)
EDTA	Merck 8418 (Darmstadt)
ethanol (100%)	Merck (Darmstadt)
ethidiumbromid 1% solution	3.8-Diamino-5-Ethyl-6-Phenylphenanthridinium-Bromid (10mg/ml), Carl Roth GmbH + Co. KG (Karlsruhe)
formaldehyd (35%)	Merck, 1.04001 (Darmstadt)
formaldehyd (37%)	Merck, 1.04094 (Darmstadt)
glacial acetic acid (100%)	Merck, 1.00063 (Darmstadt)
HCL (5M)	Combi Titrisol, Merck (Darmstadt)
H_2O_2-urea	Merck, 818356 (Darmstadt)
H_2SO_4	Merck, 100731 (Darmstadt)
hexamere	Pharmacia 27-2166-01 (Freiburg)
iso-2-propanol	Merck, 9634 (Darmstadt)
Kathon	pH 5.0, Rohm & Haas Ltd., 62368 (U.K.)
methanol	Merck, 1.06009 (Darmstadt)
$MgCl_2$	Merck (Darmstadt)
MOPS	Roth, 6979.2 (Karlsruhe)
NaCl	Merck (Darmstadt)
Na_2CO_3 x 10 H_2O	Merck, 6391(Darmstadt)
$NaHCO_3$	Merck, 6329 (Darmstadt)
Na_2HPO_4 x 2 H_2O	Merck, 6580 (Darmstadt)
NaOH	Merck (Darmstadt)
paraffin	Vogel-Histo-Comp, Vogel (Gießen)
PCR- H_2O	Sterile, deionized DEPC-H_2O
PeqGold TriFast	Peq Lab (Erlangen)
petroleum ether	Scharlau (Barcelona, Spain)
picric acid	Merck, 1.00621 (Darmstadt)

RNA-H_2O	Sterile, deionized DEPC-H_2O
TMB	Boehringer, 784974 (Mannheim)
Tris(hydroxymethyl)-amiomethan	Riedel-deHaën, 33742 (Seelze)
0,05% Tween-80	Sigma, P 1754 (Taufkirchen)
citric acid x 1 H_2O	Merck, 244 (Darmstadt)

3.2.7 Reagents

coating buffer	4.29 g/l Na2CO3 x 10 H_2O
	2.93 g/l $NaHCO_3$ pH 9.6
assay-buffer	7.12 g/l Na_2HPO_4 x 2 H_2O
	8.5 g/l NaCl pH 7.2
	1.0 g/l BSA (0.1%)
substrate solution A	1 g/l H_2O_2-urea
	18 g/l Na_2HPO_4 x 2 H_2O
	10.3 g citric acid x 1 H_2O
	0.1 ml Kathon
	pH 5.0 photosensitiv
substrate solution B	300 mg TMB (Tetramethylbenzidin)
	40 ml DMSO (Dimethylsulfoxid)
	960 ml H_2O
	10.3 g citric acid x 1 H_2O
	pH 2.4 photosensitiv
progesterone antibody (rat)	Sigma, P1922 (Taufkirchen)
peroxidase-labeled progesterone	Sigma, P3659 (Taufkirchen)
unlabeled progesterone	Sigma, P 0130 (Taufkirchen)

3.2.8 Kits

DNA-digestion	DNA-free™, Ambion (USA)
Light Cycler FastStart DNA Master SYBR Green I	Roche Diagnostic Gmbh (Mannheim)
RNA 6000 Nano reagents & supplies	Agilent Technologies (Waldbronn)
Wizard SV Gel and PCR Clean-Up System	Promega (Mannheim)

3.2.9 Enzymes

reverse transcriptase	Promega (Mannheim)
M-MLV RT Rnase H(-) Point Mutant	

taq-polymerase	Roche (Mannheim)

3.2.10 Markers

100bp-sizemarker	New England Biolabs 100bp DNA Ladder N3231S (Frankfurt)

3.2.11 Buffers and solutions

agarose-gel 1,5%	0.45g agarose 30ml 1x TAE-buffer 3µl Ethiduimbromid-solution
Bouin´s solution	375ml picric acid (saturated) 125ml formaldehyd (37%) 25ml glacial acetic acid
bromphenolblue/glycerin	15ml bromphenolblue solution 85ml RNA-H_2O 100ml glycerin (pure)
DEPC-H_2O	1ml DEPC in 1l H_2O bidest, incubate 24h at 4°, autoclave 20min at 121°C
dNTP-working solution	100µl dATP (100mM) 100µl dCTP (100mM) 100µl dGTP (100mM) 100µl dTTP (100mM) 600µl DEPC H_2O
Formaldehyde-Loading Dye	Ambion (USA)
Methanol - glacial acetic acid	methanol : glacial acetic acid (2:1)
10x MOPS	0.2M MOPS 50mM Natriumacetat 10mM EDTA autoclave 20min at 121°
RNA-Gel	0.4g Agarose MP 33.8ml Rnase free H_2O 4ml MOPS 10x conz. 2.2ml formaldehyde 37% 2µl ethidiumbromid-solution
50x TAE-buffer	2M Tris(hydroxymethyl)-amiomethan

	1M acetic acid
	90mM EDTA
Tris-buffer	50mM Tris, pH 8.0 adjust with HCL

3.2.12 Primersequences and polymerase chain reaction (PCR) products

Forward (for) and reverse (rev) primers were designed either by colleges of the institut or by myself or taken out of literature and produced by MWG-Biotech AG (Ebersberg).

primer 5'----------3'	reference/ Acc. No	length of PCR-product [bp]
Housekeeping Genes		
ß-Actin for AACTCCATCATGAAGTGTGACG rev GATCCACATCTGCTGGAAGG	NM_001033618	233
GAPDH for GTCTTCACTACCATGGAGAAGG rev TCATGGATGACCTTGGCCAG	U85042 U94889	197
Histone for ACTGCTACAAAAGCCGCTC rev ACTTGCCTCCTGCAAAGCAC	AF469469	233
Ubiquitin for AGATCCAGGATAAGGAAGGCAT rev GCTCCACCTCCAGGGTGAT	Z18245	198
Matrixmetalloproteases		
MMP-1 for GAGGAGACGCTCATTTTGATG rev ACTGGCTGAGTGGGATTTTG	AF134714	235

MMP-2 for CCCAGACAGTGGATGATGC rev TTGTCCTTCTCCCAGGGTC	NM_174745	248
MMP-9 for GACCAGGACAAGCTCTACGG rev CAGAAGCCCCACTTCTTGTC	NM_174744	203
MMP-14 for ACTTGGAAGGGGGACACC rev AGGGGGCATCTTAGTGGG	AF144758	236
MMP-19 for TTTCAAGGGGGACTATGTGTG rev CAATAGAGAGCTGCATCCAGG	X92521	240
TIMP-1 for CATCTACACCCCTGCCATG rev CAGGGGATGGATGAGCAG	AF144763	231
TIMP-2 for GGGTCTCGCTGGACATTG rev TTGATGTTCTTCTCCGTGACC	AF144764	256
Plasminogen activator system		
tPA for GGGGAAGCACAACCACTG rev AGCTGATCAGGATCCCCC	X85800	263
uPA for TGCAGCCATCTACAGGAGG rev TGGTGAGCAAGGCTCTCC	X85801	240
uPAR for TGTTTCCAGAAACCGCTACC rev AAGTGGAAGGTGTGGTTGTTG	S70635	234
PAI-1 for CAGCGACTTACTTGGTGAAGG rev TCCAGGATGTCGTAGTAACGG	BC103451	231

PAI-2 for TACGGGCTTGGAGATGCTAG rev GGTCATTCTTTCCCGACATG	AF416234	218
Monocytes chemoattractant proteins		
MCP-1 for CTCACAGTAGCTGCCTTCAGC rev GCTTGGGGTCTGCACATAAC	L32659	205
Apoptotic- and anti-apopotic factors		
Bax for TCTGACGGCAACTTCAACTG rev AAGTAGGAGAGGAGGCCGTC	L22473 U92569	194
BCL-X_L for GGCATTCAGCGACCTGAC revCCATCCAAGTTGCGATCC	AF245487	203
Caspase3 for AACCTCCGTGGATTCAAAATC rev TTCAGGRTAATCCATTTTGTAAC	NM-214131 NM-012922	114
Caspase6 for TGTTCAAAGGAGACAAGTGTCAG rev CAGAGTAGCACATGAGGAAGTC	(Sakumoto *et al.* 2000)	206
Caspase7 for CTCTTCAAGTGCTTCRAARC rev TTCTCTARCAGGGTTTTGCATC	NM-033338 AF072124	241
Fas for AGAAGGGAAGGAGTACAYMGA rev TGCACTTGTATTCTGGGTCC	NM-000043 NM-007987	124
FasL for CATCTTTGGAGAAGCAAATAG rev GGAATACACAAAATACAGCCC	X89102	205

p53 for CATGACGGAGGTTGTGAGG rev TGGTGCACTCAGAGTCGATC	NM_174201	191
Smac for AGGAAGATGAGGTGTGGCAG rev AACTGGATGTGATTCCTGGC	AF203914	184
Survivin for AGCTCTACCTCAAGGACCACC rev CTTCTATGGGGTCGTCATCTG	AY606044	195
TNFα for TAACAAGCCGGTAGCCCACG rev GCAAGGGCTCTTGATGGCAGA	AF011926	277
TNFR1 for CACCACCACCATCTGCTT rev TCTGAACTGGGGTGCAGA	(Sakumoto *et al.* 2000)	257
TNFR2 for AGCAGCACGGACAAGAGG rev CTGTGTCCCTCGTGGAGC	AF031589	220

Since there were no sequence information about cow in this specific gene regions, the author designed multispecies primers fitting for either human, rat or mouse.

Abbreviations in the primer sequences:

r = a or g

y = c or t

m = a or c

4 Methods

4.1 Development of a method to gain uterus milk *in vivo*

Investigations during the preimplantation periode of pregnancy is a difficult undertaking in the bovine species. To gain specific samples like uterus tissue, concepti or uterus milk, pregnant cows are slaughtered at certain time points after artificial insemination. Nowadays exists a national attempt to reduce the number of animals being dispachted in animal experiments. Therefore I attempted to develop an *in vivo* method, which enables me to take uterus milk samples and embroys at specific time points after artificial insemination without slaugthering the cows. Also this would give the possibility to reduce the number of animals being used for experiments. I also wanted to investigate if it was possible able to detect progesterone, oestradiol-17β, PGF2α and VEGF in the gained uterus milk.

Experimental setup

Nine about two years old Brown Swiss heifers were taken as experimental animals (Az: 209.1/211-2531.3-13/04). They were cycle synchronised using the OvSynch method followed by a double artificial insemination. Blood plasma samples were taken from the first day of the synchronisation till day 33 of gravidity for the measurement of progesterone. Within the first 24 days of gravidity the ipsilateral and contralateral uterus horn were flushed each five times with 13ml sterile 0.9% NaCl using a disposable sterile baloon embryo transfer catheter. The catheter was placed 1cm cranial of the bifurcatio uteri in both horns. The flushing fluid of the ipsilateral horn was inspected for an embryo under the microscope. Viable embryos would have been separated from the fluid and frozen in a specific RNA-Later at –80°. After that a prostaglandin stabilisator (200μl /10ml) was given to the regained flushing fluid of both horns, which was aliquoted and frozen at –20°.

- *OvSynch synchronisation of nine Brown Swiss heifers*

  day 0: each 3.5ml Receptal i.m.
  day 7: each 2.5ml Estrumat i.m.
  day 9: each 3.5ml Receptal i.m. plus first artificial insemination
  day 10: second artificial insemination

- *Blood withdrawal from the vena jugularis via EDTA-Vacuette:*

  During the first 24 days, blood samples were taken every second day. From day 26 till day 33 blood samples were taken daily to see if the heifers were still ipsilateral. The blood was centrifugated for 15min at 2000g and 4°. The gained blood plasma was aliquoted and frozen at –20°.

- *Uterus flushing of the ipsilateral and contralateral horn via disposable balloon embryo catheter:*

  - Time points:

    day 5 of gravidity
    day 7
    day 12
    day 17
    day 24

  - Epiduralanaesthesia:

    An about 10cm x 15cm large area was shaved at the sacrococcygeal region and disinfected with ethanol 70%. 5ml Procasel 2% was injected into the epidural space between the last sacral and the first tail vertebra.

  - Ipsi- and contralateral uterus horn:

    Both ovaries were transrectally palpated. The uterus horn belonging to the CL bearing ovary was refered to as ipsilateral horn. The other uterus horn was designated as contralateral horn. Dry cleaning of the vulva area and disinfection with ethanol 70% followed.

  - Flushing:

    A sterile embryo catheter was rinsed thoroughly with 3ml of sterile 0.9% NaCl and strengthened with a steel mandrin. After spreading of the labiae vulvae the catheter was brought in to the vagina under rectal control. Passing the cervix it was first placed in the contralateral horn 1cm cranial the bifurcatio uteri. The

balloon was inflated with 10ml air to seal the uterus horn from caudal. The mandrin was removed from the catheter. 13ml of sterile 0.9% NaCl was injected into the catheter using a sterile 20ml syringe. The uterus horn was massaged gently and an aspiration of the injected fluid followed. For flushing the ipsilateral horn in the same way, the balloon was deflated, the catheter removed from the vagina, reinforeced with the mandrin and placed into the ipsilateral horn. The flushing was done as discribed above. 200µl of stabilisator per 10ml was given to the gained fluid. The samples of the contralateral horn were kept in falcon tubes on ice, the samples of the ipsilateral horn were collected in cultur plates on ice. This procedure was kept for all nine heifers. A new catheter was used for each heifer, the mandrin was disinfected with ethanol 70% before each use.

- Microscopical inspection of the ipsilateral horn flushing fluid:

  From day 7 on all gained flushing fluids from the ipsilateral horns were inspected under the microscope for embryos. A migration of the embryo during the preimplantation periode into the contralateral horn is very seldom (Rüsse and Sinowatz 1991b), so that the flushing fluids from these horns were not inspected. Located embryos were charactarised according to the developing stage and viability. Afterwards they were removed from the flushing fluid and viable ones were kept in RNA-Later and frozen at –80°. The flushing fluid was pipetted into a falcon tube and kept on ice.

- Aliquotation:

  All samples were aliquoted and frozen at –20°.

4.1.1 Enzyme immuno assay (EIA) of progesterone, oestradiol-17β and PGF2α

The frozen flushing fluid samples of both horns were defrosted at 37° in a water bath. Progesterone, oestradiol-17β and PGF2α concentration were measured using an EIA with second antibody technique (Prakash *et al.* 1987; Meyer *et al.* 1990; Meyer *et al.* 1989).

Coating of the microtiter plates:

Firstly the microtiter plates were coated with sheep anti-rabbit IgG (100µl/well), which was diluted in coating buffer (1µg IgG /100µll) followed by an overnight incubation at 1°C. After the incubation the coating buffer was tipped over and 250µl of a BSA containing assay buffer was given on the wells. BSA is supposed to block unspecific bindings sites on the surface of the wells. The plates were put on a shaker and incubated for 15-30 minutes at room temperature. After tilting the buffer the plates were washed twice in Tween-80 0.05% and beaten well.

Test procedure:

For each measurement a standard curve was established using unmarked progesterone, oestradiol-17β or PGF2α. The concentration ranged for progesterone from 0.05 to 25ng/ml, for oestradiol-17β from 0.1 to 50pg/50µl and for PGF2α from 60 to 70000pg/ml. A dilution of 1:20000 was produced with horseradish peroxidase (HRP) conjugated progesterone, 1:12000 for oestradiol-17β and 1:50000 for PGF2α (progesteron-6β-hydroxyhemisuccinat-HRP, oestradiol-6-carboxy-methyl-oxim-HRP and PGF_{2a}-Tris-HRP). Rabbit anti-progesterone, anti-oestradiol-17β and anti-PGF2α (antibody against progesterone-7α-carboxyethylthioether-BSA, 6-Keto-17β-oestradiol-6-carboxymethyloxim-BSA and PGF 2α) were diluted like following: 1:150000, 1:400000 and 1:40000.

20µl of undiluted sample or standard were pipetted in each well. Afterwards 100µl with horseradish peroxidase marked progesterone, oestradiol-17β or PGF2α and 100µl of the second antibody are given to each well. The unmarked protein of the sample and standard respectively competes against the enzyme marked protein for free binding sites on the first antibody. To estimate unspecific bindings assay buffer without anit-progesterone, anti-oestradiol-17β or anti-PGF2α was taken instead of the sample. The microtiter plates are incubated over night in the dark at 4°, slightly shaking. In the morning they were tilted and washed four times with 400µl Tween-80 0.05% for each well. For starting the colouring 11ml of substrate A and B were mixed together and 150µl per each well was given on the plates. After a incubation at 37° for 40min in the dark the colouring was stopped with 50µl of 4N H_2SO_4 per each well.

Analysis:

The extinction of the colouring was measured at 450nm by a photometer and analysed by a specific computer programm (SLT-Easy-Fit, SLT, Crailsheim). The more the samples contain of progesterone, oestradiol-17β or PGF2α the lower is the extinction. The ED_{50} for

progesterone was 6 ng/ml, for oestradiol-17β 3.5 pg/ml and for PGF2α 245pg/ml. The intra-assay variance was 4-5% for progesterone, 6-7% for oestradiol-17β and 7% for PGF2α. The inter-assay variance rest with 8-9% for progesterone, 9-10% for oestradiol-17β and 13% for PGF2α.

4.1.2 Radio immuno assay (RIA) of VEGF

VEGF protein was measured using a RIA according to the method described by Berisha et al. (2000).

4.2 Expression of housekeeping genes, proteases, apoptotic and anti-apoptotic factors in the CL during oestrous cycle and induced luteolysis

4.2.1 Transvaginal ovariectomy

The perianal regions of the cows were shaved, cleaned and desinfected. The cows were sedated each with 0.8ml Rompun i.v. An epidural anaesthesia was given with 6ml Procasel 2%. The vagina was flushed and desinfected with 1L of Vet-Sept solution. Pulling the cervix caudal with a cervix clamp a crest of the vaginal mucosa was created on the dorsal part of the vagina. An about 3cm large hole was cut into this crest with a Höhne knife. Either the ovary bearing the CL was pulled through this hole and clipped with the effeminator or it was cut off in the abdominal cavity. The stumps of the ovary vessels were compressed for further four minutes with the effeminator to minimise the bleeding risk. The hole in the vagina was closed with 2-3 stitches using an absorbable suture material and the ligature carrier by Deschamps. Several swamps were saturated with Procillin and left in the vagina for two days to prefent an infection of the wound. Furthermore a systemic antibioticum (30ml Cobactan 2.5%) and 40ml Vetalgin were given.

4.2.2 Immunohistochemistry

4.2.2.1 Fixation and Paraffin embedding

The CLs were cut in slices and labeled. One part was fixated in Bouin´s solution, the other part in methanol-glacial acetic acid according to the following time table:

- 12h in Bouin´s solution or methanol-glacial acetic acid, slightly swivelling

- Dehydration, 2h for each step:
  - ethanol 70%, 90%, 96% and 100%
  - ethanol 100%: tertiary butanol (1:1)
  - tertiary butanol at 40°

After dehydration the CL slices were kept for 12h in liquid paraffin at 60° in the warming cuboard. Afterwards the slices were embedded in paraffin. Therefore the slices were placed in specific embedding forms and covered with hot liquid paraffin. The cooling process was slowed down by touching the wax surface with a hot metal loop, so that wax block cooled down constantly. At the end an embedding slide was placed on the still hot surface and the block was given into crashed ice to cool down totally. The completed paraffin blocks are stored at 4°.

4.2.3 Molecular techniques

4.2.3.1 Enzyme immuno assay (EIA)

To be sure that all five cows were synchronised properly and that all of them had developed a mature CL, an analysis of the progesterone level was made by an enzyme-immunoassay (EIA) (Prakash *et al.* 1987). The EIA was performed as described in chapter 4.1.1. As it was necessary to have a shorter incubation time, a goat anti-rat IgG and a rat anti-progesterone was used. The incubation time for the competition of the enzyme labeled progesterone and the samples changed to 2h in the dark at 20° and for the substrate reaction to 20min at 20° in the dark.

4.2.3.2 RNA extraction

RNA with its short half-life is quickly degraded by ubiquitous ribonucleases (RNases). This enzymes are found on all surfaces, especially on hands. Thus, in order to prevent degradation, RNA has to be stored at -80°C where RNase activity is reduced to a minimum.

Handling RNA samples induces the use of one-way gloves, autoclaved materials and RNase-free DEPC-water. RNA of CL tissues was extracted with Peq Gold RNAPure. For 100mg tissue 1ml Peq Gold was given into a FastPrep tube filled with 1g inert beads. The small tissue-samples itself were extracted through cutting pieces of the big tissue under -

25°C and inserting them as fast as possible into the provided tubes. Subsequently, samples are homogenised in the FastPrep FP120 with 6.5m/s and 45sec for two times and afterwards incubated at room temperature for five minutes. After addition of 200µl chloroform the mixture was vortexed for 10 seconds and incubated yet another 10 minutes. Through the following centrifugation (15min) with 12000rpm at 4°C the upper phase containing water and RNA is seperated from the phenolphase. The upper phase is then collected and the same volume of isopropanol is added in order to precipiate RNA. The tube was inverted several times and incubated at room temperature for 10 minutes. After a 10min centirfugation (4°C, 12000rpm) the solution is discarded and the pellet washed two times with 500µl 75% ethanol. Following, the pellet is dried for 10min at 37°C to completely evaporate remaining alcohol. The dry pellet is then resuspended in 50µl RNA-H_2O and incubated at 55°C for 5min in order to ensure complete and homogenic solvation. The extracted RNA-solutions are finally stored at -80°C until further usage.

4.2.3.3 DNA digestion

Because of the lack of DNA digestion during the RNA extraction, DNA attendance is quite possible. The disposed DNA-free kit is designed to remove contaminating DNA from RNA preparations and to subsequently remove the DNase and divalent cations from samples. The protocol of this method allows a removal of trace to moderate amounts of contaminating DNA (up to 50µg DNA/ml RNA) from pirufied RNA to a level that is mathematically insignificant by RT-PCR. At first, 0.1 volume 10x DNase buffer and 1µl rDNase are added to the RNA, the sample is mixed gently and incubated at 37°C for half an hour. Afterwards, 0.1 volume DNase inactivation reagent is added and again mixed well. During adjactant incubation at room temperature for about 2 minutes, it is important to mix the contents well in order to redisperse the DNase inactivation reagent. Finally, the sample is centrifuged at 10000g for 1,5min. The supernatant, which contains the RNA, is then added into a fresh tube, the pelleted inactivation reagent is discarded for it would disturbe further enzymatic reactions.

4.2.3.4 Control of the RNA quantity and quality

4.2.3.4.1 Quantity

Quantification of the total RNA concentration is done photometric at a wavelenght of 260nm, which is the absorption maximum of nuclein acids. In order to fit the measurement range, all samples have to be diluted to absorption levels of $0.05<A_{260}<0.8$.

Simultaneously, the absorption of aromatic amino acids in proteins at a wavelenght of 280nm is measured in order to achive the quality of the samples. Ratio of A_{260}/A_{280} should be at least 1.6 (measurement against water). A third measurement at 230nm furthermore identifies possible sample contaminations with salts or phenol residues. All samples are measured three times and middled in order to retain precise values and minimise calculation errors. Additionally, the photometer is leveled to the blank value (water) before each measurement in order to ensure unique circumstances and thus allows a comparison of all samples.

4.2.3.4.2 Quality

RNA-gel

Denaturing RNA-agarose-gelelectrophoresis is used in order to determine RNA quality. The 1% agarose gel contains ethiduimbromid, 10x MOPS buffer and formaldehyde and is left to polymerise in a gel chamber that had previously been incubated for 20min with 0.1M NaOH to inactivate RNases. One volume RNA sample (containing 1µg RNA) is mixed with two volumes of 1x Formaldehyde Loading dye, denaturated for 5min at 95°C and then placed on ice immediately. Afterwards, the gel is applied into the running chamber (filled with 1x MOPS as running buffer) and loaded with the samples. Electrophoresis is conducted for 1.5 hours at 30 volts. After the run, the quality fo the RNA is evaluated under UV-light: The 28S rRNA und 18S rRNA have to be visible as defined, clearly marked bands (fig. 4). Smear of the same intensity throughout the covered distance in the gel represents degraded RNA pieces of all lengths, remaining nuclein acids in the gel bags are contaminating DNA.

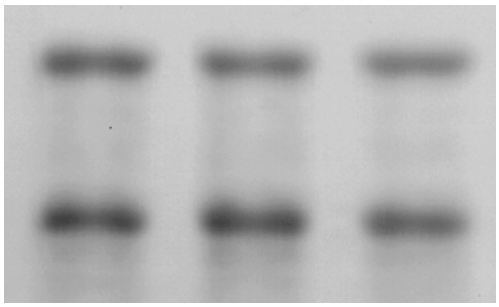


Figure 4: RNA agarose gel 1% with 1μg RNA per pocket (CL induced luteolysis samples)

Bioanalyzer

The bioanalyzer provides a framework for the standardisation of ribonucleic acid (RNA) quality control. Therefore RNA samples are electrophoretical separated on a microfabricated chip and subsequently detected with laser induced fluorescence detection. Each chip contains an interconnected set of microchannels that is used for separation of nucleic acid fragments based on their size as they are driven through it electrophoretically. An electropherogram and a gel like image are generated by the bioanalyzer software and results such as sample concentration and the so-called ribosomal ratio are displayed. The RNA 6000 ladder standard is used as a reference for data analysis. It contains six RNA fragments with following sizes: 0.2 kb, 0.5 kb, 1.0 kb, 2.0 kb, 4.0 kb and 6.0 kb. The software compares the unknown samples to the ladder fragments to determine the concentration and to identify the ribosomal RNA peaks of the unknown sample (Bioanalyzer Service). Major features of a successful ladder run are: six RNA peaks and one marker peak. The Bioanalyzer electropherogram of total RNA shows two distinct ribosomal peaks corresponding to either 18S and 28S for eucaryotic RNA and a relatively flat baseline between the 5S and 18S ribosomal peaks. Major features for a successful total RNA run are: two ribosomal peaks and one marker peak, the baseline between 27 seconds and the 18S rRNA is relatively flat as well as free of small rounded peaks corresponding to smaller molecules.

The electropherogram provides a detailed visual assessment of the quality of a RNA sample. The RNA Integrity Number (RIN) software algorithm allows for the classification of eucaryotic total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact (Mueller O *et al.* 2004). Figure 5 shows an example for two different RNA qualities. A further quality-indicator is the ratio of 28S rRNA

amount to 18S rRNA (this is provided through the area under the correlating peaks), which should be 2 at its best.

The measurement preparation starts out with the application of 9µl dye-gel into the lading-well, which is then pressed into the chip channels using the loading-station. As a pool, two more wells are filled with dye-gel furthermore. Following, 1µl RNA-sample and 5µl marker are pipetted into one sample-well respectively and 1µl ladder with 5µl marker into the ladder-well. The loaded chip afterwards is vortexed for one minute and then applied into the bioanalyzer.

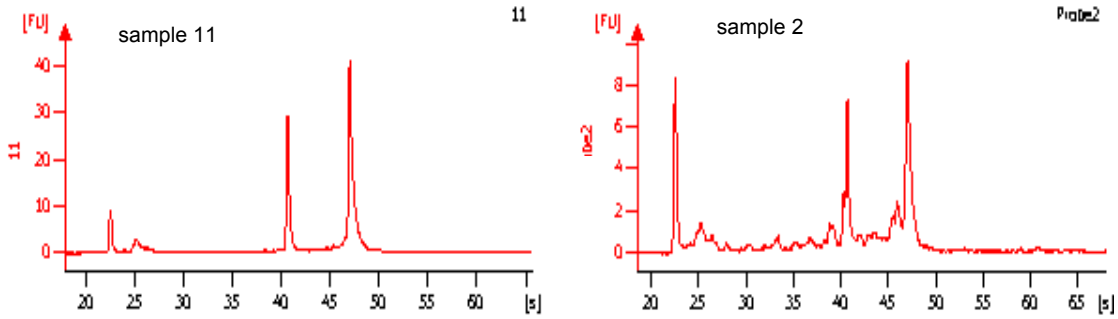


Figure 5: Two electropherograms; FU = fluorescence, S = second, first peak = marker, peak at 25s = 5S rRNA, peak at 40s = 18S rRNA, peak at 47s = 28S rRNA, RIN value of sample 11 = 10,0, RIN value of sample 2 = 7,5

4.2.3.5 Reverse transcription

To be able to use extracted mRNA for PCR based expression studies a reverse transcription (RT) reaction is performed with the total RNA of each sample. In this reaction the enzyme reverse transcriptase generates double stranded complementary DNA (cDNA) from single stranded RNA. In an optimal reaction one molecule cDNA is produced for each molecule mRNA, rRNA and tRNA. To enable the RT reaction the enzyme has to be in a correctly buffered solution and supplied with random primers and dNTPs. As preparation for the RT, 1µg total RNA are diluted in 41µl Rnase-free water then heated for 5min with 65°C and immediatly put on ice. This provides the linearisation of the RNA (i.e. no secondary structures) needed for the further transcription process. Following, 19ml mastermix (tab. 2) were added to each reaction.

	1x volume [µl] for RT	1x volume [µl] for neg. RT
5x first strand buffer	12	12
10mM dNTPs	3	3
50µM hexamere	3	3
M-MLV RT Rnase H(-) Point Mutant	1	-

Table 2: RT-mastermixes

To acquire a control for following PCR-steps, which should grant the absence of genomic DNA in the samples, one so called negative RT sample completes the procedure: A pool of all samples is generated and 1µg total RNA of this pool then diluted (the same way than all other samples). This neg. RT is added with a mastermix of 19µl (tab.2) in which the enzyme is displaced by an equal amount of RNase-free H_2O.

The RT programm starts out with the incubation of all samples 10min at 21°C in order to have an optimised primer annealing, and continues with 48min at 48°C for the RT reaction. At the end of the process, the enzyme is inactivated and RNA templates are disrupted through a heating step of 2min at 90°C. The resulting cDNA is stored at -20°C.

4.2.3.6 Polymerase chain reaction (PCR) methods

4.2.3.6.1 Block PCR

Conventional block PCR is a method used to specifically amplify target genes or gene fragments. Short sequences flanking the sequence of interest on both sides are known to be able to design gene specific primers. These should be complementary to sequences located at the 5' and 3' end of the target and about 20 base pairs in lenght. Both primers take advantage of similar melting points (determined by the GC-content), which should be around 60°C, because of the PCR-reaction being temperature controlled.

Amplification of a target is conducted by a thermo stable DNA polymerase and includes the following three steps. Successive completion of these three steps is called a cycle.

Denaturation: At the beginning of each cycle the DNA is denatured at a high temperature to separate the double stranded (ds) DNA. The first cycle of the PCR starts with a long denaturation step to ensure that both strands of the template DNA are completely

separated.

Annealing: After denaturation the reaction is cooled down in order to enable primers to anneal to the complementary sequences of the template DNA. The annealing temperature is primer specific and has to be optimized for each PCR product.

Elongation: At the optimal temperature for the polymerase (usually at around 72°C), the enzyme can bind to the single stranded DNA and synthesise a new strand that is complementary to the template strand. Thus, a dsDNA is generated from each original strand and functions throughout the next cycle as an additional template.

This semi-conservative replication method leads to an exponential amplification of the target sequence. An optimal reaction results after n cycles in 2^n dsDNA strands. Mastermix components of conventional PCR and general parameters are shown in table 3 and table 4.

substance	1x volume [µl]
PCR-H_2O	18.9
10x buffer	2.5
dNTP's	0.5
forward primer	0.5
reverse primer	0.5
taq-polymerase	0.1
template DNA	1.5

Table 3: Master mix for conventional PCR

step	temperature [°C]	time [s]	cycles
denaturation	94	120	1
denaturation	94	45	depending on primer
annealing	depending on primer	45	
elongation	72	45	
elongation	72	120	1
hold	5	not limited	

Table 4: PCR-parameters

4.2.3.6.2 Gradient PCR:

Gradient PCR includes the same steps as conventional block PCR, but is conducted in special cyclers that allow different temperature profiles to be programmed for each cavity in the cycler. This method can be used to optimize PCR conditions with respect to the primer annealing temperature. A PCR is done with several replicates of the same sample. All samples are treated equally, only the annealing temperature is changed. In an agarose gel (see below) the PCR efficiency can then be analysed investigating intensity and integrity of the product bands.

4.2.3.6.3 Quantitativ real time PCR (qPCR)

New PCR-platforms, such as Rotor-Gene or Light-Cycler, and modern dyeing and detection systems allow visualisation of PCR reaction in real time and quantification of the DNA amount originally in a sample tube. The fluorescence dye used with the Rotor-Gene is SYBR-GREEN I, it intercalates with the minor groove of dsDNA and emits light at 530nm when excited at 497nm. Due to DNA amplification by polymerase, the amount of dsDNA doubles with each cycle, resulting in more and more intense signals (measured at the end of the elongation step). This event can be monitored for each sample with analysing software even while the PCR is still in progress. This advantage gives the possibility to continue PCR reaction over the exponential phase (in contrast to conventional PCR). Figure 6 displays a characteristic amplification curve calculated by the Rotor-Gene software V5.0, which can be divided into four phases. Phase 1 is described by an exponential amplification and the fluorescence signal below background correction, phase 2 is the exponential amplification phase, phase 3 the linear amplification phase

where the PCR loses efficiency and phase 4 the plateau phase which is limited by inhibition or lack of chemicals (Pfaffl M.W. 2005).

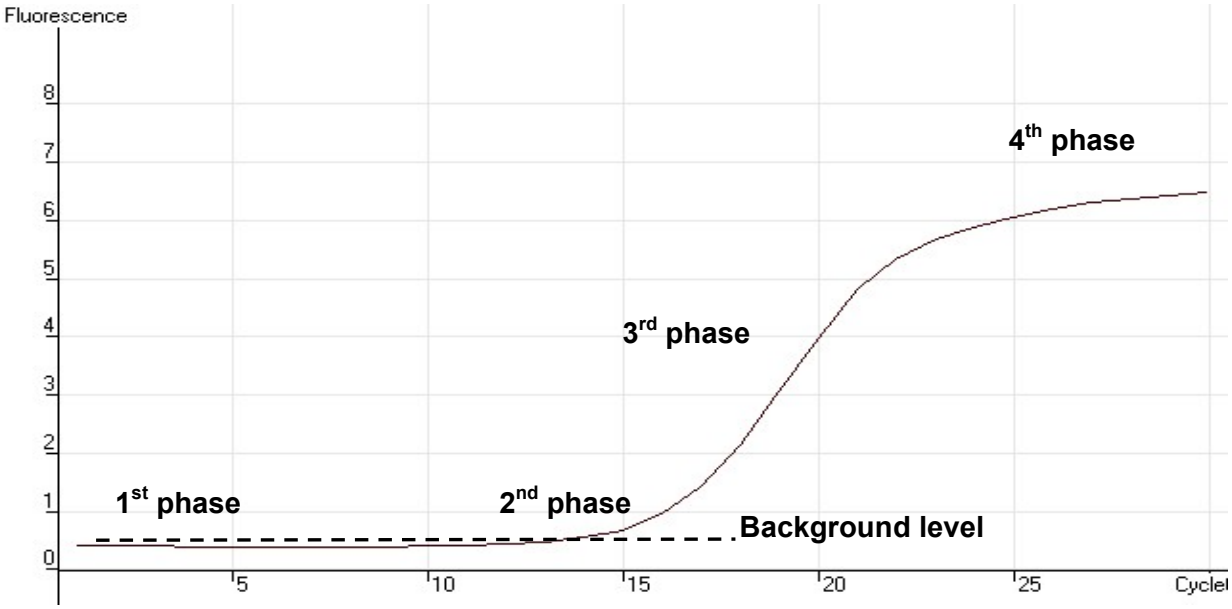


Figure 6: Fluorescence data of a real time RT-PCR

The Rotor-Gene offers as another feature, the determination of the melting point. After amplification the samples are heated from 60°C to 99°C to separate the two DNA strand of the PCR-product, which results in a loss of fluorescence. During this heating process the fluorescence signal is measured continuously to generate a melt curve. This results in a curve that shows a sudden drop in signal intensity at the melting temperature for each amplicon, which can also be displayed as a peak (fig. 7). This melting point is depending on the guanin- and cytosin bases in the PCR-product (GC content) and on the product length. After an initial product size verification in an agarose gel, this graph can be used to check if the right product was amplified or if unspecific product information occurred.

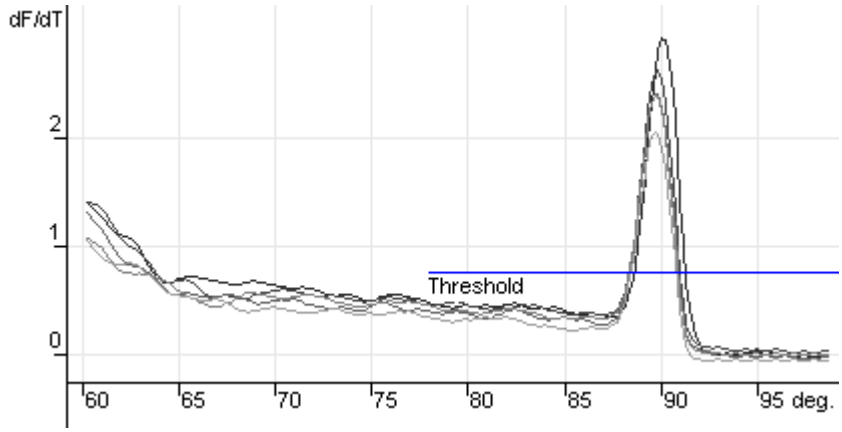


Figure 7: Melt curve analysis calculated by the Rotor-Gene Analysis Software V5.0

Table 5 shows the parameters used for qPCR experiments. Annealing temperatures are primer specific. The optimal annealing temperatures have been determinded preliminary using the gradient PCR and were further optimised for the qPCR (fig. 7).

	temperature [°C]	time [s]	cycles
denaturation	95	600	1
denaturation	95	10	40-45, product specific
annealing	primer specific	10	
elongation	72	15	
melt	60-99	0.5°/sec	1
hold	40	not limited	1

Table 5: qPCR parameters

Duration of the single steps is decreased comparing to conventional PCR. This is effected through the minimized reaction volume of 10µl and the effective heating and cooling system which allows quick acquiring of target temperatures. Since the method being extremely sensitive, 9µl master mix are added to only 1µl template cDNA. Contents of qPCR master-mix are listed in table 6.

	1x volume [µl]
cDNA	1
PCR-H_2O	6.4
$MgCl_2$ 3mM	1.2
forward primer	0.2
reverse primer	0.2
LCM (including SYBR-GREEN)	1

Table 6: qPCR master mix

Primer	annealing temperature [°C]	melting temperature [°C]
β-Actin	63	91.3-91.5
Histone	60	88.7-89.4
GAPDH	60	89.9-90.3
Ubiquitin	60	90.2-91.6
MMP-1	60	87.8-88.5
MMP-2	60	90.4-90.8
MMP-9	60	93.6-94.0
MMP-14	60	91.3-91.4
MMP-19	60	88.8-89.0
TIMP-1	60	89.9-90.2
TIMP-2	60	90.1-90.3
tPA	60	93.5-93.7
uPA	60	89.5-89.9
uPAR	60	90.4-90.8
PAI-1	60	90.3-90.5
PAI-2	60	86.0-86.1
MCP-1	60	88.4-88.9
Fas	60	83.4-84.0
FasL	60	86.5-87.0
TNFα	66	92.2-92.6
TNFR1	68	95.59-95.8
TNFR2	60	94.5-94.7
p53	62	89.8-90.1

Bax	62	91.8-92.0
BCL-X_L	60	89.4-90.0
Smac/Diablo	60	88.5-88.7
Survivin	60	92.5-92.8
Caspase3	60	80.4-80.9
Caspase6	60	91.3-91.8
Caspase7	60	85.4-86.1

Table 7: Product-specific annealing and melting temperatures of the examined factors for qPCR

The tubes for Rotor-Gene qPCR are closed after Mastermix and DNA sample have been added and put into the rotor-chamber, the program given above is started. After the run has ended, crossing point (CP) values were acquired by using the "comparative quantitation" method of the Rotor-Gene Analysis Software V5.0. The CP is a value for the target cDNA/mRNA content of the sample. Since the increase in fluorescence is due to increase in DNA, a sample initially containing more DNA subsequently reaches fluorescence above background earlier (and thus has a lower CP value) than a sample containing less DNA. Theoretically the amplification rate for the PCR product in each cycle is 2. But, with the reaction conditions being dependend on the investigated tissue and primers, the amplification is seldom optimal. Therefore the efficiency of each run is acquired using a standard curve. It is calculated via the inclination of the standard curve according to the following formula (Rasmussen R 2001):

$$E = 10^{[-1/\text{Steigung}]}$$

For the standard curve the cDNA was diluted to the following concentration: 33, 6, 1, 0.2 and 0.05ng/μl. A standard curve was established for each primerpair. An example is given in figure 8 and 9.

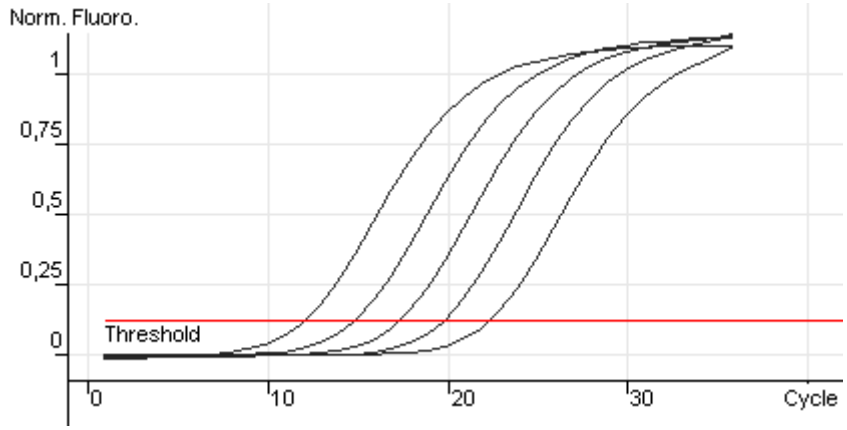


Figure 8: An example of a sigmoid amplification curve using a dilution series of TIMP-1; dilution concentrations from left to right: 33, 6, 1, 0.2 and 0.05 ng/µl cDNA; the CP value of each amplification curve is caluculated where the threshold crosses the curves.

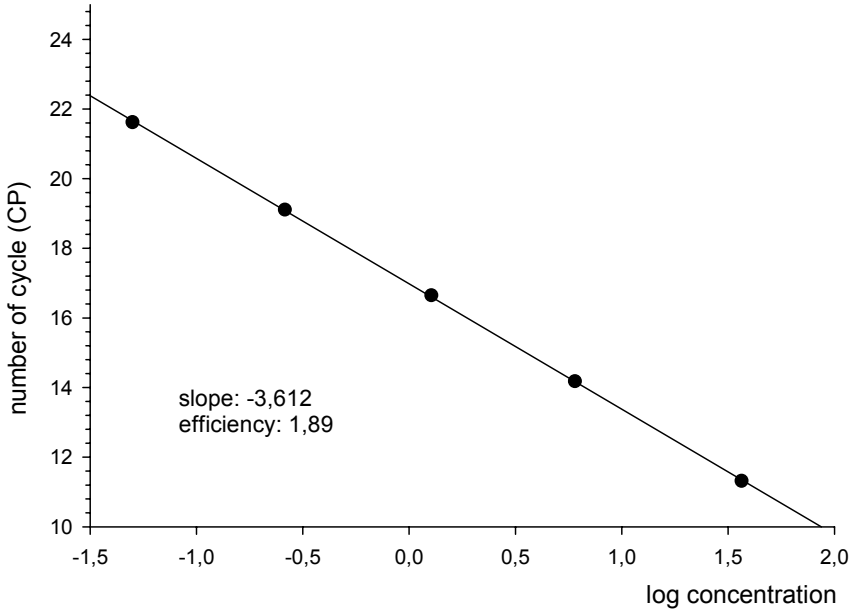


Figure 9: Determination of the slope of a dilution series for calculation of the PCR-efficiency of TIMP-1.

4.2.3.7 Primer design and optimation

New primers were designed using the HUSAR software (DKFZ, Heidelberg). For the optimal primer pair design the following parameters were considered: length of the PCR-product about 180-250 bp, primer length 18-24 bp, GC content 40-55%, similar melting temperature of the forward and reverse primer, no annealing between both primers, no

primer self-annealing. For the design of multi species primer a homolog region of the coding sequenze (CDS) of different species was taken. The primers were first optimised by block PCR using a gradient cycler. Afterwards the primers were tested by real time PCR and adjusted further if necessary.

4.2.3.8 Analysis of the PCR-product

For established primer pairs the PCR-product was identified by the charactaristic melting point and by the specific product length in an agarose gel. The melting curve should show only one melting point and the agarose gel only one band. For new primers the PCR-product was furthermore extracted by a DNA extraction kit using a filter columm and several washing steps (Manual of the Wizard SV Gel and PCR Clean-Up System, Promega (Mannheim)) and sent for sequencing (Medigenomix Gmbh (Martinsried)). The received cDNA sequence was compared with the bovine sequence in the EMBL database using BLAST software (www.ncbi.nlm.nih.gov/BLAST/). If the primers were not made of a bovine sequence the gained sequence was compared with the sequence the primers were made of. The resulting new bovine sequence was published in the EMBL database.

4.2.4 Data processing

To make sure that different CP values are only caused by different amounts of total RNA and that not other factors are able to distinguish samples from each other, four HKGs were used as a reference. HKGs are genes supposed to be not regulated in tissue, this might be genes of nuclein acid synthesis like GAPDH as an example of glycolysis or structure proteins like Histone. In this experiment Ubiquitin, Histone, GAPDH and β-Actin were chosen as reference HKGs. An index was calculated using the CPs of all four housekeeping genes using the Bestkeeper software (Pfaffl *et al.* 2004). With these index a normalisation of the retained qPCR CP-values was done by the ΔΔCP methode (Livak and Schmittgen 2001) using the following formula:

$$\Delta\Delta CP = (CP_t - CP_i)_{Control} - (CP_t - CP_i)_{Time\ x}$$

(t: target gene; i: bestkeeper index)

The CI luteolysis group 0.5h after PGF2α injection was only investigated when the group 2h showed a significant difference to the control. The statistical analysis of the data was done with the software “SIGMA STAT 3.0” after normalisation. For calculation of significances, the “one-way ANOVA” was disposed. If data reveal normal distribution, significances have been acquired through multiple comparison with the “Holm-Sidak”-test method. Differences are estimated significant, if the significance level P is ≤ 0.05. All datas are shown as mean values ± standard error of means (SEM). The expression x-fold is equal to $2^{\Delta\Delta CP}$.

5 Results

5.1 Development of a method to gain uterus milk *in vivo*

5.1.1 Flushing of the uterus horns

The amount of regained flushing fluid ranged between 3ml and 13ml. As can be seen in the follwing table 8, the flushing was sometimes not possible in two heifers, because the cervix was to narrow. On day 7 of gravidity we were able to gain one embryo, which was already dead since three days. Several flushing fluids were contaminated with sanies at different time points. All heifers having an endometritis, except one, which showed signs of oestrus and slight inflammation. Heifers were treated with 40ml Genta-Sleecol 5 i.v. From day 17 to day 24 all nine heifers were in oestrus.

Heifer	day 5	day 7	day 12	day 17	day24
1	NAD	NAD	NAD	S, G	NAD
2	NAD	embryo ✞	NAD	S, O	NAD
3	NAD	NAD	NAD	S, G	NAD
4	NAD	NAD	NAD	S, G	NAD
5	NAD	NAD	NAD	O	NAD
6	NAD	missing	S	missing, G	NAD
7	NAD	missing	S	S, G	NAD
8	NAD	NAD	NAD	S	S, G
9	NAD	NAD	S	S, G	NAD

Table 8: Schedule of flushings and special features; NAD= no abnormality detected; missing= flushing was not possible; S= sanies; G= Genta-Sleecol 5; O= oestrous

5.1.2 Progesterone analysis

5.1.2.1 Blood plasma:

The progesterone levels in the blood plasma were not measured, because all nine heifers were in oestrus between day 17 and day 24 after artificial insemination. Non of the heifers were gravid after day 21.

5.1.2.2 Uterus milk

It was possible to detect progesterone in the uterus milk of the contralateral (A) and ipsilateral (B) horn. The progesterone value ranged from <0.1 to 0.4 ng/ml in both horns.

5.1.3 Oestradiol-17β analysis in the uterus milk

We were able to detected oestradiol-17ß values between 2pg/ml and 333pg/ml. The values between the different animals and between the ipsi- and contralateral horn showed great differences, so that no correlation could be seen. A further test revealed that 0.9% NaCl with EDTA-stabilisator contains 138 pg/ml oestradiol-17β, whereas 0.9% NaCl without stabilisator only contains <2 pg/ml.

5.1.4 VEGF analysis in the uterus milk

The detected VEGF values ranged between 0.06ng/ml and 2.58ng/ml, whereas the values of both horns were partly similar during the different time points.

5.1.5 PGF2α analysis in the uterus milk

It was possible to detect PGF2α values between 0.23ng/ml and >70.00ng/ml. A level increase can be seen at the beginning and during endometritis, which is reduced after medical treatment and oestrus (tab. 9).

Cow	PGF2α (ng/ml)				
	day 5	day 7	day 12	day 17	day 24
1A	6.81	2.52	30.00	73.00 *	16.20 **
1B	3.75	2.70	28.00	54.00 *	12.90 **
2A	6.70	1.30	67.70	3.40 * °	1.18
2B	19.60	3.50 ~	21.30	4.40 * °	0.23
3A	2.60	19.00	29.50	25.60 *	6.64 **
3B	5.00	22.00	28.90	21.70 *	6.86 **
4A	5.10	5.94	26.70	20.50	0.78 **
4B	6.80	6.88	38.60	59.70	0.82 **
5A	14.40	21.00	42.70	14.20 °	0.69
5B	10.80	34.50	30.60	32.70 °	0.93
6A	7.60	missing	15.40 *	missing	9.60 **
6B	16.20	missing	>70.00 *	missing	9.50 **
7A	18.00	missing	44.60 *	2.97 *	3.27 **
7B	23.30	missing	36.60 *	15.60 *	9.70 **
8A	11.50	10.80	26.40	20.60 *	51.00 *
8B	13.30	24.50	19.60	12.60 *	25.00 *
9A	11.80	18.50	32.00 *	10.50 *	4.60 **
9B	7.60	26.70	38.00 *	>70.00 *	4.80 **

Table 9: PGF2α concentration in the uterus milk of the contralateral (A) and ipsilateral (B) uterus horn. *= sanies; **= after gentamicin treatment; °= in oestrus; ~ = embryo rinsed

5.1.6 Recapitulating comment to 5.1.

We were able to gain uterus milk in a sufficient concentration and amount to dectect progesterone, VEGF and PGF2α within the sensitivity of EIA and RIA. Oestradiol-17β levels were also detected via EIA, but these seem to be erratic due to the used EDTA-stabilisator. The ballooned embryo catheter turned out to be a good instrument for gaining uterus milk in a not too invasive kind of way.

5.2 Expression of housekeeping genes, proteases, apoptotic and anti-apoptotic factors in the CL during oestrous cycle and induced luteolysis

5.2.1 Enzyme immuno assay (EIA) of progesterone

The progesterone level was analysed in blood plasma of five Brown swiss cows to see if their oestrous cycle was correctly synchronised. The progesterone level of all five cows was below 0.2ng/ml three days after the second PGF2α injection. 13 days after these injection the level of all five cows ranged between 2.79ng/ml to 4.08ng/ml indicating a functional CL.

5.2.2 RNA quality control by the Bioanalyzer

The RNA qualitiy of the CL samples during oestrous cylce on day 1-12 and the CL samples during induced luteolysis 0h, 0.5h, 2h, 4h, 12h, 24h, 48h and 64h were verified. The CI oestrous cycle and CL luteolysis samples showed a good to very good RNA integrity and qualtity with RIN values between 10.0 to 7.5 and 9.4 to 6. respectively (tab. 10).

Group CL cycle	RIN value	SEM	Group CL luteolysis	RIN value	SEM
1-2	8.88	0.52	**0h**	8.46	0.47
3-4	9.53	0.23	**0.5h**	7.74	0.40
5-7	9.77	0.12	**2h**	8.38	0.13
8-12	9.65	0.17	**4h**	8.72	0.15
			12h	8.42	0.24
			24h	7.98	0.06
			48h	8.58	0.12
			64h	8.38	0.21

Table 10: RIN values of the CL oetrous cycle samples day 1-12 and CL induced luteolysis samples 0h, 0.5h, 2h, 4h, 12h, 24h, 48h and 64h

5.2.3 Sequencing of PCR-products

No bovine sequences were available for MMP-19 and Smac, so that the primers were designed using human or mouse sequences, respectively. The bovine MMP-19 sequence showed 93% homology to the human sequence. The bovine Smac sequence was 87%

homologous to the mouse sequence and was published in the EMBL database under the no. AJ867847.

5.2.4 Quantitative real time PCR (qPCR)

5.2.4.1 Housekeeping genes

5.2.4.1.1 Follicle phase and CL during oestrous cycle

The housekeeping genes Histone, β-Actin, GAPDH and Ubiquitin were investigated in the follicle 20h after GnRH for comparable reasons and during oestrous cycle in the CL. The relative quantitation is shown in reference to the follicle group 20h after GnRH application and as logarithm to the basis 2. The mRNA expression datas of Histone showed no significant differences between the groups. β-Actin was significantly up-regulated at days 1-7. GAPDH reveals nearly the same expression pattern like β-Actin, except that day 1-2 is not only significantly increased to the follicle group and day 8-12 but also to day 5-7. Ubiquitin was not regulated during follicle phase and CL oestrous cycle (fig. 10).

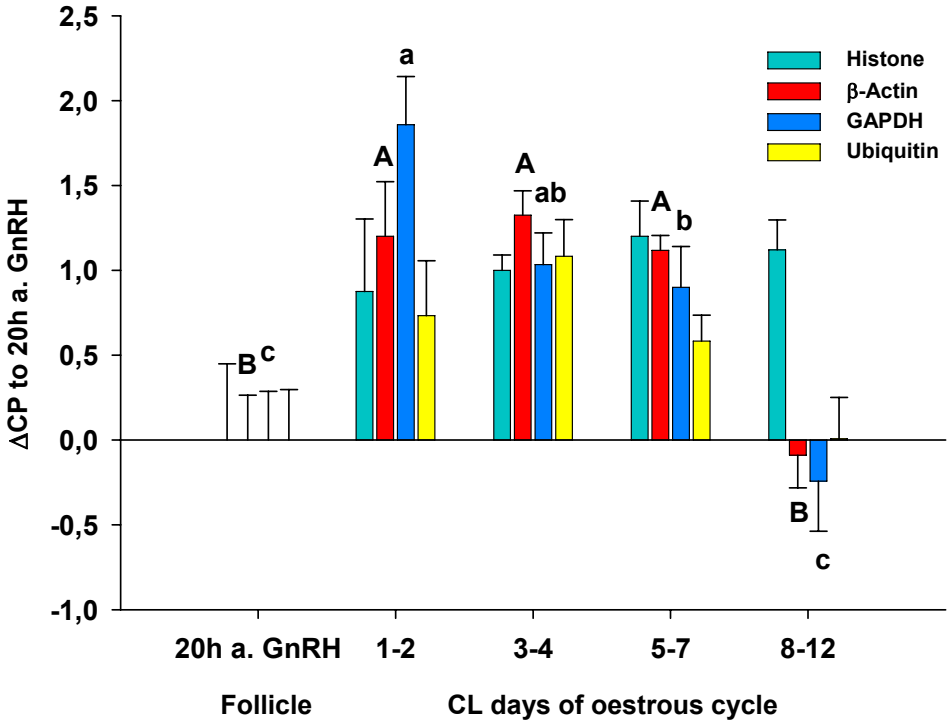


Figure 10: mRNA expression data of four housekeeping genes Histone, β-Actin, GAPDH and Ubiquitin in follicle (20h after GnRH) and in the CL during different days of the oestrous cycle in the CL; data are shown as ΔCP ± SEM to the follicle group. Different superscript letters indicate significant differences ($P < 0.05$).

5.2.4.1.2 CL during induced luteolysis

The housekeeping genes Histone, β-Actin, GAPDH and Ubiquitin were also investigated during luteolysis in the CL. The relative quantitation is shown in reference to the control group (day 8-12 = 0h) and as logarithm to the basis 2. The mRNA expression data of Histone showed slight, but significant differences from 12h after PGF2α till 64h. The β-Actin expression increased significantly to control after 0.5h and reached the control level again after 12h. At 24h a siginificant up-regulation to all groups was seen, which decreased again to the level seen at 2h. GAPDH was not significantly regulated during induced luteolysis. Ubiquitin showed a significant down-regulation at 48h and 64h as compared to all other groups (fig. 11).

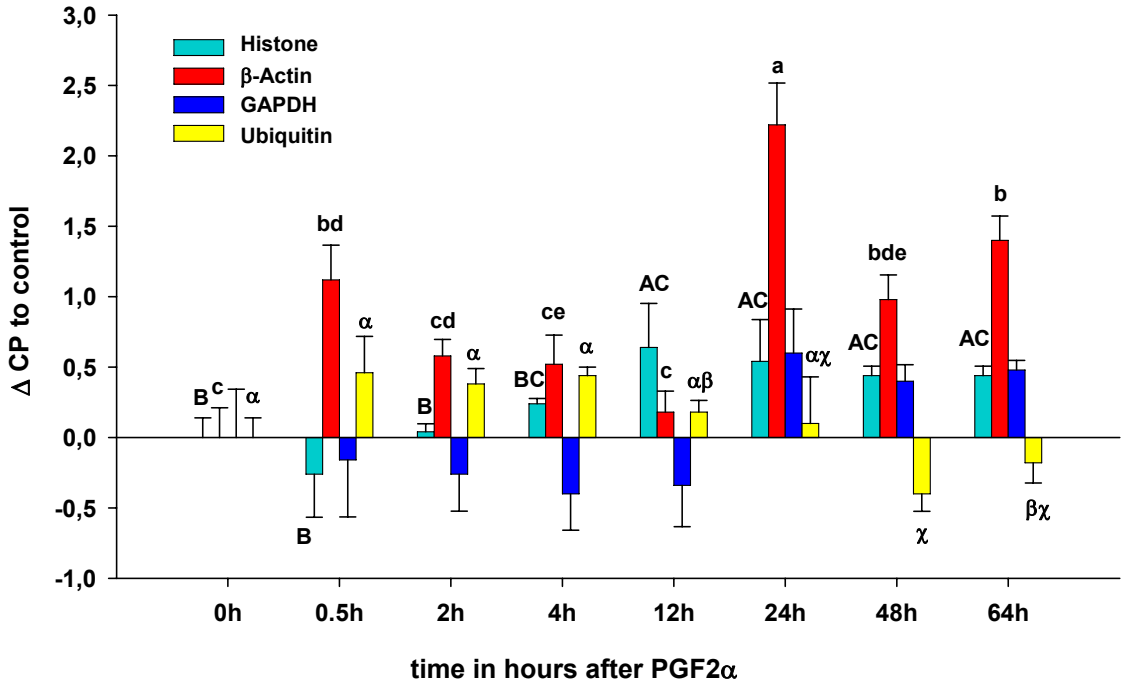


Figure 11: mRNA expression data of four housekeeping genes Histone, β-Actin, GAPDH and Ubiquitin in the CL during specific hours after PGF2α injection; data are shown ΔCP ± SEM to 0h (control). Different superscript letters indicate significant differences ($P < 0.05$).

5.2.4.2 Extracellular matrix proteases

5.2.4.2.1 MMPs and TIMPs during follicle phase and CL during oestrous cycle

The mRNA expression of MMP-1 (fig. 12), MMP-2 and MMP-14 showed no significant regulation during follicle phase and CL oestrous cycle. The mRNA expression of TIMP-2 was constantly increased from 20h after GnRH till day 8-12 of the oestrous cycle in the CL. The follicle phase showed the lowest expression and was significant to the groups day 3-4, day 5-7 and day 8-12 of the oestrous cycle, while all other time points of the oestrous cycle were also significantly lower to day 8-12 (fig. 13). MMP-9 was significantly up-regulated from day 1-12 compared to 20h after. Day 8-12 was also significant higher than all other groups. MMP-19 also revealed significant increase from day 1-12 compared to 20h after GnRH with the highest expression from day 3-12. TIMP-1 showed a significant up-regulation from day 1-12 compared to the follicle group. (fig. 14).

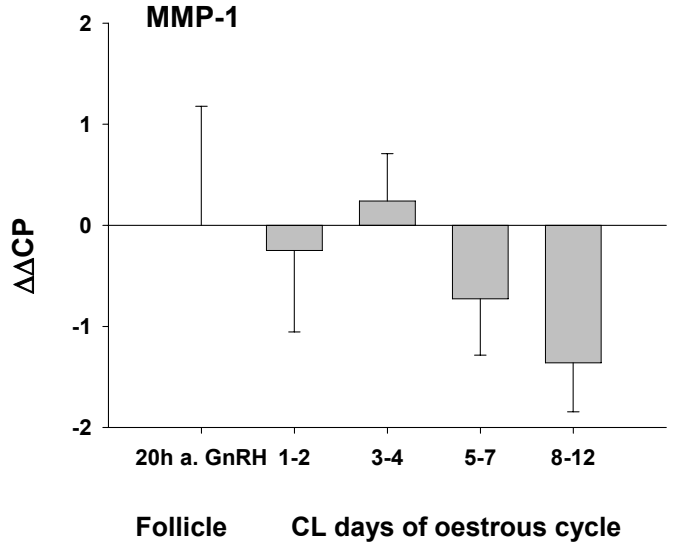


Figure 12: mRNA expression of MMP-1 in follicle group and CI during oestrous cycle; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to the follicle group, where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP = \Delta CP_{(goup\ I=follicle)}$ - $\Delta CP_{(goup\ II-V)}$. The statistic analysis were determined from ΔCP values (n=4-5). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$). CP value of the follicle group was 28.09.

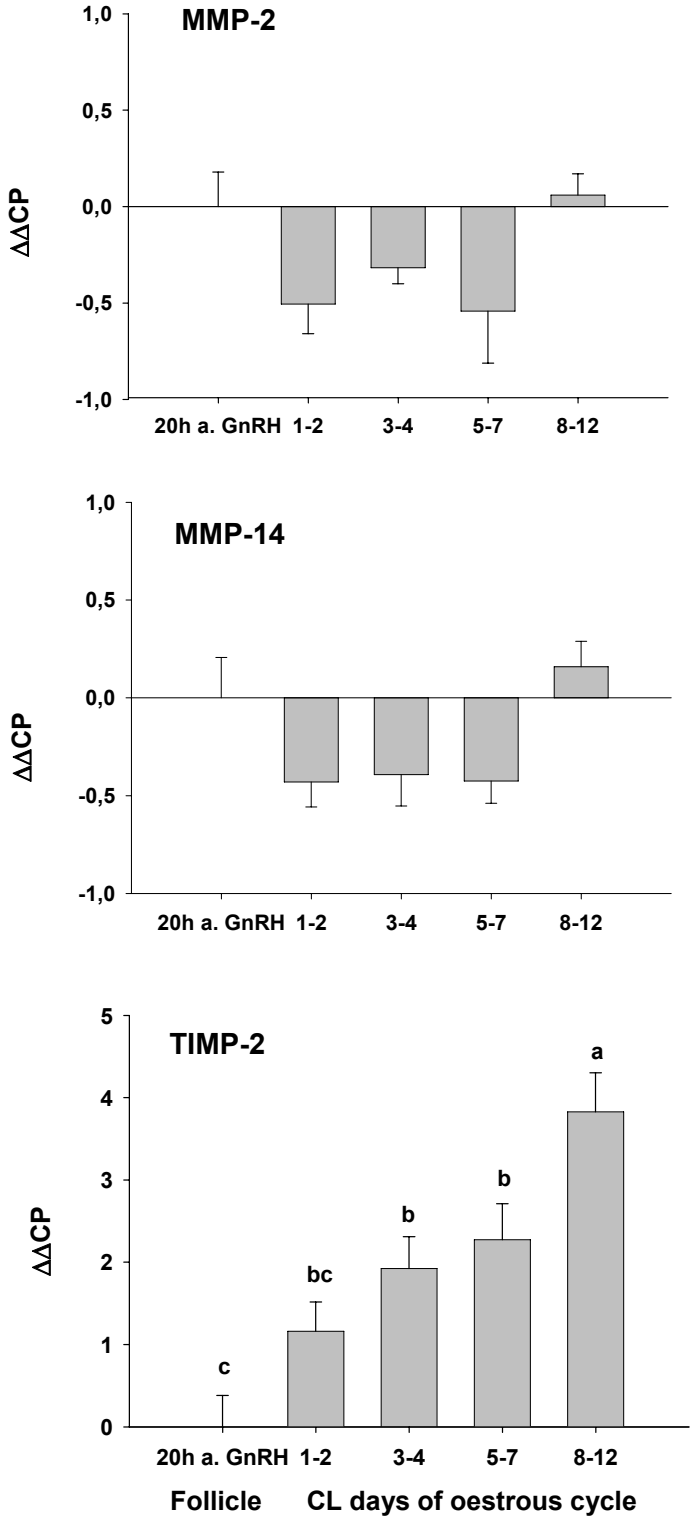


Figure 13: mRNA expression of MMP-2, MMP-14 and TIMP-2 in follicle group and Cl during oestrous cycle; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to the follicle group, where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP = \Delta CP_{(goup\ I=follicle)} - \Delta CP_{(goup\ II-V)}$. The statistic analysis were determined from ΔCP values (n=4-5). Results are presented as expression changes $\Delta\Delta CP \pm SEM$. Different superscript letters indicate significant differences ($P < 0.05$). CP values of the follicle group were 19.80, 23.45, 17.91.

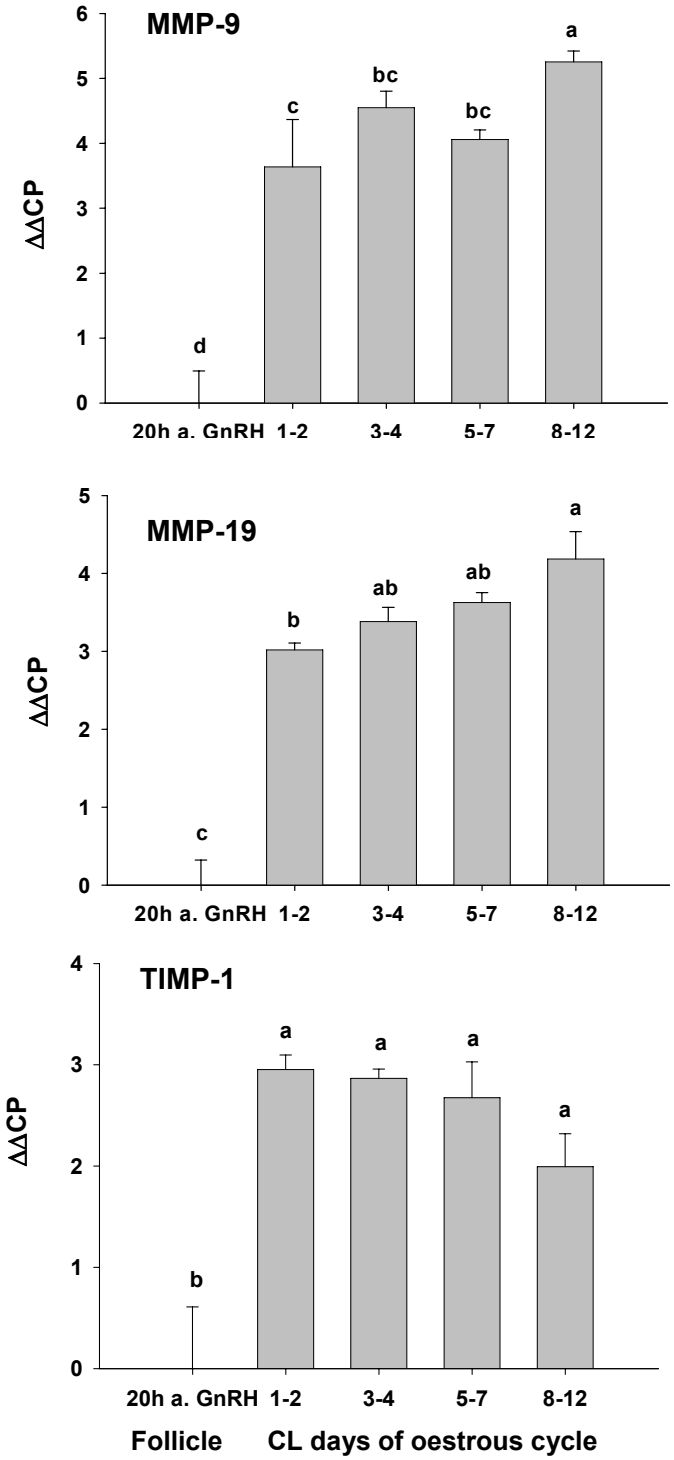


Figure 14: mRNA expression of MMP-9, MMP-19 and TIMP-1 in follicle group and CI during oestrous cycle; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to the follicle group, where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP = \Delta CP_{(goup\ I=follicle)} - \Delta CP_{(goup\ II\text{-}V)}$. The statistic analysis were determined from ΔCP values (n=4-5). Results are presented as expression changes $\Delta\Delta CP \pm SEM$. Different superscript letters indicate significant differences ($P < 0.05$). CP values of the follicle group were 30.20, 26.00, 16.88.

5.2.4.2.2 PA system during follicle phase and CL during oestrous cycle

tPA showed no significant regulation during follicle phase and CL oestrous cycle (fig. 15). The mRNA expression of uPA was constantly increased from the follicle phase to day 8-12 of the oestrous cycle. The follicle phase showed the lowest expression with significant differences to all days of the oestrous cycle. Day 1-2 was also significantly lower expressed than day 8-12. uPAR expression showed nearly the same expression pattern like uPA, but day 8-12 was significant lower expressed than day 5-7 (fig. 16). The mRNA expression of PAI-1 was significantly increased during the first seven days of the oestrous cycle compared to the follicle phase and day 8-12 of the oestrous cycle. PAI-2 revealed the same expression pattern as PAI-1, but the increase from day 1-2 to day 5-7 was much higher than for PAI-1 (fig. 17).

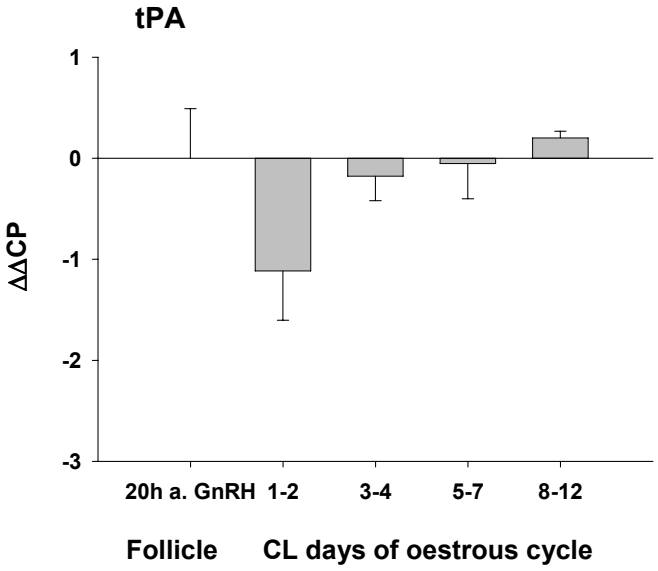


Figure 15: mRNA expression of tPA in follicle group and Cl during oestrous cycle; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to the follicle group, where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP = \Delta CP_{(goup\ I=follicle)}$ - $\Delta CP_{(goup\ II-V)}$. The statistic analysis were determined from ΔCP values (n=4-5). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$). CP value of the follicle group was 22.68.

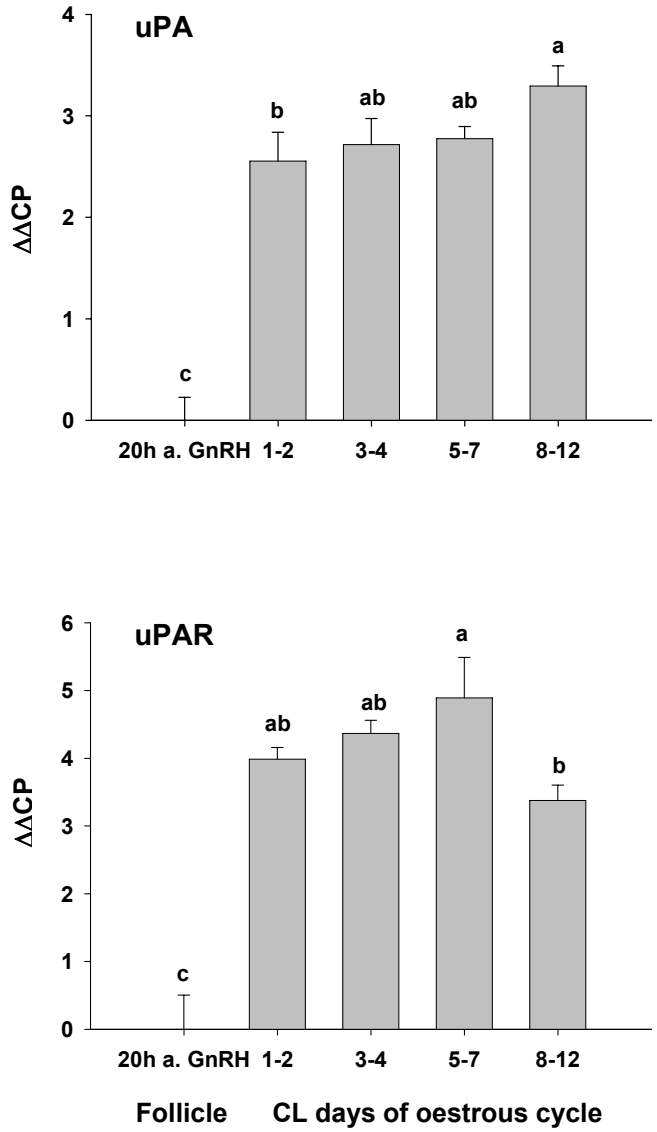


Figure 16: mRNA expression of uPA and uPAR in follicle group and CI during oestrous cycle; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to the follicle group, where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP$ = $\Delta CP_{(goup\ I=follicle)}$ - $\Delta CP_{(goup\ II\text{-}V)}$. The statistic analysis were determined from ΔCP values (n=4-5). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$). CP values of the follicle group were 28.18 and 27.37.

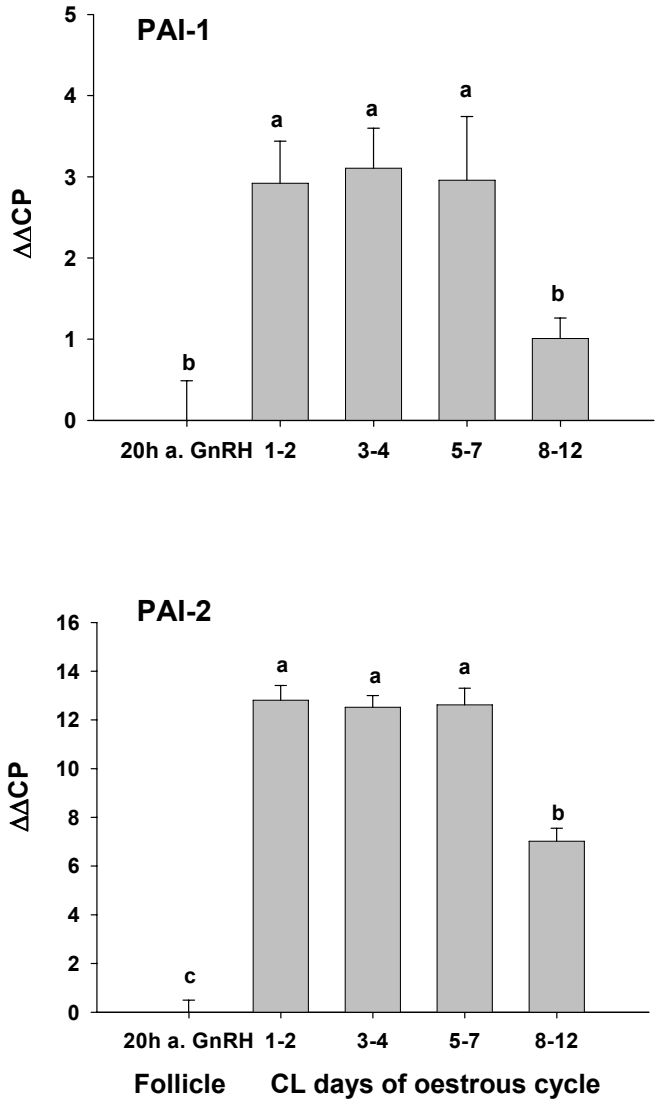


Figure 17: mRNA expression of PAI-1 and PAI-2 in follicle group and CI during oestrous cycle; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to the follicle group, where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP$ = $\Delta CP_{(goup\ I=follicle)}$ - $\Delta CP_{(goup\ II-V)}$. The statistic analysis were determined from ΔCP values (n=4-5). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$). CP values of the follicle group were 23.90 and 29.17.

5.2.4.2.3 MMPs and TIMPs during induced luteolysis

The mRNA expression during luteolysis of MMP-1 showed a highly significant 256- to 1024-fold up-regulation beginning 2h after induction of luteolysis till 64h with the highest increase at 48h. (fig 18). MMP-2 showed a significant increase 48h to 64h after PGF2α. A significantly higher expression level to control group was seen for MMP-14 from 12h on to 64h, with the highest expression rate between 24h to 64h. TIMP-2 showed the opposite expression pattern than MMP-14 with a significant down-regulation of maximal 8-fold at 48h to 64h (fig. 19). MMP-9 was steadily significantly up-regulated from 12h till 64h with the highest expression at 48h. The expression of TIMP-1 was significantly increased from 4h to 24h with a peak at 12h. MMP-19 showed an increased expression from 24h to 64h with a maximal up-regulation of 32-fold at 48h (fig. 20).

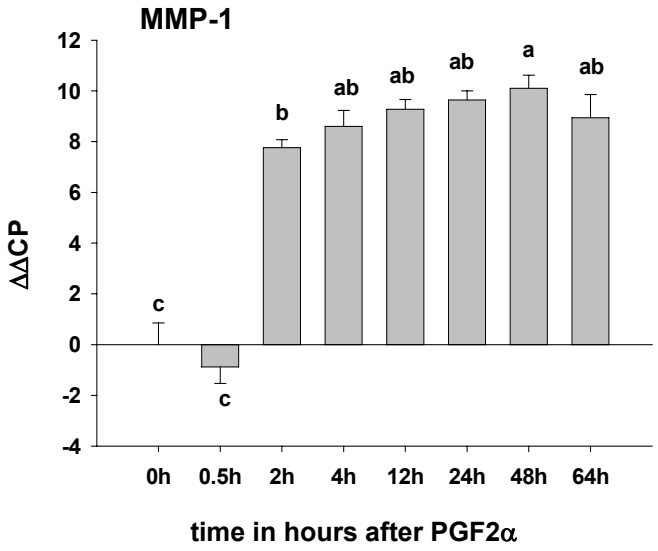


Figure 18: mRNA expression of MMP-1 in the CI during induced luteolysis; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to control (0h), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ I=control\ (0h))}$ - $\Delta CP_{(goup\ II\text{-}VIII)}$. The statistic analysis were determined from ΔCP values (n=5). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

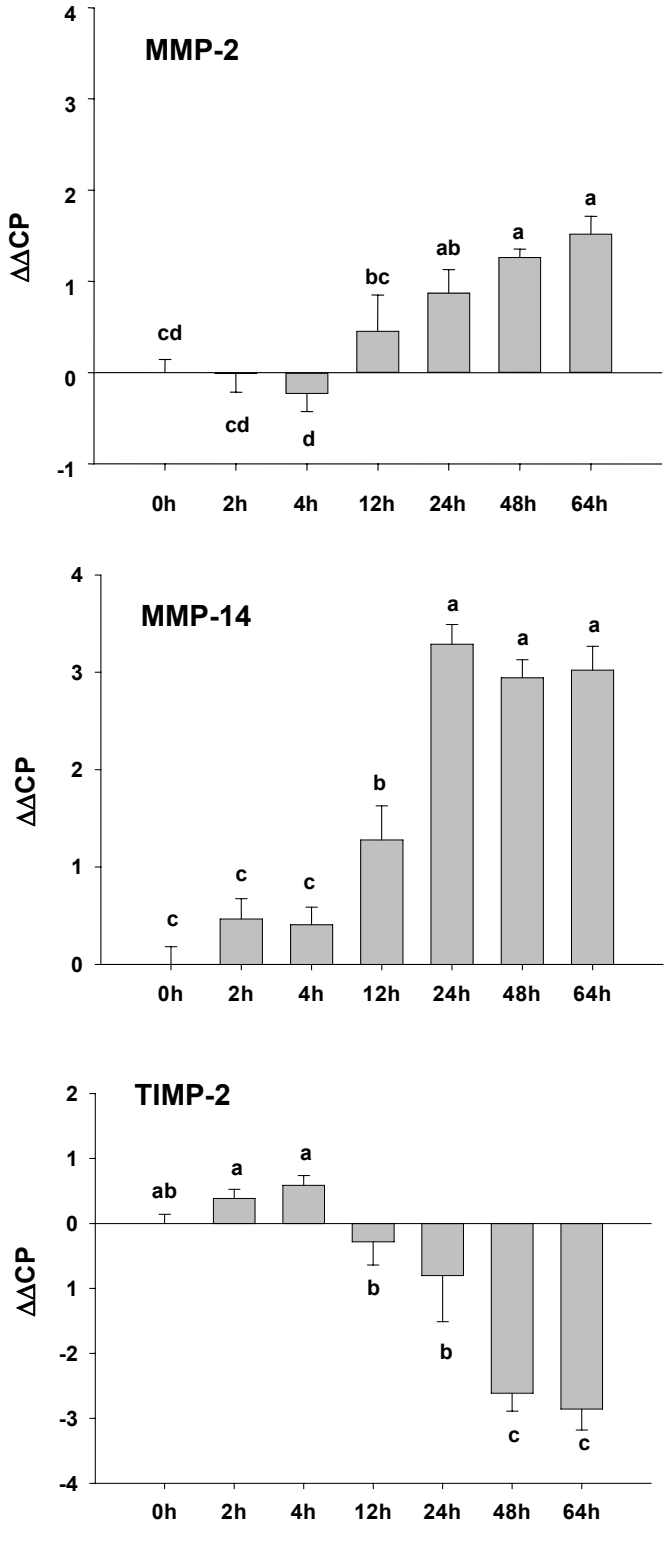


Figure 19: mRNA expression of MMP-2, MMP-14 and TIMP-2 in the CI during induced luteolysis; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to control (0h), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ I=control\ (0h))}$ - $\Delta CP_{(goup\ II\text{-}VII)}$. The statistic analysis were determined from ΔCP values (n=5). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

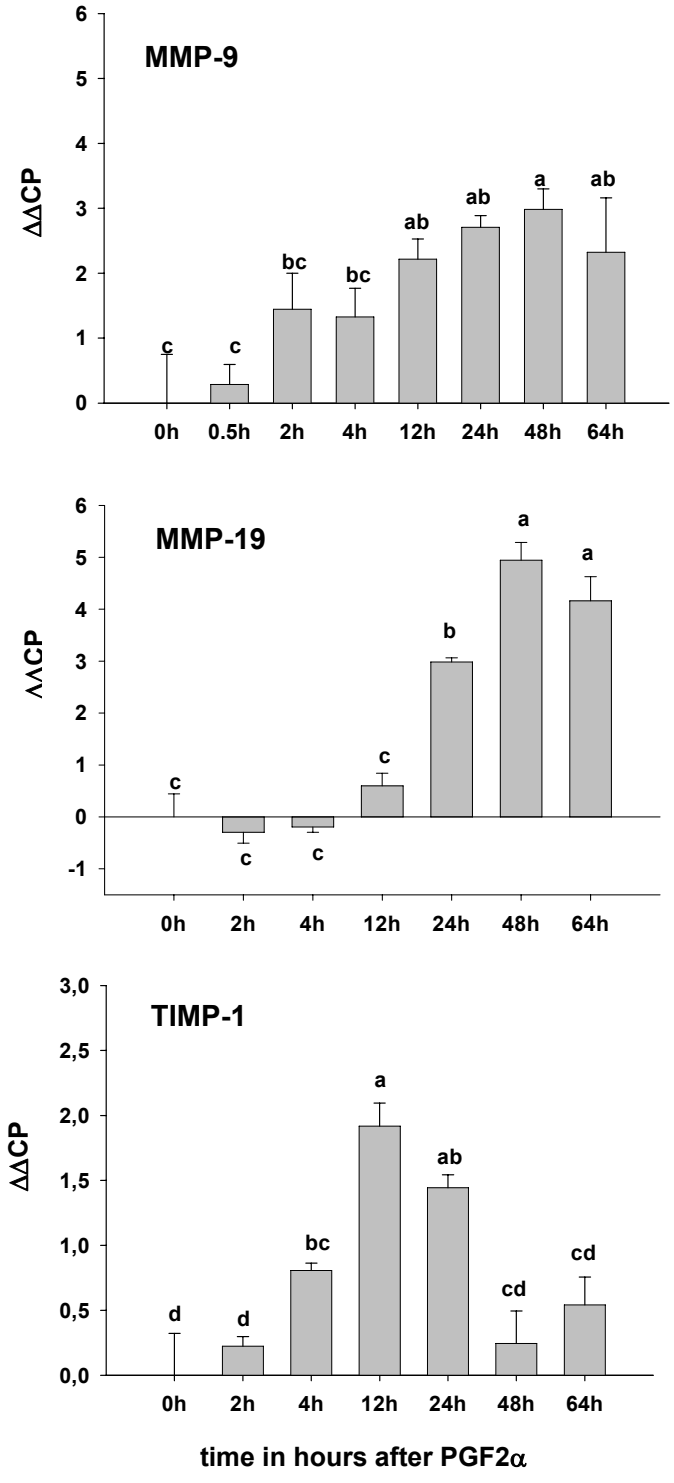


Figure 20: mRNA expression of MMP-9, MMP-19 and TIMP-1 in the CI during induced luteolysis; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to control (0h), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ I=control\ (0h))}$ - $\Delta CP_{(goup\ II\text{-}VII)}$. The statistic analysis were determined from ΔCP values (n=5). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

5.2.4.2.4 PA system during induced luteolysis

During induced luteolysis tPA mRNA expression showed a significant increase of all time points to control group with the highest up-regulation at 2h and 24h. uPA expression was significantly increased from 2h to 12h followed by a further increase from 24h to 64h. uPAR showed a significant up-regulation from 2h till 64h, which stayed on the same expression level (fig. 22). PAI-1 revealed a significant rising increase from 2h to 24h, which decreased to a lower expression level from 48h to 64h, which was still significantly up-regulated to control. The expression of PAI-2 was quite similar to PAI-1 with a significant increase on the same level from 2h to 12h, but increase decreased already at 24h to a lower expression level till 64h. This level was still significantly up-regulated to control (fig. 23).

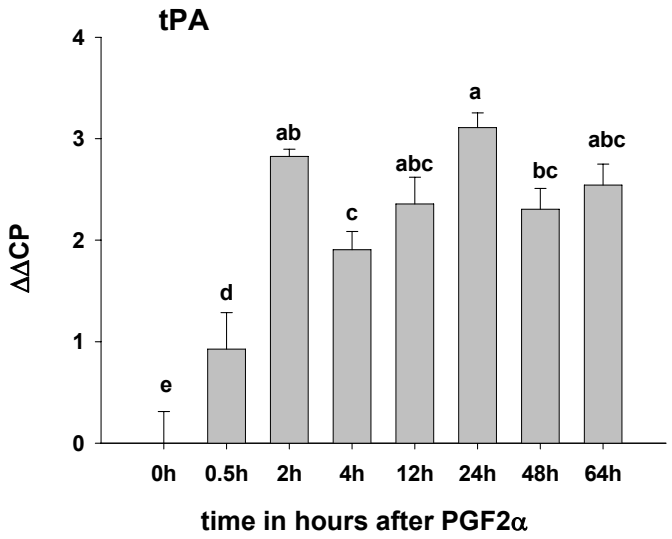


Figure 21: mRNA expression of tPA in the CI during induced luteolysis; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to control (0h), where $\Delta CP=CP_{target} - CP_{index}$ and $\Delta\Delta CP=\Delta CP_{(group\ I=control\ (0h))} - \Delta CP_{(goup\ II\text{-}VIII)}$. The statistic analysis were determined from ΔCP values (n=5). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

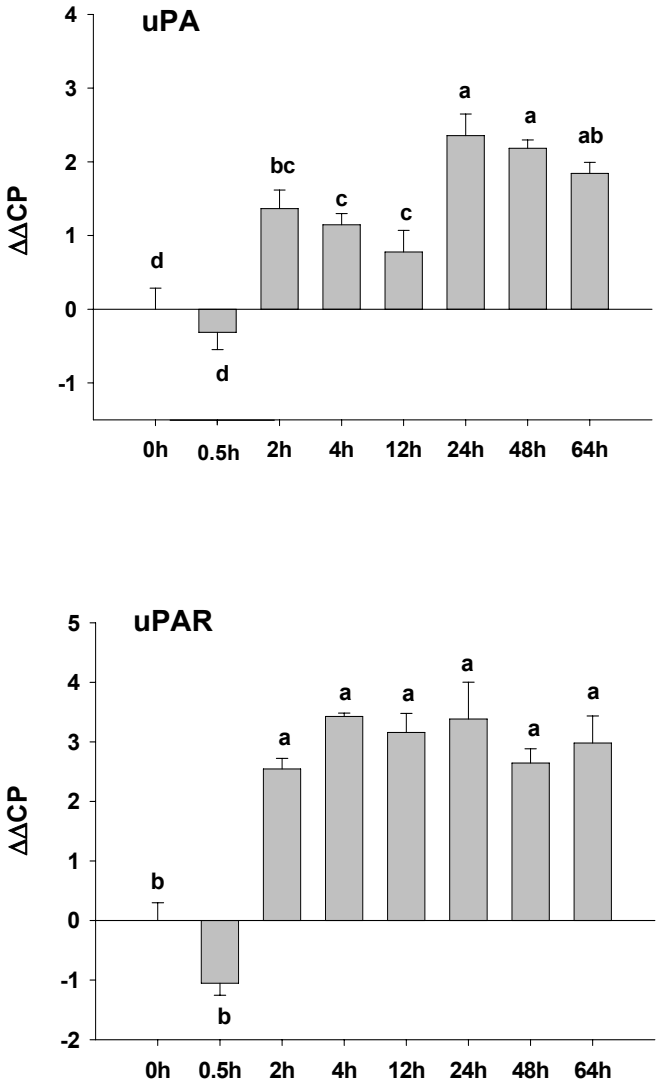


Figure 22: mRNA expression of uPA and uPAR in the CI during induced luteolysis; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to control (0h), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ I=control\ (0h))}$ - $\Delta CP_{(goup\ II\text{-}VIII)}$. The statistic analysis were determined from ΔCP values (n=5). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

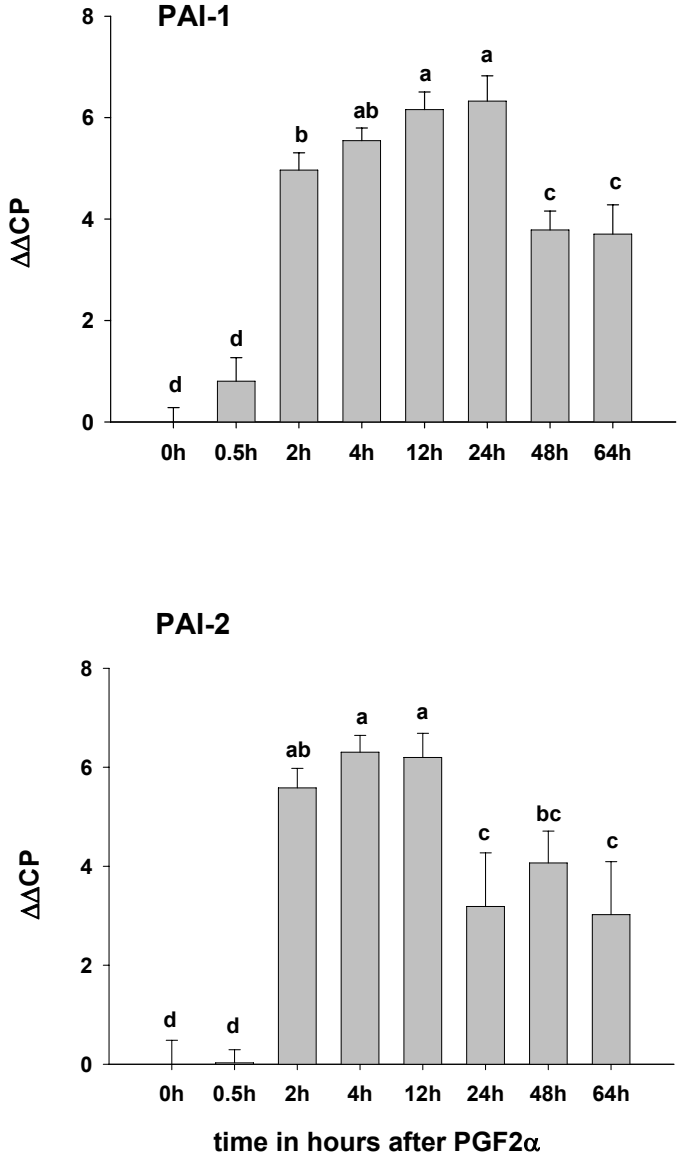


Figure 23: mRNA expression of PAI-1 and PAI-2 in the CI during induced luteolysis; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to control (0h), where $\Delta CP=CP_{target}$ - CP_{index} and $=\Delta CP_{(group\ I=control\ (0h))}$ - $\Delta CP_{(goup\ II-VIII)}$. The statistic analysis were determined from ΔCP values (n=5). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

5.2.4.3 Monocyte chemoattractant protein-1 (MCP-1)

5.2.4.3.1 CL during oestrous cycle

MCP-1 mRNA expression was not regulated during oestrous cycle (fig 24).

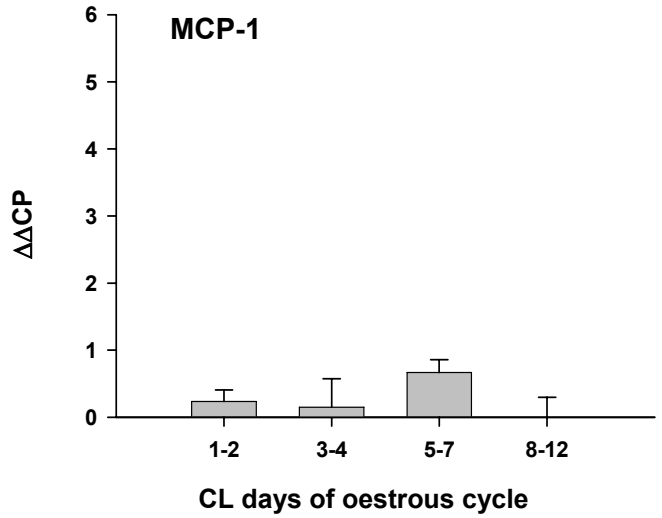


Figure 24: mRNA expression of MCP-1 in CI during oestrous cycle; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to the day 8-12 (group IV), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ IV=day\ 8\text{-}12)}$ - $\Delta CP_{(group\ I\text{-}III).}$ The statistic analysis were determined from ΔCP values (n=4). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

5.2.4.3.2 CL during induced luteolysis

All time points after PGF2α treatment showed a significant increase of mRNA for MCP-1 compared to control group. The first increase is seen 0.5h, which is significantly lower then the next increase from 2h to 12h. From 24h to 48h the expression rices again significantly higher than from 2h to 12h with a decline at 64h to the leve seen at 4h.

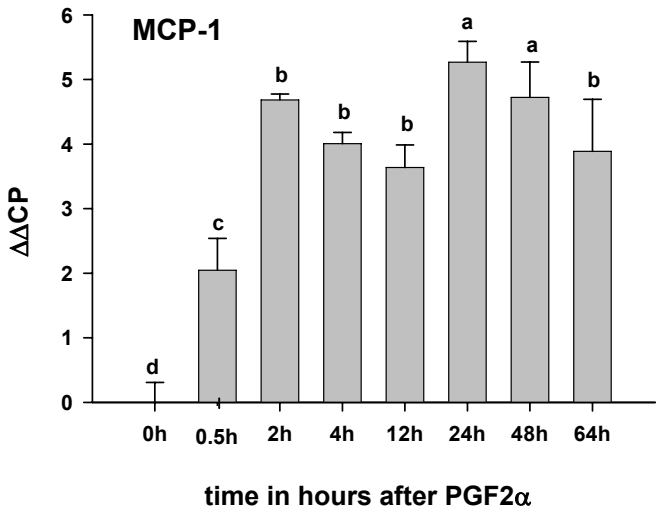


Figure 25: mRNA expression of MCP-1 in the CI during induced luteolysis; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to control (0h), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ I=control\ (0h))}$ - $\Delta CP_{(group\ II\text{-}VIII)}$. The statistic analysis were determined from ΔCP values (n=5). Results are presented as expression changes ΔΔCP ± SEM. Different superscript letters indicate significant differences ($P < 0.05$).

5.2.4.4 Apoptosis

5.2.4.4.1 Extrinsic pathway during oestrous cycle in the CL

The mRNA expression of Fas showed no significant regulation in the CL during oestrous cycle (fig. 26). Fas-Ligand, TNFα and TNFR1 revealed the same expression pattern with a significant down-regulation from day 1 to 7 compared to day 8-12. TNFR2 on the other hand was significantly decreased from day 1 to 4 during oestrous cycle (fig. 26, fig. 27).

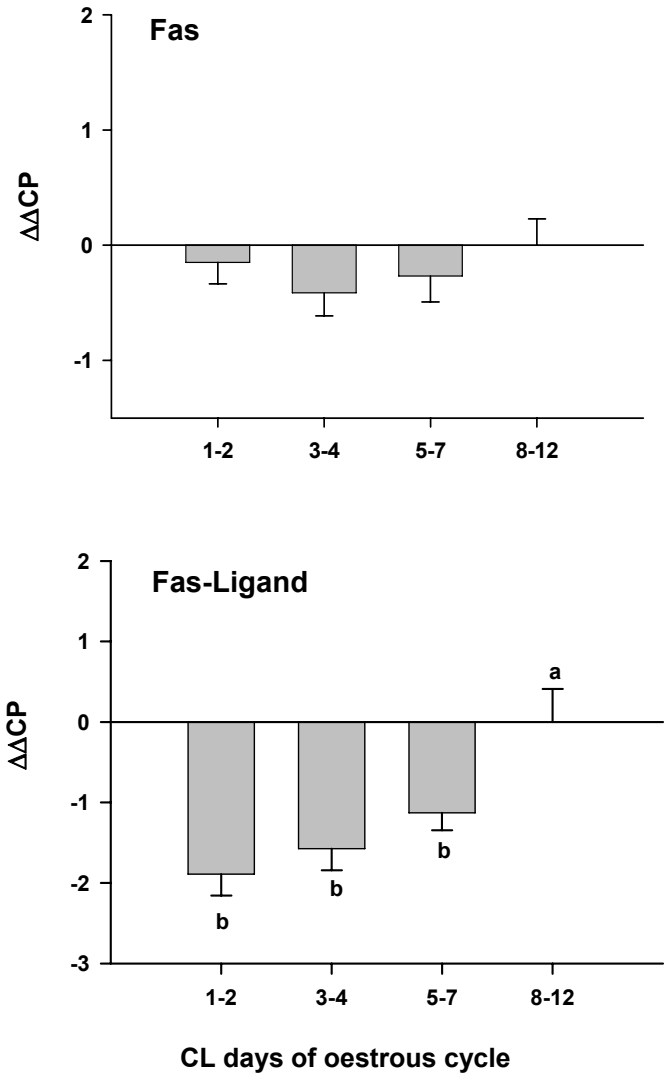


Figure 26: mRNA expression of Fas and Fas-Ligand in CI during oestrous cycle; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to the day 8-12 (group IV), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ IV=day\ 8\text{-}12)}$ - $\Delta CP_{(group\ I\text{-}III).}$ The statistic analysis were determined from ΔCP values (n=4). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

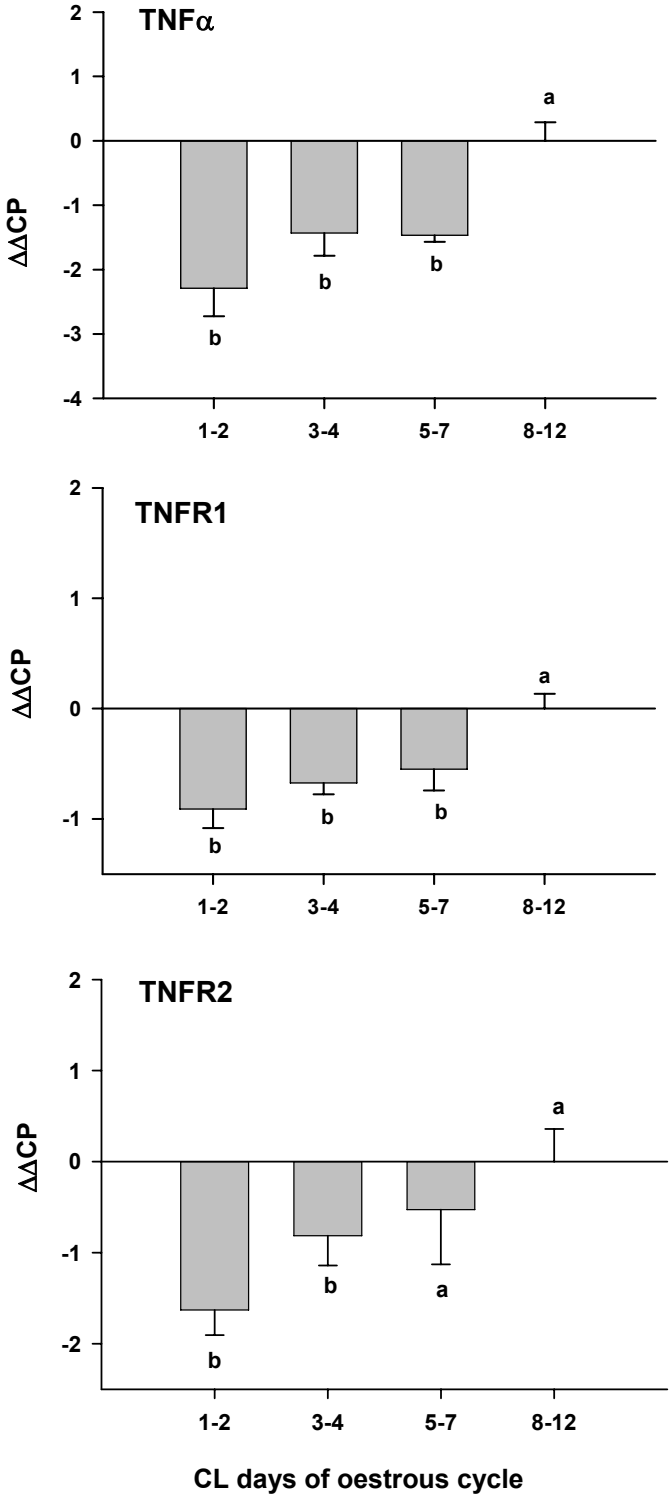


Figure 27: mRNA expression of TNF-α, TNFR1 and TNFR2 in CI during oestrous cycle; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to the day 8-12 (group IV), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ IV=day\ 8\text{-}12)}$ - $\Delta CP_{(group\ I\text{-}III).}$ The statistic analysis were determined from ΔCP values (n=4). Results are presented as expression changes ΔΔCP ± SEM. Different superscript letters indicate significant differences ($P < 0.05$).

5.2.4.4.2 Intrinsic pathway during oestrous cycle in the CL

The mRNA expression of p53, Bax and Smac revealed no significant regulation during oestrous cycle. Bcl-X_L was significantly downregulated from day 1 to day 7 compared to the midluteal phase. The expression of Survivin was significantly increased during the first seven days of oestrous cycle (fig. 28, fig. 29).

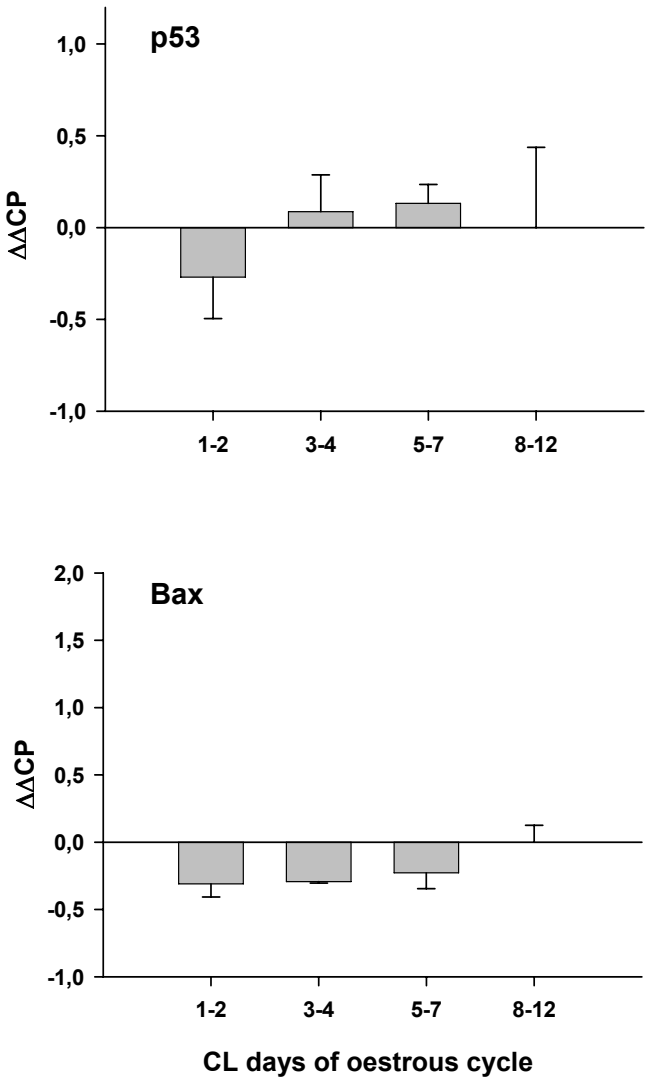


Figure 28: mRNA expression of p53 and Bax in CI during oestrous cycle; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to the day 8-12 (group IV), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ IV=day\ 8\text{-}12)}$ - $\Delta CP_{(group\ I\text{-}III).}$ The statistic analysis were determined from ΔCP values (n=4). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

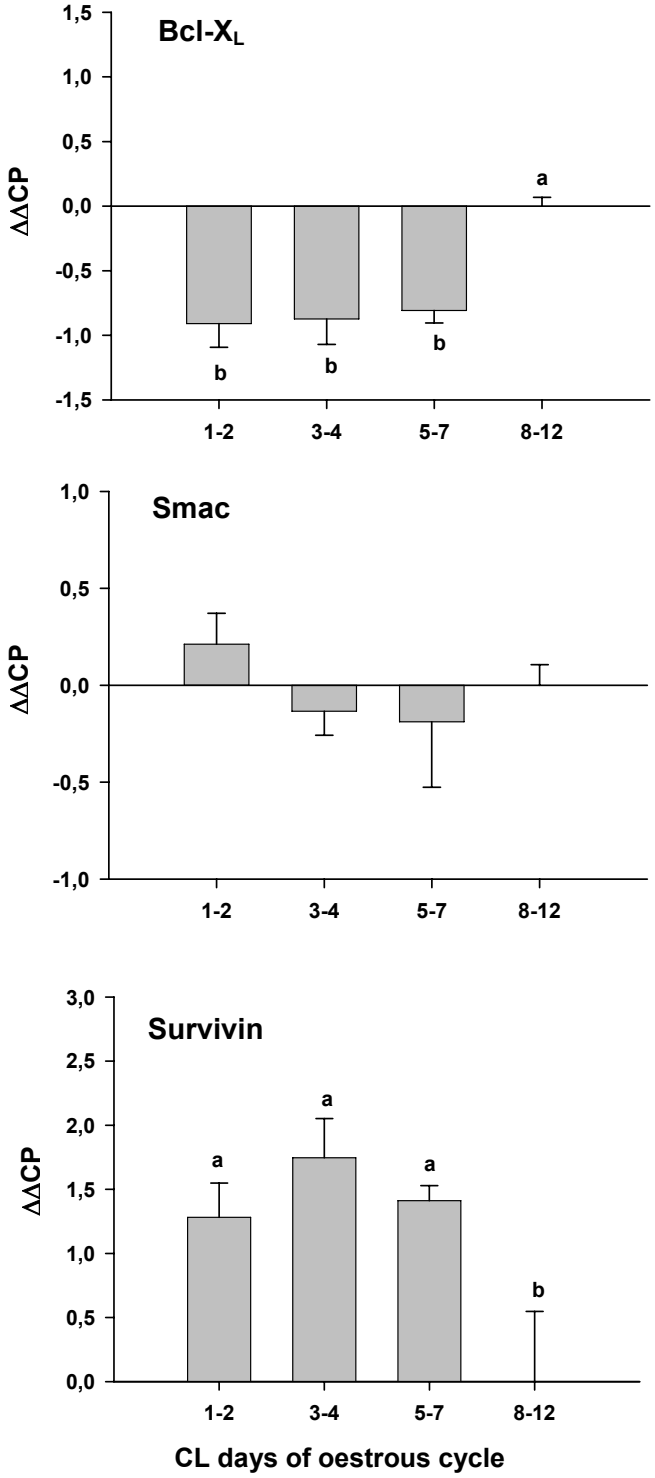


Figure 29: mRNA expression of Bcl-X_L, Smac and Survivin in CI during oestrous cycle; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to the day 8-12 (group IV), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ IV=day\ 8\text{-}12)}$ - $\Delta CP_{(group\ I\text{-}III)}$. The statistic analysis were determined from ΔCP values (n=4). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

5.2.4.4.3 Caspase3, -6, -7 during oestrous cycle in the CL

Caspase3 and caspase7 showed no regulation during oestrous cycle (fig 30). The mRNA expression of caspase6 was significantly down-regulated from day 1 to day 7 compared to the mid luteal group. The expression during the first two days was also significant lower than at day 5-7 (fig 31.).

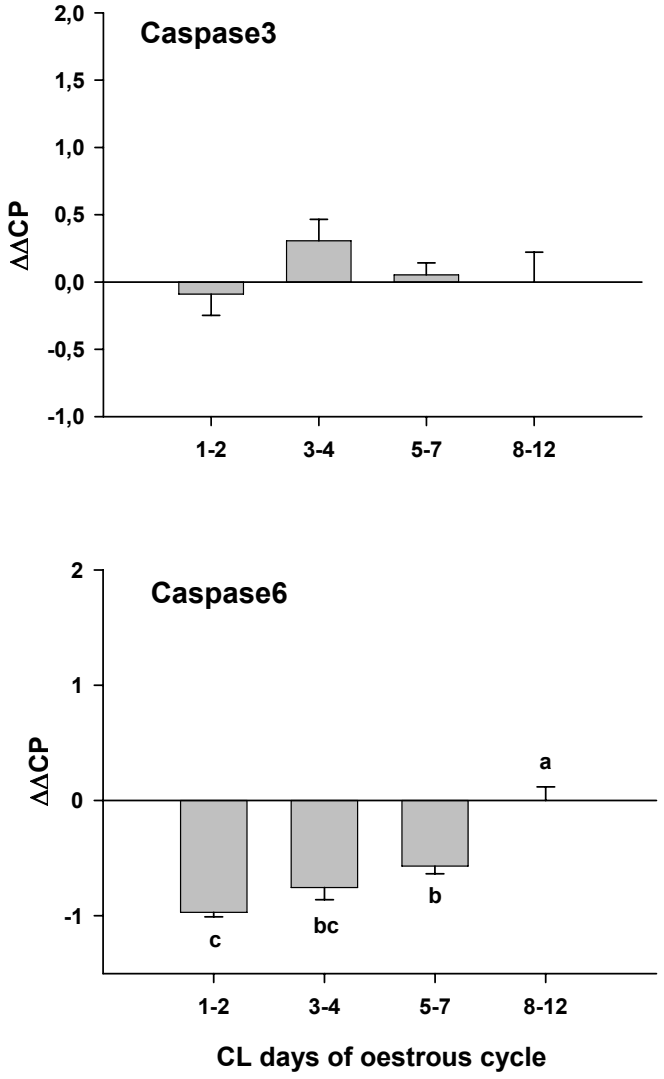


Figure 30: mRNA expression of caspase3 and caspase6 in Cl during oestrous cycle; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to the day 8-12 (group IV), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ IV=day\ 8\text{-}12)}$ - $\Delta CP_{(group\ I\text{-}III)}$. The statistic analysis were determined from ΔCP values (n=4). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

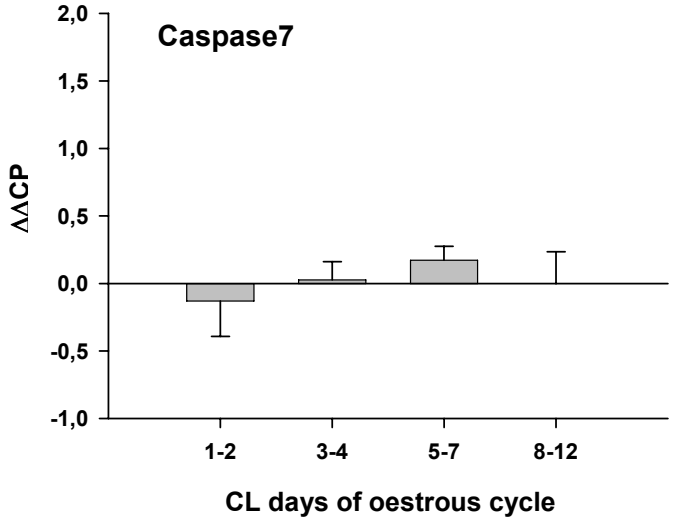


Figure 31: mRNA expression of caspase7 in CI during oestrous cycle; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to the day 8-12 (group IV), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ IV=day\ 8\text{-}12)}$ - $\Delta CP_{(group\ I\text{-}III).}$ The statistic analysis were determined from ΔCP values (n=4). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

5.2.4.4.4 Extrinsic pathway during induced luteolysis in the CL

The expression of Fas in the CL during induced luteolysis was constantly increased from 2h onwards till 64h. All time points were significantly up-regulated to control group. Fas-Ligand was significantly up-regulated after 0.5h with a further significant increase to 12h and 24h. These increase was reduced at 48h nearly to the level of 0.5h again with a slight increase at 64h (fig. 32). For TNFα a significant increase of 16-fold was seen after 0.5h, which increased to a significant expression level of 32-fold at 2h. All other time points were significantly less expressed compared to 2h, but still significantly increased compared to control group. TNFR1 was not regulated during induced luteolysis. The expression of TNFR2 was significantly increased from 2h to 64h on a constant level (fig. 33).

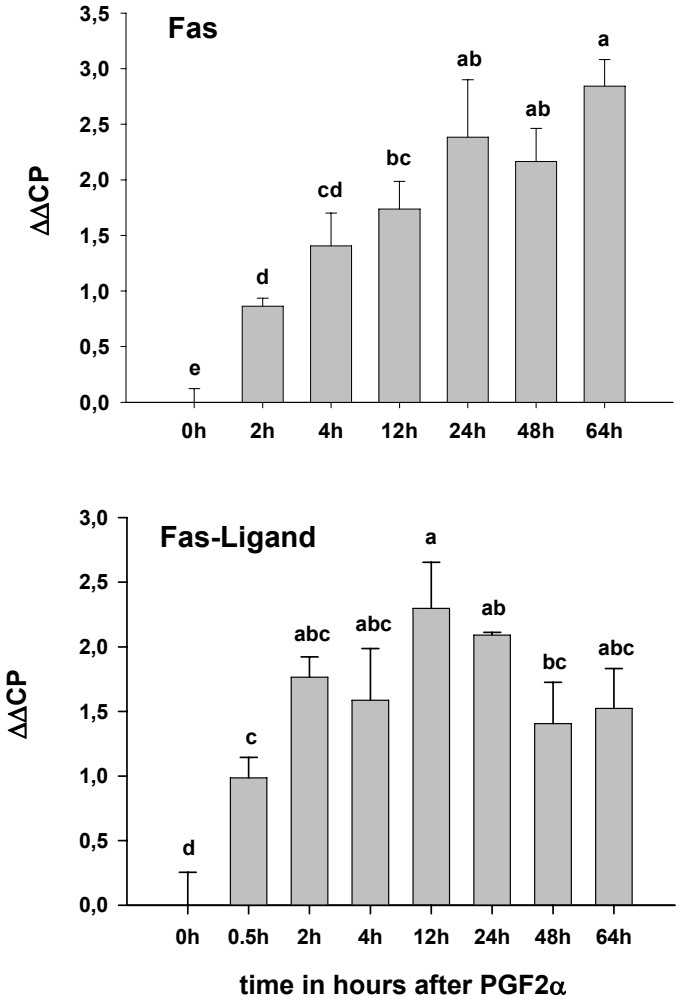


Figure 32: mRNA expression of Fas and Fas-Ligand in the Cl during induced luteolysis; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to control (0h), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ I=control\ (0h))}$ - $\Delta CP_{(group\ I\text{-}VIII)}$, The statistic analysis were determined from ΔCP values (n=5). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

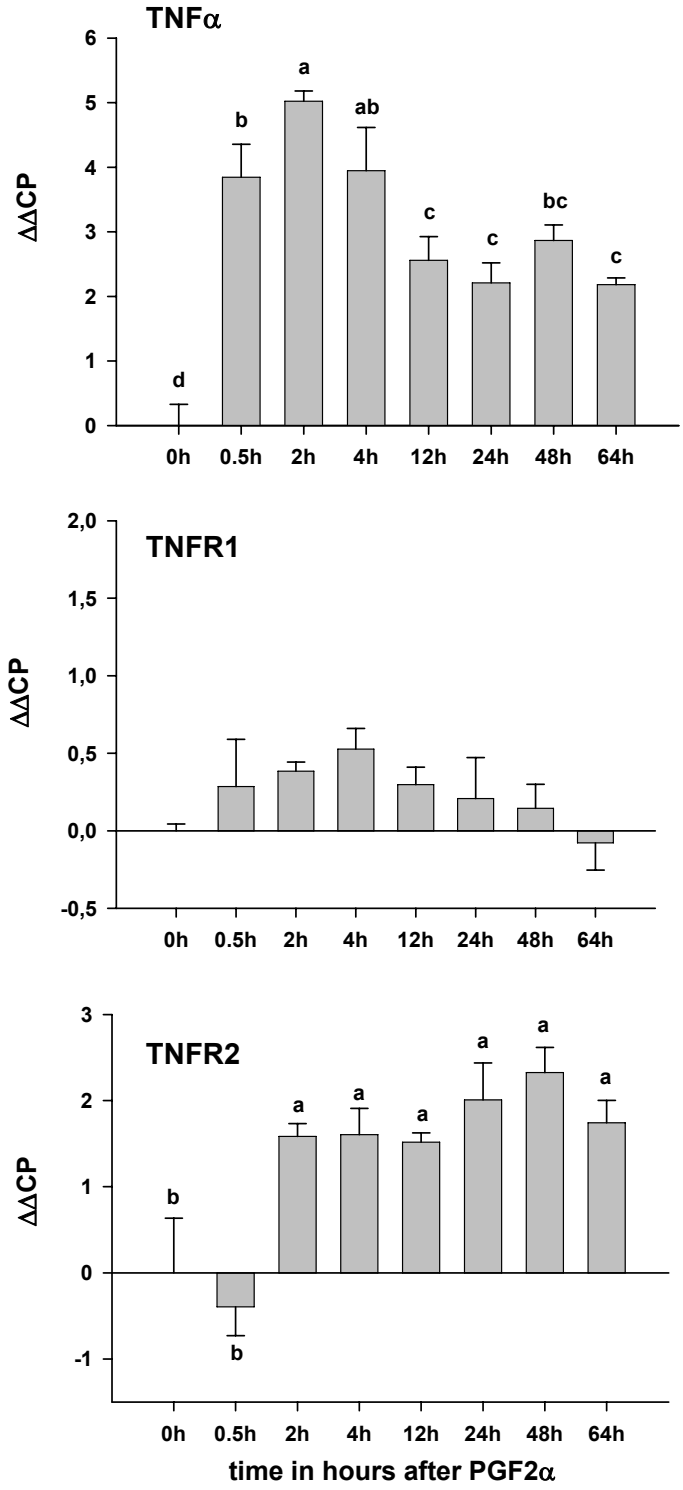


Figure 33: mRNA expression of TNFα, TNFR1 and TNFR2 in the CI during induced luteolysis; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to control (0h), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ I=control\ (0h))}$ - $\Delta CP_{(group\ I\text{-}VIII)}$. The statistic analysis were determined from ΔCP values (n=5). Results are presented as expression changes ΔΔCP ± SEM. Different superscript letters indicate significant differences ($P < 0.05$).

5.2.4.4.5 Intrinsic pathway during induced luteolysis in the CL

During induced luteolysis only Smac showed no significant regulation in the CL at all. The mRNA expression of p53 increased significantly from 24h to 64h compared to control group. Bax showed the same expression pattern than p53 with a significant up-regulation from 24h to 64h (fig. 34). In contrast to Bax, Bcl-X_L revealed a significantly increased expression only from 48h to 64h. Survivin was significantly downregulated from 2h to 12h after PGF2α, with an increase to control level from 24h to 64h (fig. 35).

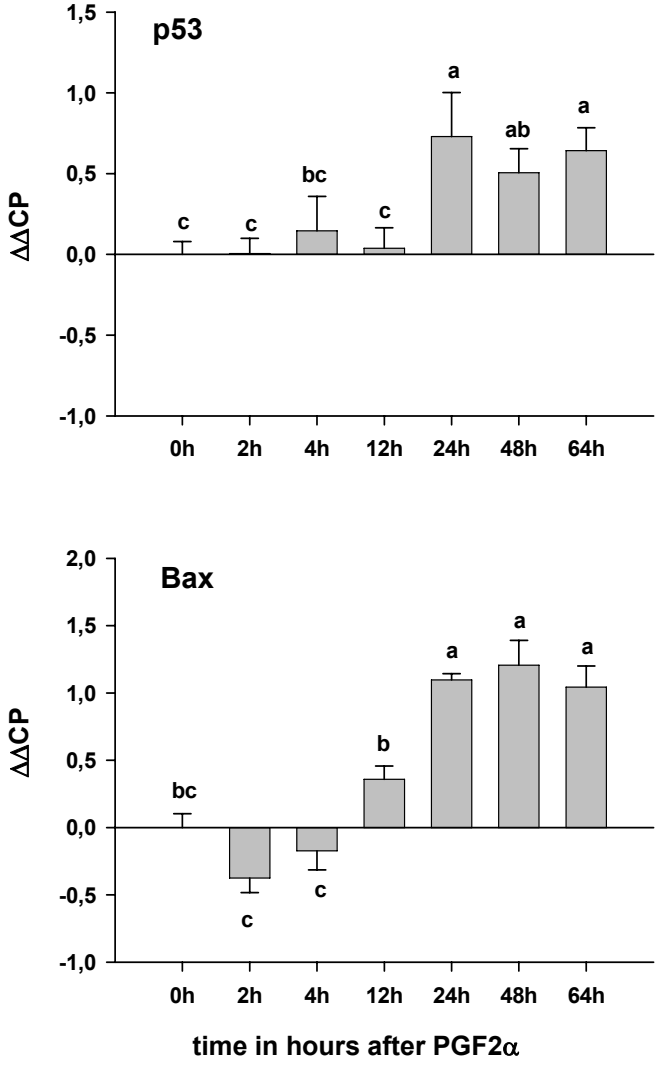


Figure 34: mRNA expression of p53 and Bax in the CI during induced luteolysis; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to control (0h), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ I=control\ (0h))}$ - $\Delta CP_{(group\ I\text{-}VII)}$. The statistic analysis were determined from ΔCP values (n=5). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

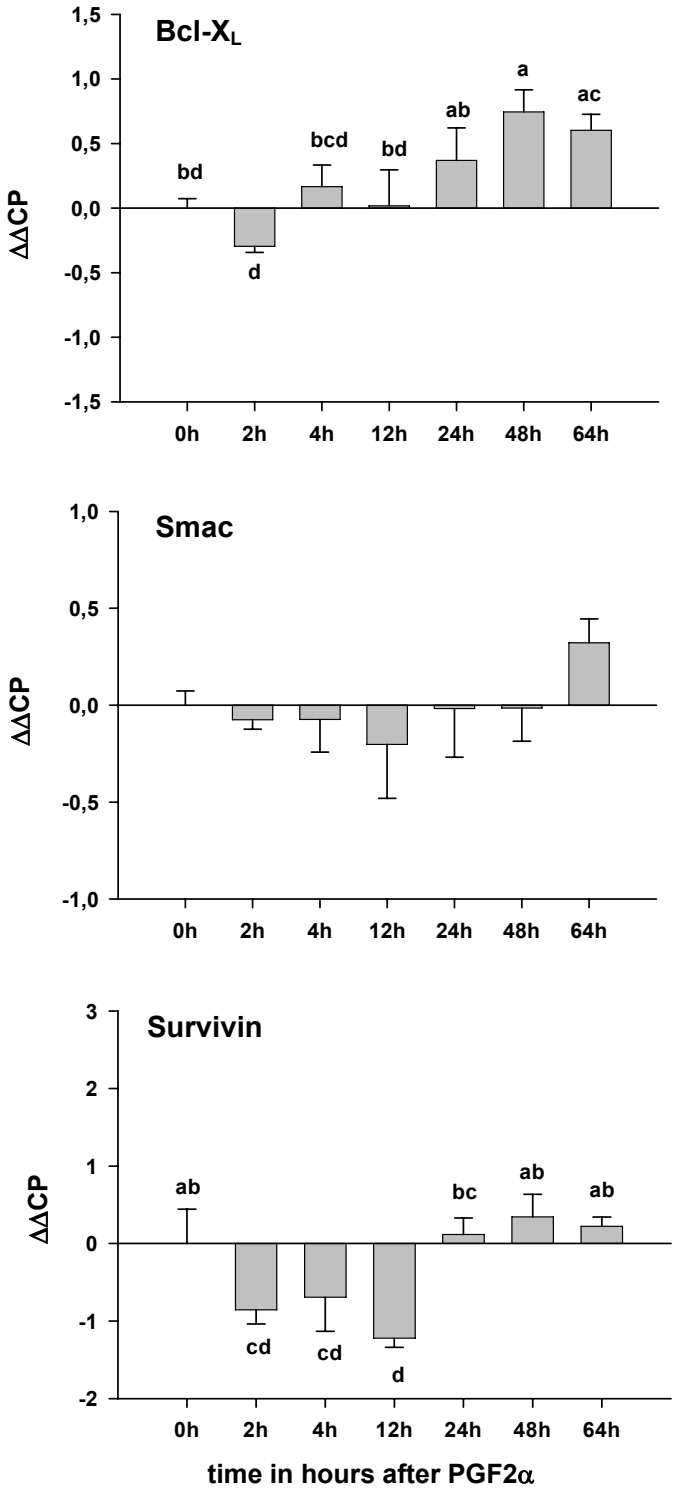


Figure 35: mRNA expression of Bcl-X_L, Smac and Survivin in the CI during induced luteolysis; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to control (0h), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ I=control\ (0h))}$ - $\Delta CP_{(group\ I\text{-}VII)}$. The statistic analysis were determined from ΔCP values (n=5). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

5.2.4.4.6 Caspase3, -6, -7 during induced luteolysis in the CL

Caspase3 mRNA expression during induced luteolysis showed a significant increase from 12h to 64h with the highest expression at 64h. Caspase6 showed a rising up-regulation from 2h to 64h, which was significant to control group at all time points (fig. 36). The expression of caspase7 was significantly increased from 24h on with the highest up-regulation of 45-fold at 64h (fig. 37).

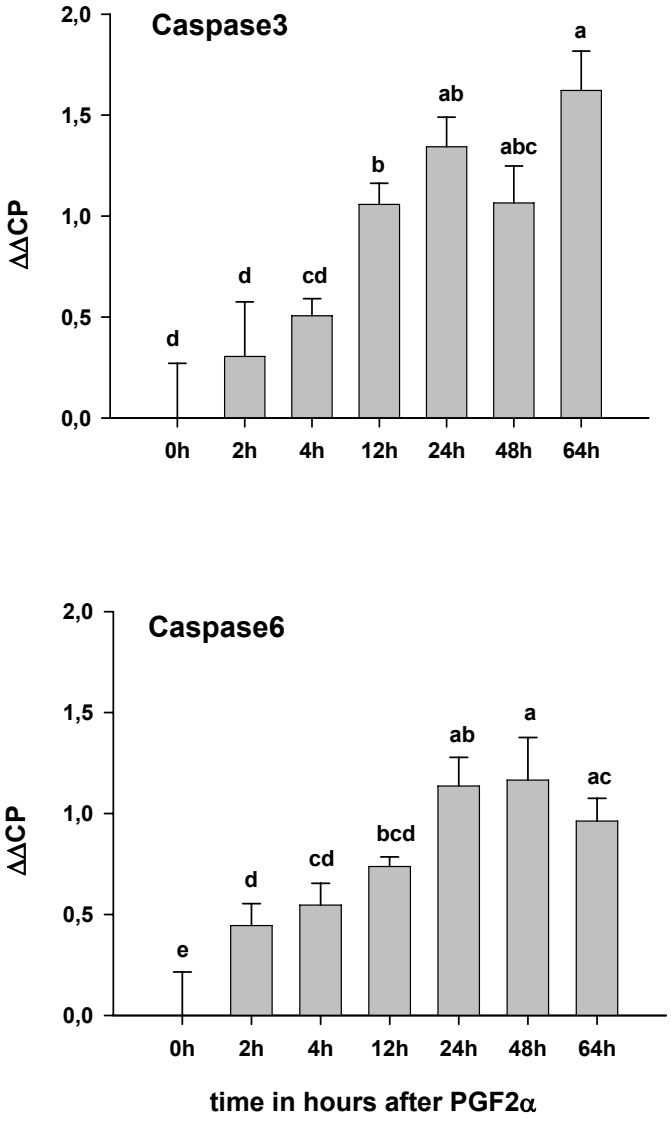


Figure 36: mRNA expression of caspase3 and caspase6 in the CI during induced luteolysis; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to control (0h), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ I=control\ (0h))}$ - $\Delta CP_{(group\ I\text{-}VIII)}$. The statistic analysis were determined from ΔCP values (n=5). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

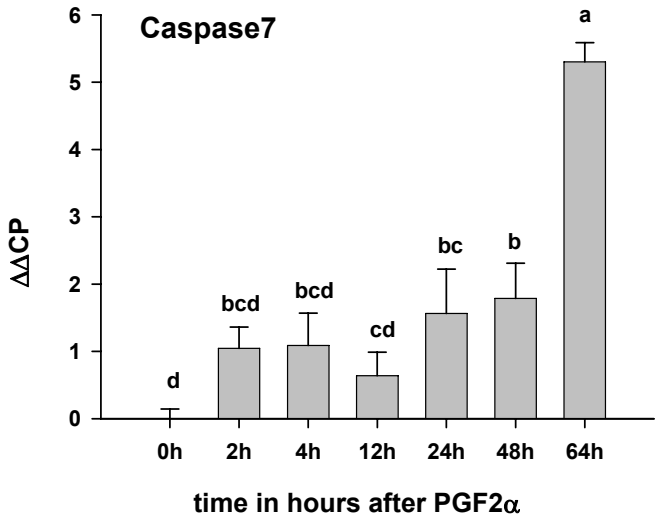


Figure 37: mRNA expression of caspase7 in the CI during induced luteolysis; the changes in mRNA expression for the different groups were calculated by normalisation with the Bestkeeper index and relative to control (0h), where $\Delta CP=CP_{target}$ - CP_{index} and $\Delta\Delta CP=\Delta CP_{(group\ I=control\ (0h))}$ - $\Delta CP_{(group\ I\text{-}VIII)}$. The statistic analysis were determined from ΔCP values (n=5). Results are presented as expression changes $\Delta\Delta CP \pm$ SEM. Different superscript letters indicate significant differences ($P < 0.05$).

6 Discussion

6.1 Development of a method to gain uterus milk *in vivo*

6.1.1 Method

The aim of the first part of these study was to develop a method to gain uterus milk *in vivo* at specific time points during the preimplantation periode in cattle. Therefore a technique used for flushing embroys during embryo transfer with a specific baloon catheter for heifers was modified. The first concern was to evaluate the correct volume of the used flushing fluid. As less as possible should be used for not disturbing the development of the embryo till day 24 of pregnancy, but the regained volume had to be enough for the investigations via different biomolecular methods. Normaly flushing of embryos is made with 5 x 50ml flushing fluid for each uterus horn (H. Thenhumberg personal notice). As no flushing of the embryo should occur till day 24, the volume of the catheter was evaluated, which was 3ml, and it was decided to try the flushing with a total volume of 13ml per horn. It was possible to gain between 3ml to 13ml back, which was enough for the measurment of different factors, placing the catheter 1cm cranial to the bifurcatio uteri and massaging gently. But despite using sterile instruments and fluid, and working as clean as possible an infection of the uterus occurred in three heifers after the second and in five heifers after the third flushing. In one heifer no infection was found. The flushing fluids of all these heifers were contaminated with sanies. After treatment with Genta-Sleecol 5 the flushing fluid was clear again. One embryo was gained by chance at day 7 of gravidity, which showed no viability and the developing stage of day 4 of gravidity. All heifers showed signs of oestrus between day 17 and 24 of gravidity. Therefore the value of progesterone in the blood plasma for confirming the gravidity was not evaluated. For the evaluation of prostaglandins an EDTA-stabilisator was given to the gained flushing fluid, which was found by further tests to desturbe the oestradiol-17β evaluation via EIA. The first attempt of developing an *in vivo* method for gaining uterus fluid showed positive and negative aspects, so that further changes are necessary. The biggest problem was the infection of the uterus using these flushing method five times in a row at different time points during preimplantation, which led to a resorbtion of the embryo. To prevent these infection, just one flushing should be done per gravidity time point with gaining of the embryo afterwards for confirmation of pregnancy, so that the heifer can be inseminated again after two oestrous cycles. Again both horns should be flushed, whereas one sterile disposable catheter should be used for each horn and the flushing should be devided into two parts: first flushing with 13ml of sterile 0.9% NaCL for biomolecular methodes, then flushing of the embryo using 5 x 50ml sterile PBS buffer. With this procedure the embryo and the

uterus milk can be gained at the same time, which might be necessary for the investigation of the source of proteins found in the uterus milk. The embryo can be classified by the developing stage and stored in RNA-Later at –80° for further biomolecular investigations. The received flushing fluid should be separated in two aliquotes, whereas one is used for the oestradiol-17β measurement and must not contain EDTA-stabilisator. These two aliquotes should be centrifugated and the fluid should be stored seperatly at –20° from the received cell pellet. Part of the cell pellet can be used for the identification of the cells found in the uterus lumen and the rest stored in RNA-Later at –80° for further biomolecular investigations. For the evaluation of prostaglandins in the uterus milk it should be considered to treat the heifers with a prostaglandin synthetase inhibitor shortly before flushing. It was reported in pony mares that cervical stimulation elevates the intrauterin PGF level. This increase was inhibited by treatment with flunixin-meglumine before flushing (Berglund *et al.* 1982).

6.1.2 Results

We were able to measure progesterone, oestradiol-17β, PGF2α and VEGF in the uterus milk. It was seen that the concentration of the measured factors were in the detectable range of the used EIA and RIA. It should be kept in mind that the uterus milk was diluted in 13ml of flushing fluid, so that all values are higher in the undiluted uterus milk. As the uterus infection occurred after the second and third flushing respectively, no statistic evaluation was made. It was seen that progesterone ranged between <0.1 and 0.4ng/ml, whereas no correlation to the inflammation was found. The pattern of oestradiol-17β was erratic, because the oestradiol-17β levels of the flushing fluid from heifers with no infection till day 12 of gravidity could not be related to each other. This could be due to the fact, that a EDTA-stabilisator was given to the samples, which seems to influences the EIA measurement of oestradiol-17β (erratic higher level), which we evaluated in further tests. VEGF was evaluated by RIA and revealed levels between 0.08 and 2.58ng/ml, whereas the higher levels were seen during inflammation. The level of PGF2α showed a clear correlation to the infection. The differences seen between the different heifers or even between the two uterus horns of one animal during the first flushing might be also due to the different massage intensity or sensitivity of the uterus horns while flushing, which can induce prostaglandin secretion (Berglund *et al.* 1982). In general we can assume that the hormone levels in undiluted uterine milk are about 10-fold higher according to the diluting effect.

6.2 Expression of housekeeping genes, proteases, apoptotic and anti-apoptotic factors in the CL during oestrous cycle and induced luteolysis

6.2.1 Housekeeping Genes (HKGs)

RT-PCR specific errors in the quantification of mRNA transcript are easily generated by any variation in the amount of starting material between samples. These can happen through pipetting failures or by uncorrect photometric measurement of the total RNA amount per µl. The quantification errors are especially relevant when the samples have been obtained from different individuals like in this experiment, and can result in misinterpretation of the expression profiles of the target genes. Consequently, the question of what constitutes an appropriate standard arises (Thellin *et al.* 1999). The accepted method for minimising these errors and correcting for sample to sample variation is to amplify a cellular RNA that serves as an internal reference against which other RNA values can be normalised (Karge *et al.* 1998). The ideal standard should be expressed at a constant level among different tissues of an organism or in the same tissue of different individuals, at all stages of development, and should be unaffected by the experimental treatment. It was not possible to find such a housekeeping gene, which is stable expressed during oestrous cycle and induced luteolysis. Therefore we decided to calculate an index out of the four different housekeeping genes Histone, β-Actin, GAPDH and Ubiquitin using the Bestkeeper software (Pfaffl *et al.* 2004) and to normalise all target gene data with these index following the ΔΔCP method (Livak and Schmittgen 2001). There is evidence that the gene expression of all these four housekeeping genes changes during different physiological stages of the cells and that they have specific cell functions (Bustin 2000; Gillespie and Vousden 2003; Thellin *et al.* 1999; Warrington *et al.* 2000). Histones are abundant, basic, structural proteins that bind to the DNA and enable a tight packing of the DNA into nucleosomes. 147bp of DNA is folded over a Histone octamer (two molecules each of the four core Histones H2A, H2B, H3 and H4) to form a nucleosome. In between two nucleosomes another Histone (H1) bindes to 20bp of DNA to enable the spiralisation of the DNA into solenoides (Koolmann and Röhm 1998). Different variants were found for the four core Histones H2A, H2B, H3, H4 and for the linker Histone H1 (Gillespie and Vousden 2003; Pusarla and Bhargava 2005). These linker variant (H1.2) was found to be a pro-apoptotic factor in cells undergoing X-ray irridation (Konishi *et al.* 2003). These group showed that H1.2 has the ability to release cytochrom c from the mitochondria, which leads to apoptosis via the intrinsic pathway. How these release actually functions is not known definitively, but it seems to be a p53 dependent mechanism (Zong 2004). The pro-apoptotic action of H1.2 could be the reason for the

increase of mRNA expression of Histone from 12h to 64h shortly before the structural luteolysis begins. p53 shows nearly the same expression pattern like Histone, which could be due to its posssible regulatory action on Histone. β-Actin, a structur protein of the cytosceleton, is up-regulated during the first 7 days of the oestrous cycle and after 0.5h as well as from 24h to 64h after luteolysis. The luteal tissue of the developing CL consists of highly mitotic cells, mainly endothelial cells, which could explain the increase of expression at the early luteal stages. How β-Actin expression is regulated in apoptotic cells is presently not known. The work of Naora et al. (1995) indicates that β-Actin is differently expressed in different cells and that these expression is also dependend on the apoptotic stimulus. It is possible that PGF2α is responsible for the increased expression after 0.5h. At the time of structural luteolysis, 24h after PGF2α, apoptotic cells are found in the CL of marmoset monkeys (Young *et al.* 1998) showing the formation of apoptotic bodies. These formation effects the cytosceleton of the cells and is associated with the depolymerisation of Actin, which also plays a particular role in the formation and maintenance of these apoptotic bodies (Suarez-Huerta *et al.* 2000). These results might indicate the reason for the up-regulation of the β-Actin expression during structural luteolysis. GAPDH is an enzyme necessary for the glycolysis and gluconeogenesis. It shows an increased expression during the first seven days of the oestrous cycle, which could be due to the higher mitotic cell rate and the higher metabolic rate of these cells compared to the midluteal stage. It is reported that GAPDH expression is different during the cell cycle (Mansur *et al.* 1993) and that growth hormons can activate its transcription (Freyschuss *et al.* 1994). Two groups (Qi and Sit 2000b) also reported that GAPDH is up-regulated during apoptosis and that it is regulated by p53 (Chen *et al.* 1999). Our data show that GAPDH is not regulated during luteolysis, although an increase of expression can be seen from 24h onwards just like the expression of p53. It might be due to the greater variances in the groups that no significancies were found. Ubiquitin is necessary for the degradation of proteins and acts as regulator of all aspects of cell biology, including cell division, growth, communication, movement and apoptosis (Johnson 2002; Pickart 2001). It is reported that Ubiquitin is up-regulated during PGF2α induced luteolysis in marmoset monkeys (Young *et al.* 1998) and in sheep (Murdoch *et al.* 1996). This is in contrast to our data, which revealed a slight but significant decreased expression of Ubiquitin from 48h to 64h during induced luteolysis. The reason for these down-regulation is not know and has to be futher investigated. For the evaluation of factors in tissues undergoing apoptosis it seems to be necessary to normalise these datas with more than one HKG, because of their specific roles during apoptosis.

6.2.2 Extracellular matrix proteases in the CL during oestrous cycle and induced luteolysis

6.2.2.1 CL during oestrous cycle

6.2.2.1.1 Matrix metalloproteases (MMPs)

After ovulation, the follicular tissue is reorganised as granulosa cells hypertrophy, theca and granulosa-cells differentiate into luteal cells, and vasculature forms in the granulosa luteal compartiment (Young *et al.* 2002). The CL is a most highly vascularised organ and receives the greatest rate of blood flow per unit of tissue of any organ in the body (Wiltbank *et al.* 1988). Degradation of the ECM by MMPs is required in endothelial cell migration, organisation and, hence, angiogenesis (McCawley and Matrisian 2000). For the formation of new vessels, the basement membrane of capillaries must be degraded (fig. 38) to allow migration of endothelial cells from the theca interna compartment into the surrounding avascular granulosa luteal tissue. Even if the expression of MMP-1 during 20h after GnRH and during the first seven days of oestrous cycle is not significantly regulated, which might be caused by a higher variance within the groups, there is evidence that MMP-1 is able to facilitate the *in vitro* migration of microvascular endothelial cells, and is therefore associated with advancing angiogenesis (Partridge *et al.* 2000). Although a number of studies on the regulation of *in vitro* endothelial MMP expression by angiogenic growth factors have shown an induction of MMPs. bFGF and VEGF have been reported to induce MMP-1 expression, whereas VEGF also increases MMP-2 expression (Lamoreaux *et al.* 1998; Sato *et al.* 2000; Unemori *et al.* 1992). As shown for the bovine CL (Berisha *et al.* 2000) VEGF is up-regulated in the first seven days of the oestrous cycle, while bFGF (FGF-2) increases at day 1-2 in the bovine CL (Schams *et al*. 1994), so that a regulatory effect of these growth factors on MMPs could be assumed. Not only MMP-1, but also MMP-2, its receptor MMP-14 and inhibitor TIMP-2 may play a critical role in angiogenesis. Even though no statistically significant regulation for MMP-2 and MMP-14 could be evaluated during oestrous cycle, TIMP-2 mRNA expression was significantly increased during the first seven days. Our hypothesis is, that at the beginning of CL formation not all bindings site of MMP-14 are blocked by TIMP-2, because of its reduced expression compared to the mid luteal phase. This could suggest, that MMP-2 might not be inhibited at the beginning of vessel sprouting in the CL, but during mid luteal phase, when angiogenesis is completed. Our findings are in agreement with recent data (Goldberg *et al.* 1996; Smith *et al.* 1996; Zhang *et al.* 2002; Zhang *et al.* 2005). MMP-2, MMP-14 and TIMP-2 are localized in bovine luteal endothelial cells during early and mid cycle (Zhang *et al.* 2002, Zhang *et al*. 2003), which enables these cells to digest collagen

type IV, a major component of the basement membrane (Moses 1997), which has to be penetrated to ensure their migration. A lack of MMP-14 leads to insufficient angiogenesis in mice (Zhou *et al.* 2000) and a neutralising MMP-2 antibody could inhibit capillary tube formation by bovine endothelial cells, an indispendable step in angiogenesis (Zhang *et al.* 2005). In ewes, administration of a MMP-2 antibody results in incomplete CL formation, whereby the build up of normal vasculature is blocked (Gottsch 2001). All these findings suggest, MMP-2 play an essential role in neovascularisation events in the developing CL. MMP-2, MMP-14 and TIMP-2 were also found to be expressed in large luteal cells and the activation of MMP-2 during CL development enables these cells to degrade the pericellular ECM, which is necessary to accommodate the enlargement of these cells during this time (Zhang *et al*. 2003). The same action as activated MMP-2 on basal membrans is thought of MMP-19, which was detected in endothelial and vascular smooth muscle cells (Djonov *et al.* 2001; Kolb *et al.* 1999). In the rat CL the lowest expression of MMP-19 mRNA was seen during day 2 to 8 of the oestrous cycle (Jo and Curry, Jr. 2004). TIMP-1 is able to counteract with MMP-19 (Kolb *et al.* 1999), which could be possible in the developing bovine CL to hold the action of MMP-19 in bay. MMP-9 shows an steadily increased expression 20h after GnRH till the mid luteal phase. This expression pattern could be caused by invading macrophages, which are known to produce MMP-9 (Welgus *et al*. 1990).

6.2.2.1.2 Tissue inhibitors of matrix metalloproteases (TIMPs)

The massive capability of proteases to degrade the ECM must be hold in bay by inhibitors like TIMPs. These inhibitory action of TIMP-1 and TIMP-2 can be assumed in the developing CL, where TIMP-1 is, among others, expressed in vascular smooth muscle cells. These cells play a critical role in vascular maturation and survival. TIMP-1 might be a protectiv factor for newly formed capillaries by curbing MMP-19 acitvity (Nehls *et al.* 1992; Zhang *et al.* 2003). TIMP-1 deficient mice display reduced serum progesterone levels during CL development, which can be linked to its ability to regulate MMP action (Nothnick 2003). Additionaly to their inhibitory activities TIMP-1 and TIMP-2 stimulate the growth of endothelial cells and fibroblasts as well as other cell types unrelated to their MMP inhibitory activity (Hayakawa *et al.* 1992; Hayakawa *et al.* 1994). In the bovine, ovine and porcine CL TIMP-1 is expressed in large luteal cells during the oestrous cycle (Pitzel *et al.* 2000; Smith *et al.* 1994a; Zhang *et al.* 2003). These presence of TIMP-1 in steroidogenic cells may be associated with its ability to enhance steroid production during maturation of the CL, which could explain the higher mRNA expression in the first seven

days of CL development additionaly to its ability to inhibit MMP-19.

6.2.2.1.3 Plasminogen activator system (PAs)

Another important proteolytic system, which influences the rapid angiogenesis in the CL are the plasminogen activators tPA and uPA with its receptor uPAR and their inhibitors PAI-1 and PAI-2. Our data revealed a constant expression of tPA, which was not regulated during oestrous cycle, while uPA, uPAR, PAI-1 and PAI-2 levels were increased from 20h after GnRH onwards till the mid luteal phase, respetively till day 5-7 for PAI-1. Previous studies in the rat (Bacharach *et al.* 1992), mouse (Liu *et al.* 2003) and monkey (Liu *et al.* 1997) showed a distinct expression of uPA and PAI-1 in the developing CL. uPA was found to be expressed by endothelial cells along the route of capillary extensions, while PAI-1 was expressed in fibroblasts in the environments of uPA expressing capillary like structures. These results suggest, that PAI-1 may protect neovascularised tissues from excessive proteolysis (Bacharach *et al.* 1992; Bacharach *et al.* 1998). Interaction between uPA and its receptor appear to be mandatory for the angiogenic effect of uPA as was shown with monoclonal antibodies anti-uPA and anti-uPAR that blocked the proangiogenic effects of uPA at the endothelial cell level (Fibbi *et al.* 1998). In addition to their proteolytic impact uPA, uPAR and PAI-1 seem to play an essential role in cell migration. Both uPAR and PAI-1 are able to bind to vitronectin (Okumura *et al.* 2002), an adhesive glycoprotein in the ECM, which attachs cells to the ECM. uPA increases the affinity of uPAR to vitronectin and at the same time enables PAI-1 to compete with uPAR for the binding site on vitronectin. This process leads to endocytosis of the uPA/uPAR/PAI-1 complex and detachment of the cells from their environment, which is necessary for migration. It is thought that PAI-1 is only able to detach cells, if cell surface uPAR is increased over the engaged integrins. This hypothesis is supported by the observation that overexpression of uPAR in CHO cells (i.e. increased ratio of uPAR to integrins) led to a pronounced increase in the efficiency of PAI-1 to detach the cells from fibronectin (Czekay *et al.* 2003). A recent study (Isogai *et al.* 2001) revealed that endothelial cells escape the vitronectin rich environment of their perivascular space and penetrate the unvascularised and fibronectin rich stroma. In terms of these data it seems possible that endothelial cells in the CL expressing uPA/uPAR interact with PAI-1 secreted from fibroblast, become detached from the ECM and are now able to migrate into the unvasculated stroma to form new capillaries, which is the fundamental step to enable the supply of luteal cells. One could assume an angiogenic effect during oestrous cycle due to the high expression of uPA, its receptor uPAR and PAI-1 during the time of

angiogenesis, which is normally completed at days 5-7 of the oestrous cycle (Berisha *et al.* 2000). Like some MMPs the PAs are induced in endothelial cells upon stimulation with angiogenic factors like VEGF and bFGF (Mandriota and Pepper 1997). PAI-2 shows an equal expression pattern like PAI-1. PAI-2 is known to be expressed by monocytes (von Heijne *et al.* 1991) and eosinophils (Swartz *et al.* 2004). Eosinophils seem to play an essential role in neovascularisation of the newly formed CL (Murdoch and Steadman 1991). Immediately before ovulation a recruitment of eosinophils into the junction between the theca and granulosa cell layer of the dominant follicle takes place. Our data suggest, that this happens between 20h after GnRH and ovulation. After follicle ruptur high numbers of eosinophils gather in the former follicle antrum and are accumulating within and around dilated vessels in the developing bovine CL (Reibiger and Spanel-Borowski 2000). In sheep, eosinophils are assumed to influence angiogenesis, because cortisol induced eosinopenia causes small-sized corpora lutea with an underdeveloped vascular bed and thus insufficient endocrine function (Murdoch and Steadman 1991). Having these circumstances in mind it seems possible that the increased mRNA expression of PAI-2 is due to the invasion of eosinophils into the developing CL. The role of PAI-2 in eosinophils is not clear thus a protective function against degradation of their cell membran through uPA being released from endothelial cells seems possible.

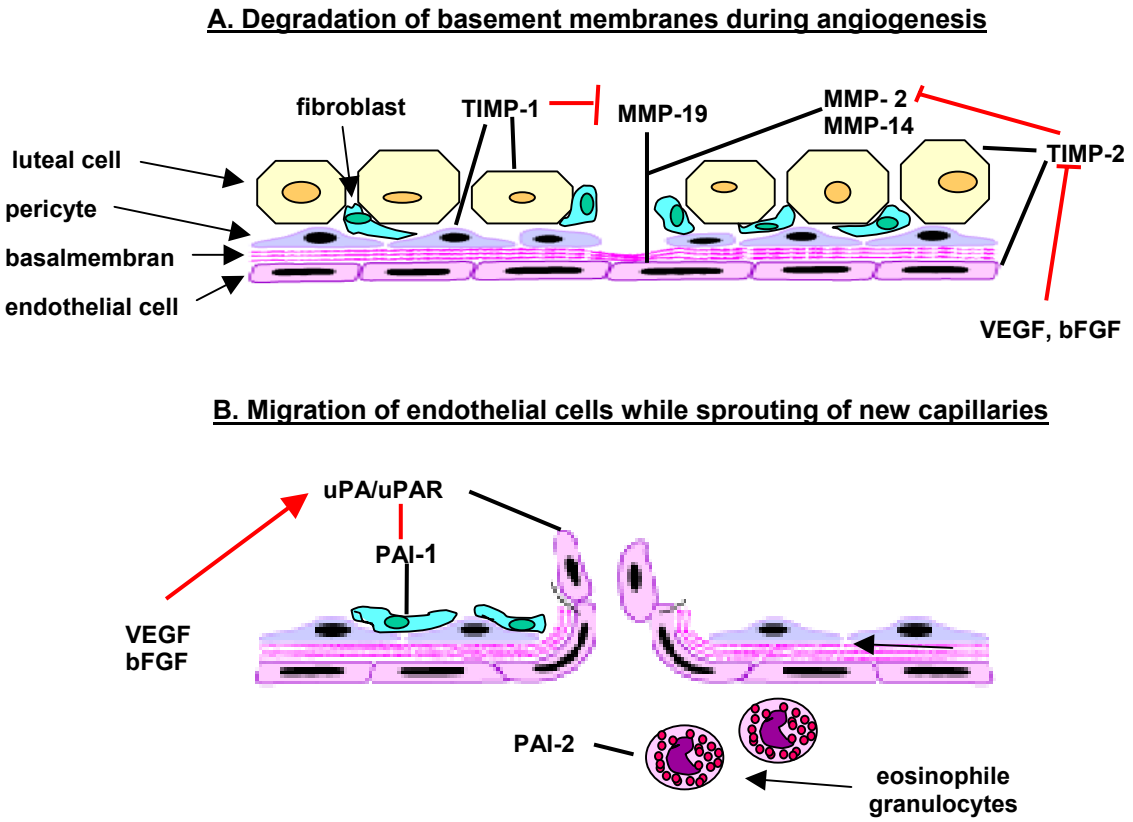


Figure 38: Schematic presentation of cells, which express matrix metalloproteases, their inhibitors and the plasminogen activator system during angiogenesis in the bovine CL.

6.2.2.2 CL during induced luteolysis

6.2.2.2.1 Matrix metalloproteases (MMPs) and tissue inhibitors of matrix metalloproteases (TIMPs):

A complete different mRNA expression pattern then during the oestrous cycle was seen for all investigated factors during PGF2α induced luteolysis. To our astonishment MMP-1 mRNA expression was 256-fold up-regulated within two hours after PGF2α application and remained constant at this high level till 64h with a peak of 1024-fold increase at 48h compared to control group. MMP-1 and the plasminogen activator system were the only

ones of the investigated factors showing an increased expression already at the beginning of luteolysis 0.5h and 2h after PGF2α injection, respectively. These synergistic expression might be linked to the infiltration of T lymphocytes and macrophages into the regression CL as was demonstrated in cattle (Penny *et al.* 1999), rat (Townson *et al.* 1996), pig (Standaert *et al.* 1991) and human (Brannstrom *et al.* 1994b). Recently, the expression of the monocyte chemoattractant protein-1 (MCP-1) by cells of the CL has been postulated as mechanism for the recruitment of monocytes/macrophages within the CL during luteolysis (Townson *et al.* 2002), which have been implicated in phagocytosis of luteal cells (Paavola 1979), degradation of ECM (Endo *et al.* 1993) and secretion of proinflammatory mediators (Benyo and Pate 1992; Rueda *et al.* 2000). Our own study of MCP-1 as well as results from other groups (Bowen *et al.* 1996; Penny *et al.* 1998; Tsai *et al.* 1997) shows a significant increase of mRNA expression from 0.5h until 64h after induction of luteolysis with a 16- to 32-fold up-regulation at 2h and 24h. MCP-1 is expressed in bovine endothelial cells in the CL as well as in fibroblasts, monocytes and T lymphocytes (Penny 2000). We hypothesise that the early increase of MCP-1 after 0.5h might be due to expression by endothelial cells, which recruit monocyctes from the circulation into the cellular compartment of the CL. Monocytes themselves express MCP-1, which could lead to a higher expression level seen from 2h onwards after induction of luteolysis. They are also thought to contribute to the loss of connective tissue component of the ECM through production of MMP-1, MMP-9, MMP-2 (Welgus *et al.* 1990) and the plasminogen activator system (Sitrin *et al.* 1994) while migrating from blood vessels into the surrounding tissue. There is evidence that collagen I, which is a major component of the ECM in the CL (Luck and Zhao 1993) induces the expression of MMP-1 in macrophages already after 2h in culture (Shapiro S.D. *et al.* 1993) and that laminin up-regulates uPA as well as MMP-9 expression after 1h and 4h, respectively (Khan and Falcone 1997). Collagen I is degraded primarily by MMP-1 whereas MMP-9 is involved in degrading collagen I that has been denatured by the initial cleavage by MMP-1 (Zhou *et al.* 2003). In context of these data the chronology of MMP-1 and MMP-9 expression during luteolysis could be explained.

Considering all these data monocytes/macrophages seem likely to play an important role in degradating the ECM in the regressing CL through their ability to secrete different proteases. There is evidence that TNFα, INFγ, IL-β and GM-CSF enhance the protein secretion of MMP-1, MMP-9, uPA and uPAR in monocytes when they are differently combined among each other (Kirchheimer *et al.* 1988; Sitrin *et al.* 1994; Zhang *et al.* 1998; Zhou *et al.* 2003). The same regulatory mechanism through inflammatory cytokines like TNFα, INFγ and IL-β can be hypothesised in the regressing CL. All of these cytokines

show an increased expression during luteolysis in cattle and are known to be produced by macrophages, lymphocytes, fibroblasts and endothelial cells all of them being abundant in the CL (Neuvians *et al.* 2004). Another MMP regulated by TNFα is MMP-2. Different groups showed in cell culture experiments that TNFα up-regulates the MMP-2 expression in luteal cells in porcine (Pitzel *et al.* 2000) and bovine (Zhang *et al.* 2005) species in a time and concentration depending manner. In bovine luteal cells mRNA expression of MMP-2 is significantily increased after 12h to 48h, which is in contrast to our *in vivo* data showing a significant up-regulation only at 48h and 64h after induced luteolysis. This could be due to lower TNFα concentration acting in the *in vivo* system than used in cell cultur. The connecting link between TNFα and MMP-2 is p53, a tumor suppressor gene, which expression is increased by TNFα (Bian and Sun 1997b; Gotlieb *et al.* 1994; Yeung and Lau 1998). p53 acts as a transcription factor on the promotor of MMP-2 and thereby increasing the MMP-2 mRNA expression (Bian and Sun 1997). We could show during induced luteolysis that the p53 mRNA expression pattern is identic to the MMP-2 expression. This congruence in mRNA expression might give evidence of a connection between ECM degradation and apoptosis of cells, which is also necessary for the regression of the CL. Detachment of cells from the ECM results in apoptosis (Alexander *et al.* 1996), whereas endothelial cells expressing MMP-2 in the regressing CL (Duncan *et al.* 1998; Ricke *et al.* 2002b; Young *et al.* 2002; Zhang *et al.* 2005) seems to be the first cells to undergo separation from the ECM and apoptosis (Modlich *et al.* 1996). At the beginning of structural regression of the CL an alteration on the vascular elements, which includes detachment of endothelial cells from the basal membrane and occlusion of small blood vessels, occurs (Davis *et al.* 2003). MMP-2 is known to degrade basement membrans and is therefore necessary for detaching endothelial cells (Moses 1997). The activation of MMP-2 is regulated through the expression of its receptor MMP-14 and TIMP-2, which either enhances or suppresses the MMP-2 activation depending on the concentration of TIMP-2 (Curry, Jr. and Osteen 2003). At the time of structural regression after 12h of induced luteolysis an up-regulation of MMP-14 and simultaneous decrease of TIMP-2 till 64h can be seen. It might be that TIMP-2 being high expressed at the midluteal phase suppresses MMP-2 activation and as recently as its expression decreases an activation of MMP-2 can occur while structural regression. Our data concerning TIMP-2 are in contrast to older findings (Juengel *et al.* 1994), who showed an up-regulation of TIMP-2 mRNA expression at 8h and a decrease at 48h after induced luteolysis. This could be due to different detection methodes. The same action as activated MMP-2 on basal membrans is thought for MMP-19, which was detected in endothelial and vascular smooth muscle cells (Djonov *et al.* 2001; Kolb *et al.* 1999). In the rat ovary the highest expression

of MMP-19 mRNA was localised in the regressing CL (Jo and Curry, Jr. 2004), which is in agreement with our mRNA expression data. TIMP-1 is able to counteract with MMP-19 (Kolb *et al.* 1999), which can be assumed till 12h after induced luteolysis when TIMP-1 is high and MMP-19 still low expressed. MMP-19 expression starts to increase after the beginning of structural luteolysis, which could potentiate the degradation of laminin and therefore the detachment and apoptosis of endothelial cells.

6.2.2.2.2 Plasminogen (PA) activator system

The plasminogen activator system, especially uPA, uPAR and PAIs, is supposed to play a important role in enabling monocytes, neutrophils and activated T cells to migrate through basal membrans by degrading laminin (Khan and Falcone 1997). Migrating monocytes polarise uPAR at the leading edge focusing the chemotactic gradient and thereby binding uPA on their specific site of the plasma membran (Plesner *et al.* 1997). It has been shown that monocytes synthesise PAI-2, which function extracellulary to limit uPA activity and thus regulate the invasive capabilities of monocytes (Estreicher *et al.* 1990). The vast majority of PAI-2 synthesized by monocytes, however, remains intracellular (von Heijne *et al.* 1991). Activation of monocytes by inflammatory agents such as TNFα induces a rapid accumulation of high levels of PAI-2 in the cytoplasma that does not appear to be correlated with uPA synthesis (Vassalli *et al.* 1992) and may play a functional role in regulating apoptotic cell death in these cells (Chapman *et al.* 1990; Dickinson *et al.* 1995). Chapman et al. (1990) revealed that macrophages also produce PAI-1, which is mainly found in the extracellular space. This indicates that PAI-1 is the major extracellular PA inhibitor which is able to retard tPA and uPA. An increased expression of tPA was recently shown in monkey and rat CL during luteolysis (Liu *et al.* 1996; Liu *et al.* 2003). The source of tPA seem to be luteal cells (Liu *et al.* 2003) and macrophages (Hart *et al.* 1989). The functional role of tPA while luteolysis is not clear, but addition of exogenous tPA to cultured rat or monkey luteal cells significantly decreased their progesterone production (Feng *et al.* 1993; Liu *et al.* 1995). This suggests that tPA might be not only a molecule that participates in the extracellular matrix degradation during luteal tissue regression, but also a molecule that may have other activities to inhibit progesterone synthesis via possible autocrine and paracrine pathways.

6.2.3 Apoptosis in the CL during oestrous cycle and induced luteolysis

6.2.3.1 CL during oestrous cycle

6.2.3.1.1 Fas antigen (Fas) and Fas-Ligand (FasL)

The Fas antigen is a member of the tumor necrosis factor family of cell surface receptors (Nagata and Golstein 1995) and engagement of Fas with its ligand induces apoptosis. FasL is expressed at high levels on activated T lymphocytes (Suda *et al.* 1993) and mediates apoptosis of target cells (Nagata and Golstein 1995). Expression of Fas and FasL mRNA has been demonstrated in CL of mice, rats and humans (Sakamaki *et al.* 1997; Roughton *et al.* 1999; Kondo *et al.* 1996). Moreover, FasL has been shown to enhance mouse structural luteolysis *in vivo* (Sakamaki *et al.* 1997). The present study demonstrates that Fas mRNA expression is not regulated during the first twelve days of oestrous cycle as is in agreement with the data of Taniguchi et al. (2002), who found an up-regulation only during regression of the bovine CL. Fas is expressed on human granulosa cells (Quirk *et al.* 1995) as well as on bovine (Taniguchi *et al.* 2002) and mouse luteal cells (Quirk *et al.* 2000b), which became only sensible to Fas mediated apoptosis when treated with INFγ alone or with INFγ in combination with TNFα. These cytokines are expressed by activated T cells and macrophages (Pate 1995), which are located in the bovine CL during the whole oestrous cycle. But an activation of these cells with a greater increase in numbers occurs not before day 14 (Penny *et al.* 1999). Also progesterone and growth factors like insulin-like-growth-factor (IGF) suppress the up-regulation of Fas expression, proving an anti-apoptotic function of growth factors and progesterone (Kuranaga *et al.* 2000; Quirk *et al.* 2000). Our data reveal that FasL is significantly up-regulated on day 8-12, which could be interpreted as a slight increased numbers of invading T cells.

6.2.3.1.2 Tumor necrosis factor alpha (TNFα), TNF receptor 1 and 2 (TNFR1, TNFR2)

The expressions of TNFα and its receptors, TNFR1 and TNFR2, show a equaly higher level during the midluteal phase compared to the early luteal phase in the bovine CL. TNFα is known to have luteotropic as well as luteolytic functions in the CL, depending on its concentration. A high dose of TNFα leads to a prolongation of the luteal lifespan, whereas a low dose induces luteolysis and shortens the oestrous cycle (Skarzynski *et al.* 2003). TNFα, TNFR1 and TNFR2 are evident in the early stage CL in cows (Sakumoto *et al.* 2000; Skarzynski *et al.* 2003), pigs (Wuttke *et al.* 1997; Miyamoto *et al.* 2002) and

human (Vaskivuo *et al.* 2002). TNFα is a potent stimulator of luteal prostaglandins (PGs) including PGF2α and PGE2 (Benyo and Pate 1992; Sakumoto *et al.* 2000). Luteal PGs are known to stimulate progesterone secretion from bovine CL *in vitro,* suggesting that they have luteotropic abilities (Miyamoto *et al.* 1993). Macrophages (Zhao *et al.* 1998, Hehnke-Vagnoni *et al.* 1995) and endothelial cells are sources of TNFα, and these cells infiltrate into newly formed CL concomitant with vascular angiogenesis (Reynolds *et al.* 1994). These findings could be the reason, why the mRNA expression of TNFα elevates during the development of the CL. Thus it could be assumed that TNFα contributes to the production of PGs in the early CL and may partly promote the formation of the CL. Additional studies in human luteinised granulosa cells support a luteotropic role of TNFα (Yan *et al.* 1993). This effect is not limited to steroidogenic cells since TNFα is capable of stimulating PGE2 secretion in bovine luteal endothelial cells (Okuda *et al.* 1999). Since both TNFα (Leibovich *et al.* 1987) and PGE2 (Schror 1985) are known to affect the proliferation of endothelial cells, both of these factors may have a regulatory influence on the proliferation of endothelial cells during the luteal development. The action of TNFα is mediated through its two receptors TNFR1 and TNFR2 (Okuda *et al.* 1999). In the bovine CL TNFR1 is expressed by small and large luteal cells as well as by endothelial cell. In these cells the expression was significantly higher as in the steroidogenic cells (Friedman *et al.* 2000). TNFR1 is increasing in the same pattern as TNFα during oestrous cycle, which might be due to the proliferative stage of the endothelial cells during angiogensis in the early CL. Our datas are in contrast to earlier findings of Sakumoto et al. (2000), who could show an increased expression during days 3-7 followed by a decrease at day 8-12 to the level seen at days 1-2. A higher sensitivity of the real time PCR compared to the block PCR could be the reason for these differences. TNFR2 is also expressed in bovine endothelial cells in the CL (Okuda *et al.* 1999) as well as in T cells and macrophages (Carpentier *et al.* 2004; Mukhopadhyay *et al.* 2001). In macrophages both receptors seem to be required for TNFα induced proliferation (Mukhopadhyay *et al.* 2001). If TNFR2 is also needed for endothelial cell proliferation is not known. The increased expression during oestrous cycle could be a sign of infiltration of macrophages into the developing CL.

6.2.3.1.3 p53, Bax and Bcl-X_L

The mRNA expression of p53 and Bax showed no significant regulation during oestrous cycle in the bovine CL. There are no data available how p53 is regulated in other species during oestrous cycle. But different groups reported a regulated expression of p53 in bovine luteal cells, human and rat granulosa cells during apoptosis (Nakamura and Sakamoto 2001; Herr *et al.* 2004; Amsterdam *et al.* 2003). It seem unlikely that apoptosis occurs during the the first seven days of oestrous cycle being the time were massive formation of new capillaries occurs (Berisha *et al.* 2000) as well as during days 8-12, when the CL reaches its highest secretory abilities. bFGF seems to have a suppressive action on the expression of p53 in human granulosa cells. It enhances MDM2 expression, which blocks p53 activity (Amsterdam *et al.* 2003). VEGF as angiogenic factor is also linked with p53. Wild type p53 is able to decrease VEGF expression in human leiomyosarcoma cells and to inhibit angiogenesis, which is very improtant for the development of the CL (Zhang *et al.* 2000). These data could explain, why p53 is not regulated during oestrous cycle. p53 is associated with the mitochondrial pathway during apoptosis and increases the expression of Bax, an apoptotic factor of the intrinsic pathway, and by the other hand decreases the Bcl-2 expression, which is an anti-apoptotic factor that inhibits the action of Bax (Miyashita *et al.* 1994; Miyashita and Reed 1995). Bax was found to be expressed in human granulosa and theca cells, bovine luteal cells and rat granulosa cells (Rodger *et al.* 1998; Liszewska *et al.* 2005; Tilly *et al.* 1995). We showed that Bax is not regulated during oestrous cycle as well as its enhancer p53 in the bovine CL, which was also found in the human CL during early and midluteal phase (Sugino *et al.* 2000). There is evidence that progesterone not only regulates Fas expression, but also prevents an up-regulation of Bax in bovine luteal cells during early to mid luteal phase. The same result is seen for PGE2, which stimulates the progesterone secretion in bovine CLs and so might prevent apoptosis (Liszewska *et al.* 2005). How progesterone regulates the expression of Bax is not clear, but there seems to be a possible correlation with the progesterone receptor (Marone *et al.* 1998). The opponents of Bax are either Bcl-2 or Bcl-X_L, which are forming heterodimers with Bax and preventing the activation of capsase3 through the mitochondrial pathway (Hengartner 2000). Bcl-X_L was found in rat granulosa cells and in the bovine corpus luteum during structural luteolysis (Tilly *et al.* 1995; Rueda *et al.* 1997). As for Bax, progesterone plays a regulatory role in Bcl-2 expression in bovine luteal cells. It increases the Bcl-2 expression and prevents apoptosis of these cells (Liszewska *et al.* 2005). We were not able to detect Bcl-2 cDNA in the bovine CL despite the use of serveral primer pairs, which were designed using the bovine Bcl-2 sequence

(acc. no. XM_612457), which was also used by Liszewska et al. (2005). The same experience was made by Rueda *et al*. (1997), who decided to use Bcl-X_L instead. These Bcl-2 familiy member has the same anti-apoptotic functions like Bcl-2, so that a comparable regulation could be assumed. If progesterone up-regulates Bcl-X_L in the same manner like Bcl-2, the increase of the Bcl-X_L expression during the midluteal phase could be explained by a higher progesterone level compared to the early luteal phase. Other regulatory factors of Bcl-2 and Bcl-X_L are bFGF and VEGF, which were found to increase the expression of these factors in endothelial cells (Pardo *et al.* 2002; Del Bufalo *et al.* 2003). Inhibition of Bcl-X_L with antisense oligonucleotides lead to an antiangiogenic effect with a decrease in endothelial cell proliferation (Del Bufalo *et al.* 2003).

6.2.3.1.4 Smac and Survivin

Pro-angiogenic factors seem to play a critical role in the regulation of not only p53, Bax and Bcl-X_L, but also of Smac and Survivin. Angiopoietin-1 (Ang-1) promotes endothelial cell survival during vascular development and maintains the vascular integrity (Suri *et al.* 1996; Thurston *et al.* 2000). Recent findings show that Ang-1 plays an important role in the modulaton of angiogenesis in the human and bovine CL during oestrous cycle (Wulff *et al.* 2000; Tanaka *et al.* 2004). It was found that Ang-1 is able to suppress the expression of Smac and to enhance the expression of Survivin in serum deprivated endothelial cells, whereas cell survival is mediated. These effects were only seen, when Ang-1 interacted with Tie-2 and not with integrins (Harfouche *et al.* 2002). Minimal detectable levels of Survivin are found in quiescent endothelial cells, whereas an abundantly expression is seen in endothelial cells of three-dimensional vascular tubes *in vitro* and in newly formed capillaries during angiogenesis *in vivo*. Survivin expression can be stimulated by VEGF (O'Connor *et al.* 2000) and bFGF (Tran *et al.* 1999), which is consistent with the cell cycle dependent expression of the survivin gene in G2/M phase (Li *et al.* 1998) and suggests that this pathway maintain a critical anti-apoptotic threshold at cell division. bFGF is not only able to increase Survivin, but also to decrease the release of Smac from the mitochondria of lung cancer cells (Pardo *et al.* 2003). Survivin regulates cell survival during angiogenesis either by binding to Smac, which is then not able to inhibit XIAP (Song *et al.* 2003), or by inhibiting caspase3 in a direct or indirect way (Johnson *et al.* 2002). Having this in mind it seems possible that the up-regulation of Survivin during the first seven days of the oestrous cycle is related to the massive angiogensis occuring during this time.

6.2.3.1.5 Caspase3, -6 and -7

The caspases 3, -6 and -7 belong to the so called effector caspases, which are able to cleave specific substrates during apoptosis including poly (ADP-ribose) polymerase, DNA-dependent protein kinase and structural proteins like actin and lamins (Rosen and Casciola-Rosen 1997). These caspases play an essential role in the regression of the CL as was seen in caspase3 deficient mice (Carambula *et al.* 2002). Not many data can be found about their expression, especially of caspase6 and -7, during oestrous cycle in different species. It is reported, that caspase3 protein is constantly expressed through out the oestrous cycle in the human CL (Vaskivuo *et al.* 2002). In the macacque CL immunohistochemistry revealed a constant expression of caspase3 during early and mid lutal phase with a higher expression at the onset of regression. The mRNA expression was not regulated during the whole menstrual cycle (Peluffo *et al.* 2005). This confirmes our data, which showed a not regulated expression of caspase3 during the first twelve days of the oestrous cycle. D'haeseleer *et al*. (2006) also found an expression of caspase3 in small and large luteal cells during diffurent stages of the bovine oestrous cycle- which were not significantly regulated. In the human CL only few cells are found to undergo apoptosis during early and mid luteal phase (Vaskivuo *et al.* 2002), which could be also possible for the bovine CL, so that no regulation of caspase3 and -7 can be found. Caspase6 is significantly up-regulated from the early to the mid luteal stage, which one could see as gradual progress of accumulation of mRNA for the following regression of the CL. Studies with non-pregnant and pregnant ewes showed, that such a accumulation of mRNA is not needed for caspase3 (Rueda *et al.* 1999b). Progesterone is thought to be one of the suppressors of caspase3 activation, because a specific progesterone antagonist increased the caspase3 mRNA level in bovine luteal cells of the mid luteal phase (Okuda *et al.* 2004). The same action on caspase3 activation has VEGF. It was shown that a VEGF antagonist induces apoptosis and caspase3 activation in endothelial cells of the CL in marmoset monkies (Fraser *et al.* 2005). The highest levels of VEGF mRNA and protein is seen during the first seven days with a decline in expression in the mid and late luteal stage of the bovine CL (Berisha *et al.* 2000).

6.2.3.2 CL during induced luteolysis

Structural luteolysis is a complex process that eliminates the CL from the ovary. There is evidence that structural luteal regression in the cattle occurs via apoptosis (Juengel *et al.* 1993; Zheng *et al.* 1994). This has also been demonstrated in the regression CL of rabbits (Dharmarajan *et al.* 2004), sheep (Rueda *et al.* 1995), rats (Orlicky *et al.* 1992) and

humans (Shikone *et al.* 1996). Two major intracellular apoptosis signaling cascades have been charactarised, the extrinsic pathway via the death receptors and the intrinsic pathway via factors released from the mitochondria.

6.2.3.2.1 Extrinsic pathway

Apoptosis can be initiated via several cytokine receptors including the TNF superfamily receptors (TNFRs). TNFα is acting via TNFR1, which is expressed on steroidogenic (Petroff *et al.* 2001) and endothelial cells (Friedman *et al.* 2000) of the bovine CL, and TNFR2, which was demonstrated to be expressed on the granulosa cells of porcine ovaries (Nakayama *et al.* 2003) and bovine endothelial cells (Okuda *et al.* 1999). Cytokines, especially TNFα and INFγ, produced by immune cells, which infiltrate the CL during luteolysis, seem to participate in the regression of the bovine CL (Pate and Landis 2001). The number of leukocytes (cytotoxic T cells and macrophages) increases at the time of luteolysis (Penny *et al.* 1999; Townson *et al.* 2002). Subluteolytic release of PGF2α from the uterus acts on T cells (Penny *et al.* 1999) and endothelial cells (Townson *et al.* 2002) to promote the monocyte chemoattractant protein-1 (MCP-1) production during the late luteal phase. MCP-1 is a member of the chemokine family of cytokines that is involved in leukocyte physiology and chemotaxis (Penny 2000). Provement that PGF2α is involved in the regulation of MCP-1 is given by a study in which PGF2α production was inhibited between day 16 and 18 (around natural time of luteolysis) of the oestrous cycle in cattle. No increase in MCP-1 expression or macrophage infiltration was observed in the animals in which luteolysis was inhibited (Penny 2000). These findings could explain the rapid up-regulation of MCP-1 expression seen after 0.5h of PGF2α injection. This is in agreement with data from Tsai et al. (1997), where MCP-1 expression was increased 1h, 4h and 24h after PGF2α treatment in the CL of ewes. Luteal endothelial cells may express MCP-1 in response to a product from steroidogenic luteal cells or eosinophils (Murdoch 1987). Reactive oxygen species are produced as an early response to PGF2α (Sawada and Carlson 1991) and these products have been found to stimulate MCP-1 expression in endothelial cells (Kilgore *et al.* 1997; Xu *et al.* 1996). In response to the MCP-1 action there is an influx of macrophages and cytotoxic T cells into the CL, which may also express MCP-1 and explain the further increase seen at 24h after PGF2α treatment. Macrophages and T cells secrete TNFα and INFγ (Pate and Landis 2001), which is in agreement with our data, where a rapid increase of TNFα can be seen from 0.5h after the beginning of induced luteolysis with the highest expression at 2h, which declines to a still

significantly increased expression level at 64h compared to control group. TNFα acts by binding to its two receptors TNFR1 and TNFR2, which are both expressed in bovine endothelial cells in the CL (Friedman *et al.* 2000; Okuda *et al.* 1999). Friedman et al. (2000) found that TNFR1 is higher expressed in endothelial cells than in steroidogenic cells and that apoptosis was seen in these cells after 6h incubation with TNFα. There is evidence that capillary regression and endothelial cell death is a hallmark of PGF2α induced luteal regression, these include studies in the pig (Bacci *et al.* 1996), guinea pig (Azmi and O'Shea 1984), sheep (Rueda *et al.* 1995) and human CL (Gaytan *et al.* 1999). Steroidogenic luteal cells were found to undergo apoptosis at a later stage, when a massive degeneration of capillaries had already occurred (Juengel *et al.* 1993; Bacci *et al.* 1996). We found a constant, but not regulated expression of TNFR1 during induced luteolysis as well as a significantly increased expression of TNFR2 from 2h till 64h. It could be that apoptosis of endothelial cell in the CL is triggerd through the interaction of TNF with both receptors as it was described by Fotin-Mleczek *et al.* (2002). The prestimulation of TNFR2, compared to the expression of TNFR1, can lead to its binding of tumor necrosis factor receptor-associated factor-2 (TRAF2), which is a necessary factor for the activation of the anti-apoptotic NFκB pathway by TNFR1, and lead to apoptosis of endothelial cells. Pru et al. (2003) reported that TNFα mainly acts on microvascular endothelial cells of the bovine CL, which induces apoptosis potentiated by INFγ. It was shown that progesterone is able to inhibit apoptosis in endothelial and luteal cells (Friedman *et al.* 2000; Rueda *et al.* 2000a), suggesting that progesterone may serve as a regulator of luteal cell survival. The decrease of progesterone during luteolysis is initiated by PGF2α, which also increases the production of endothelin-1 (ET-1) (Meidan *et al.* 1999) and angiotensin (Schams *et al.* 2003) in endothelial cells. ET-1 augments PGF2α induced inhibition of progesterone synthesis and then may act by stimulation of TNFα secretion from macrophages (Meidan *et al.* 1999), thereby promoting the apoptotic cascade in cells of the CL. TNFα may also induce a further increase of ET-1 (Woods *et al.* 1999) at the time of luteolysis. If progesterone is able to regulate the TNFRs is not known up to date, but there is evidence that the mRNA expression of Fas, another death receptor belonging to the TNFR familiy, can be increased by a progesterone antagonist onapristone (OP) in cultured bovine luteal cells (Okuda *et al.* 2004). This was also found in rat luteal cells, where progesterone suppresses prolactin induced steroidogenic cell apoptosis via a reduction of the Fas expression levels (Kuranaga *et al.* 2000). This could be in agreement with our findings that the expression of Fas is steadily up-regulated from 2h onwards till 64h. Fas induces apoptosis in luteal cells (Taniguchi *et al.* 2002; Kuranaga *et al.* 2000) by binding of Fas-Ligand (FasL), which is expressed on cytotoxic ($CD8^+$) T

cells (Suda *et al.* 1993). Expression of Fas and FasL has been demonstrated in the CL of mice, rats and humans (Sakamaki *et al.* 1997; Roughton *et al.* 1999; Kondo *et al.* 1996). The highest protein expression of Fas was found during regression of the CL in rats (Roughton *et al.* 1999). Moreover, FasL has been shown to enhance mouse structural luteolysis *in vivo* (Sakamaki *et al.* 1997), while Kuranaga et al. (2000) found that FasL expression on immune cells are necessary to induce apoptosis in rat luteal cells. But it was also found that FasL alone has no cytotoxic effect on luteal cells. However, treating luteal cells with FasL in combination with OP resulted in killing of 55% of the cells, while with OP alone only 30% were killed (Okuda *et al.* 2004). Other cytokines may also initiate and escalate Fas apoptotic action in the CL. The expression of Fas mRNA was increased by INFγ, and TNFα augmented the stimulatory action of INFγ on Fas expression. Moreover, apoptotic bodies were observed in luteal cells treated by FasL in the presence of INFγ and/or TNFα, showing that leukocyte-derived TNFα and INFγ play important roles in FasL/Fas mediated luteal cell death in the bovine CL (Taniguchi *et al.* 2002). The increase of Fas seen during induced luteolysis can therefore be enabled by the decrease in progesterone and further elevated through the secretion of TNFα and INFγ by invading macrophages, whereas the increased expression of FasL might be due to the infiltration of the CL by cytotoxic T cells.

6.2.3.2.2 Intrinsic pathway

The intrinsic apoptotic signaling cascade is generally thought to be activated by apoptotic stimuli that originate within a cell in response to certain drugs, radiation or growth factors withdrawal and primarily cause changes in mitochondrial permeability through alterations in the ratio of pro- and anti-apoptotic Bcl-2 family members (Adams and Cory 1998). There is evidence that the activation of luteal cell death during luteolysis can be triggered by oxidative stress (Rueda *et al.* 1995a). PGF2α increases the free intracellular calcium concentration, activates protein kinase C and affects progesterone production by inhibiting cholesterol transport or 3β-hydroxysteroid dehydrogenase (3β-HSD) expression (Niswender *et al.* 1994). PGF2α also increases the production of reactive oxygen species (ROS) (Riley and Behrman 1991; Sawada and Carlson 1991; Aten *et al.* 1992; Shimamura *et al.* 1995), while progesterone inhibits ROS production (Sugino *et al.* 1996). Decreased blood flow in the CL during regression also activates a xanthine-xanthine oxidase system and produces ROS in the presence of oxygen (Sugino *et al.* 1993). The mononuclear phagocytes produce ROS in the CL throughout the luteal phase, but this production is inhibited during the mid luteal phase by high concentrations of progesterone

(Sugino *et al.* 1996). When ROS overcomes the anti-apoptotic system, nuclear translocation of p53 is known to be induced by H_2O_2 (Uberti *et al.* 1999) and p53 induced apoptosis requires the generation of ROS (Li *et al.* 1999). It was shown that bovine luteal cells treated with H_2O_2 underwent apoptosis with an increased mRNA expression of p53 and Bax (Nakamura and Sakamoto 2001). This indicates that p53 and Bax are increased by ROS, which production is induced by PGF2α and inhibited by progesterone. It was also found that progesterone decreases Bax expression (Liszewska *et al.* 2005), which might not be possible during the beginning of structural luteolysis, when the progesterone level has declined. Our data show an increased mRNA expression of p53 and Bax from the beginning of the structural luteolysis at 24h to 64h after PGF2α treatment, while the anti-apoptotic factor Bcl-X_L only shows a slight increase from 48h to 64h. This up-regulation of p53 might be due to an increase in oxidative stress and the beginning of p53 accumulation in the nucleus of the compromised cells (Kastan *et al.* 1991). The expression of Bax shows a higher and earlier increase then Bcl-X_L, which might indicate a insufficient inhibition of Bax and therefore an activation of the intrinsic pathway leading to apoptosis of luteal cells. An up-regulation of Bax and a constant expression of Bcl-2 mRNA and protein was found in buffalo CL 4h after PGF2α treatment (Yadav *et al.* 2002). In this study a different PGF2α analogon in a higher dose was used, which might explain the more rapid increase in expression. Nevertheless it was also reported that p53 and Bax are higher expressed in the regressing than in the bovine CL during pregnancy and that the level of Bcl-X_L was unchanged (Rueda *et al.* 1997). The same was seen in the human CL (Sugino *et al.* 2000). The both opponents Smac and Survivin are expressed in the bovine CL during induced luteolysis, but Smac is not regulated, while Survivin shows a down-regulation during the first twelve hours of luteolysis. It seems that Smac as pro-apoptotic factor might be posttranscriptionally regulated, which needs to be further evaluated. Survivin is only up-regulated during cell cycle, otherwise it is constantly expressed. The down-regulation during functional luteolysis is not explainable to date.

6.2.3.2.3 Caspase3, -6 and -7:

Both apoptotic pathways lead to the activation of certain effector caspases. The intrinsic pathway leads to the activation of caspase3, -6 and -7, whereas the initiator caspase9 activates caspase3 and -7. In the extrinsic pathway the activation of caspase3 is made by caspase8, if it also activates caspase7 is not known. Caspase6 is activated in both pathways through caspase3, which is able to cleave caspase8 in positiv feedback loop.

These feedback is also seen for caspase3 by cleaving caspase9 (Slee *et al.* 1999). Among the 11 caspases presently known, the vast majority of data support a fundamental role for caspase3 in proteolytic disruption of cellular homeostasis and the ultimate dismantling of the cells destined for apoptosis (Nicholson *et al.* 1995; Casciola-Rosen *et al.* 1996; Wang *et al.* 1994). Caspase3 was found to be expressed in various cells in the ovary of different species (Carambula *et al.* 2002; Vaskivuo *et al.* 2002; Boone and Tsang 1998; Rueda *et al.* 1999; Yadav *et al.* 2005; Peluffo *et al.* 2005; Johnson and Bridgham 2000). It was found, that caspase3 mRNA expression was increased during PGF2α induced luteolysis in the ovine CL after 12h and that these increase was followed by a significant elevation of activity of caspase3. Also no changes in the mRNA expression was seen on day 12 or day 14 of the oestrous cycle or pregnancy. Thus, if induction of caspase3 is an important factor in luteolysis, changes in caspase3 mRNA level appear to thightly coincide with the actual initiation of luteolysis as opposed to being the result of a gradual process of accumulation throughout the luteal phase (Rueda *et al.* 1999). These findings are in agreement with our data, where the caspase3 mRNA expression also increases from 12h till 64h after PGF2α and was not regulated during oestrous cycle. This might indicate, that the activity of caspase3 could be equally in the cow as in the sheep CL during luteolysis. Yadav et al. (2005) report that caspase3 activation was highly increased 18h after induction of luteolysis. In the monkey CL capsase3 protein expression was highest during the regression phase (Peluffo *et al.* 2005) and caspase3 deficient mice show a resistant to PGF2α induced apoptosis (Carambula *et al.* 2003) that further confirms the involvement of these caspase in luteal cell apoptosis. Matikainen et al. (2001) showed that apoptosis in granulosa cells from caspase3 knock out mice occur later than in wild typ mice, which might be due to the stimulatory activation of caspase7 in the mitochondrial pathway. Caspase7 is later up-regulated in granulosa cells of wild type mice than caspase3, which could explain the later onset of apoptosis in the knock out mice. This might also explain the late up-regulation of caspase7 seen in the bovine CL starting at 24h with an immense increase of 23-fold at 64h. Not many data are available about caspase6 expression in ovarian cells. Only Johnson and Bridgham (2000) reported capsase6 mRNA and protein expression as well as enzyme activity in granulosa and theka cells of hen follicles. Caspase6 is activated by caspase3, both showing similar mRNA expression patterns during induced luteolysis in the bovine CL.

The infiltration of macrophages and T cells seems to be a necessary step in the induced luteolysis of the bovine CL, because of their ability to degradate components of the ECM by expressing matrix metalloproteinases and the plasmingen activator system, and through their induction of apoptosis in endothelial and luteal cells.

6.2.4 Conclusion

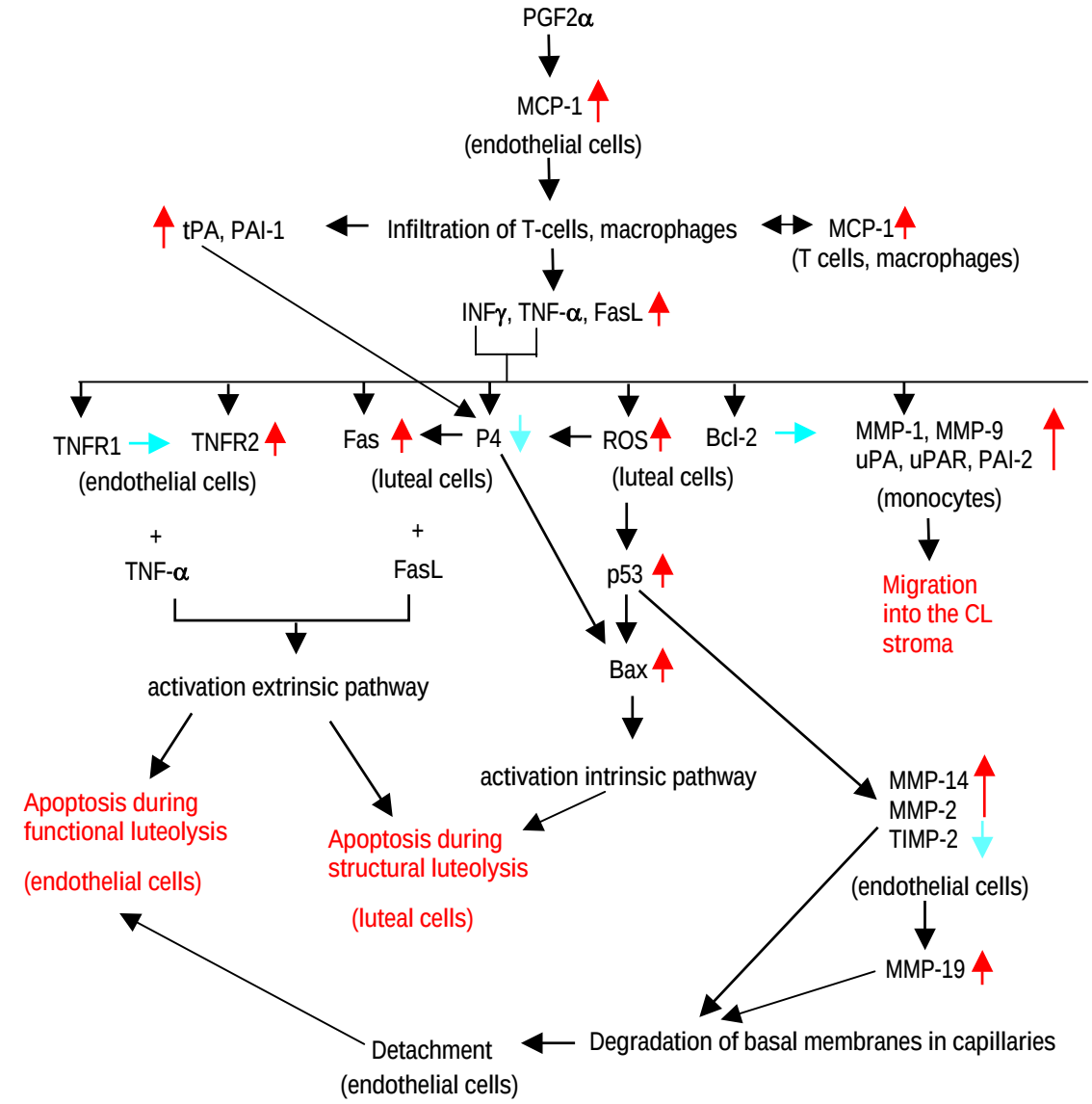
It can be ascertained by reviewing our findings and the data published by other groups that ECM degrading proteases play a critical role in the successful angiogenesis during development of the CL after ovulation. VEGF and bFGF seem to be important regulatory factors in these process. They are able to stimulate the expression of MMP-1, MMP-2 in luteal endothelial cells, which enable these cells to degrade the vascular basement membranes for sprouting new capillaries. MMP-19 is also expressed by these cells and has the same action like MMP-2 on basement membranes. The action of MMP-2 is not only regulated by VEGF and bFGF, but also by TIMP-2, which is lower expressed during angiogenesis in the CL and therefore might not block the action of MMP-2. TIMP-1 and TIMP-2 seem to be not only necessary for the inhibition of MMPs, but they are also able to enhance the proliferation of endothelial cell during angiogenesis. VEGF and bFGF also stimulate the expression of uPA in endothelial cells during angiogenesis. By forming a complex with uPAR and PAI-1 on the outer cell membrane these cells might be now able to detach from the vitronectin rich perivascular region and migrate towards the fibronection rich stroma of the CL in order to form new capillaries.

Not only angiogenesis plays a critical role during CL formation, but also the inhibition of apoptotic action during vessel sprouting. Progesterone, bFGF and VEGF seem to inhibit an increased expression of apoptotic factors like p53 and Bax, while progesterone also inhibits Fas and caspase3. Addtitionally to the suppression of apoptotic factors an up-regulation of anti-apoptotic factors like Bcl-$X_{L.}$ and Survivin occurs. TNFα, produced by invading macrophages during CL formation, acts not only as an apoptotic factor, but is also able to enhance the production of PGE2 in endothelial and luteal cells, which stimulates together with TNFα the proliferation of endothelial cells during luteal angiogenesis.

During regression of the CL the inhibitory action of progesterone, VEGF and bFGF vanish. PGF2α as luteolytic agents leads to the expression of MCP-1 on luteal endothelial cells. Thereupon monocytes and cytotoxic T cells migrate into the regressing CL and also start to express MCP-1 to recruite further mononuclear cells. For these migration into the stroma of the CL monocytes express uPA, uPAR and PAI-2. Macrophages are necessary for the phagocytosis of apoptotic cells and the degradation of ECM components in the CL during luteolysis. They are able to express different MMPs like MMP-1, MMP-9 and MMP-2. These MMPs degrade collagen I, the main structur collagen in the stroma of the CL,

which might enhance the migration abilities of these macrophages. MMP-2 is also up-regulated in microvascular endothelial cells by TNFα, which is produced by macrophages. At the same time a decrease of TIMP-2 occurs, so that no inhibition of MMP-2 happens and a detachment of the endothelial cells from the basement membrane follows. This leads to apoptosis of the endothelial cells, which seem to be the first cells to undergo cell death during luteolysis of the CL.

Endothelial cells express TNFR1 and TNFR2 on there surface during luteolysis, which leads to the activation of the extrinsic apoptotic pathwas by binding of TNFα. INFγ enhances these apoptotic action on endothelial cells. It seems possible that apoptosis of luteal cells occurs after the degradation of capillaries, which leads to an undersupply with oxygen and an increase of ROSs. These reactive oxygen spieces up-regulate p53 and Bax as apoptotic factors of the intrinsic pathway, which leads to an activation of the caspases and apoptosis of luteal cells. These apoptotic action is further enhanced by the activation of the extrinsic pathway. Luteal cells stimulated by TNFα and INFγ express Fas on their cell surface when the progesterone level declines. Cytotoxic T cells, which also migrate into the CL during luteolysis, express FasL and promote luteal cell death by activation the extrinsic pathway. This happens only when the progesterone level is decreased, which is known to happen at the end of the functional luteolysis (fig. 39).



↔ one action causes the other and vice versa

→ leads to the following action

↑ increased expression

↓ decreased expression

→ not regulated

\+ complex formation

Figure 39: Survey of the procedures during luteolysis of the CL concerning apoptotic factors, MMPs and PA system members.

7 Summary

The study is subdivided into two different parts: the first part deals with the development of a method to gain uterus milk *in vivo* during the preimplantation periode in cattle for the investigation of regulatory factors. The second part investigates different proteases in bovine follicles 20 hours after GnRH (Gonadotropin releasing hormone) injection (shortly bevor ovulation) for comparable as well as in the corpus luteum (CL) during oestrous cycle and induced luteolysis. In addition apoptotic as well as anti-apoptotic factors were evaluated in the CL during oestrous cycle and induced luteolysis.

For the development of a method for gaining uterus milk *in vivo* during the first 24 days of gravidity in cattle, nine heifers were cycle synchronised using the Ovsynch method and artificially inseminated. Before flushing an epiduralanaesthesia was given and both uterus horns were flushed with 13ml 0.9% NaCl using a balloon embryo transfer catheter at day 5, 7, 12, 17 and 24 of gravidity. The catheter was placed 1cm cranial to the bifurcatio uteri in both horns. It was possible to retrive between 3ml and 13ml of the used flushing fluid. The uterus milk from the ipsilateral horn was inspected for an embryo and an EDTA-stabilisator was given to the uterus milk of both horns. An infection of the uterus occured in three heifers after the second and in five heifers after the third flushing. In one heifer no infection was found. Between day 17 and day 24 all heifers showed clear signs of oestrus. It was possible to detect progesterone, oestradiol-17β, PGF2α and VEGF via enzyme immunoassay (EIA) and radio immunoassay (RIA), respectively. Because of the occurred infection no statistic analysis was made. But it could be seen that the level of progesterone ranged between <0.1 and 0.4ng/ml, whereas no correlation to the inflammation was found. The values of the native uterus milk should be about 10-fold higher, because of the diluting effect of the flushing fluid. The pattern of oestradiol-17β was erratic, because the oestradiol levels of the flushing fluid from heifers with no infection till day 12 of gravidity could not be related to each other. It could be shown in further tests that high oestradiol-17β levels were measured via EIA in 0.9% NaCl with EDTA-stabilisator compared to 0.9% NaCl without the stabilisator. VEGF revealed levels between 0.08 and 2.58ng/ml, whereas the higher levels were seen during inflammation. The level of PGF2α showed a clear correlation to the infection. But there were also differences seen between the different heifers or even between the two uterus horns of one animal during the first flushing, which might be due to the massage of the uterus horns while flushing, which can induce prostaglandin secretion. The method of gaining uterus milk *in vivo* should be changed as follows: to prevent an endometritis just one flushing should be done per gravidity with gaining of the embryo, so that the heifer can be inseminated again after two oestrous cycle. Again both horns should be flushed, whereas one sterile disposable catheter should be used for each horn and the flushing should be devided into two parts: first flushing with 13ml of sterile 0.9% NaCL for biomolecular methodes, then flushing of the embryo using 5 x 50ml sterile PBS buffer. The embryo should be frozen in RNA-Later at -80° for further investigations. The received flushing fluid should be separated in two aliquotes, whereas one is used for the oestradiol-17β measurement and must not contain the

EDTA-stabilisator. These two aliquotes should be centrifugated and the fluid should be stored seperatly at –20° from the received cell pellet. Part of the cell pellet can be used for the identification of the cells found in the uterus lumen and the rest stored in RNA-Later at –80° for further biomolecular investigations. For the evaluation of prostaglandins in the uterus milk it should be considered to treat the heifers with a prostaglandin synthetase inhibitor shortly before flushing.

For the investigations of different extracellular matrix proteases and apoptotic as well as anti-apoptotic factors in bovine follicles and CL, RNA samples of a previous experiment were taken. In these experiment 26 cows received luteolytic dosis of a PGF2α-analogon and the CLs were removed by transvaginal ovaryectomy 0, 2, 4, 12, 24, 48 and 64 hours after induction of luteolysis. Additionally to these experiment five more cows were ovarectomised and the CLs were taken 0.5 hours after PGF2α treatment. For the investigation during oestrous cycle CL samples were collected at the slaughter house. For comparable reasons also follicles 20 hours after GnRH injection were taken using a superovulation model. The mRNA quality was inspected using the Bioanalyzer and good to very good results were seen for all samples. The mRNA expression levels were detected using quantitativ real time PCR (Rotor-Gene with SYBR Green I). The expression datas were normalised with the Bestkeeper index of four housekeeping genes using the $\Delta\Delta$ CP method. The matrix metalloproteases (MMP-1, MMP-2, MMP-9, MMP-14, MMP-19), their tissue inhibitors (TIMP-1, TIMP-2), the plasminogen activator (PA) system (tissue –PA (tPA), urokinase-PA (uPA), urokinase-PA-receptor (uPAR), PA-inhibitor–1(PAI-1), PA-inhibitor-2 (PAI-2)), which are able degradate the extracellular matrix were investigated in follicles 20h after GnRH injection and in the CL during oestrous cycle and induced luteolysis. Furthermore the mRNA expression of the monocyte chemoattractant protein-1 (MCP-1), factors of the extrinsic apoptotic pathway (Tumor necrosis factor alpha (TNFα), TNF-Receptor-1 (TNFR1), TNF-Receptor-2 (TNFR2), Fas, Fas-Ligand (FasL)) and intrinsic apoptotic pathway (p53, Bax, Bcl-X_L, Smac, Survivin) as well as the caspses 3, -6 and 7 were evaluated in the CL during oestrous cycle and induced luteolysis. During the first seven days of the oestrous cycle angiogenesis plays a critical role in the development of the CL. Basal membranes of blood vessels have to be removed to enable endothelial cell migration, which is necessary for the formation of new capillaries. MMP-1, MMP-2 and MMP-14 were not regulated, but TIMP-2, MMP-9, MMP-19 and TIMP-1 showed an increasing expression from 20h after GnRH till day 8-12 of the oestrous cycle in the CL. VEGF and bFGF seem to be able to stimulate the expression of MMP-1 and MMP-2 in luteal endothelial cells, which enable these cells to degrade the vascular basement membranes for sprouting new capillaries. MMP-19 is also expressed by these cells and has the same action like MMP-2 on basement membranes. The action of MMP-2 is not only regulated by VEGF and bFGF, but also by TIMP-2, which is lower expressed during angiogenesis in the CL and therefore might not block the action of MMP-2. TIMP-1 and TIMP-2 seem to be not only necessary for the inhibition of MMPs, but they are also able to enhance the proliferation of endothelial cell during angiogenesis. VEGF and bFGF also stimulate the expression of uPA in endothelial cells during angiogenesis. By forming a complex with uPAR and PAI-1 on the outer cell membrane these cells might be now able to detach from the vitronectin

rich perivascular region and migrate towards the fibronection rich stroma of the CL in order to form new capillaries. Not only angiogenesis plays a critical role during CL formation, but also the inhibition of apoptotic action during vessel sprouting. Progesterone, bFGF and VEGF seem to inhibit an increased expression of apoptotic factors like p53 and Bax, while progesterone also inhibits Fas and caspase3. Addtitionally to the suppression of apoptotic factors an up-regulation of anti-apoptotic factors like Bcl-$X_{L,}$ and Survivin occurs. TNFα, produced by invading macrophages during CL formation, acts not only as an apoptotic factor, but is also able to enhance the production of PGE2 in endothelial and luteal cells, which stimulates together with TNFα the proliferation of endothelial cells during luteal angiogenesis.

During induced luteolysis all investigated MMPs showed an increased expression as well as TIMP-1. Only TIMP-2 was down-regulated till 64h after PGF2α. PGF2α as luteolytic agents leads to the expression of MCP-1 on luteal endothelial cells. Thereupon monocytes and cytotoxic T cells migrate into the regressing CL and also start to express MCP-1 to recruite further mononuclear cells. For these migration into the stroma of the CL monocytes express uPA, uPAR and PAI-2. Macrophages are necessary for the phagocytosis of apoptotic cells and the degradation of ECM components in the CL during luteolysis. They are able to express different MMPs like MMP-1, MMP-9 and MMP-2. These MMPs degrade collagen I, the main structur collagen in the stroma of the CL, which might enhance the migration abilities of these macrophages. MMP-2 is also up-regulated in microvascular endothelial cells by TNFα, which is produced by macrophages. At the same time a down-regulation of TIMP-2 occurs, so that no inhibition of MMP-2 happens and a detachment of the endothelial cells from the vessel basement membrane follows. This leads to apoptosis of the endothelial cells, which seem to be the first cells to undergo cell death during luteolysis of the CL. Apoptosis seems to play an important role during luteolysis of the CL. The extrinsic pathway mediated by TNFα, its two receptors, Fas and FasL seem to be more important than the intrinsic pathway, due to the massiv up-regulation of these factors seen during induced luteolysis. TNFα and FasL show a high increased expression from the beginning of induced luteolysis onwards, which might by due to the infiltration of T cells and macrophages expressing these factors. Endothelial cells express TNFR1 and TNFR2 on their surface during luteolysis, which leads to the activation of the extrinsic apoptotic pathwas by binding of TNFα. INFγ enhances these apoptotic action on endothelial cells. It seems possible that apoptosis of luteal cells occurs after the degradation of capillaries, which leads to an undersupply with oxygen and an increase of ROS. These reactive oxygen spieces up-regulate p53 and Bax as apoptotic factors of the intrinsic pathway, which leads to an activation of the caspases and apoptosis of luteal cells during induced luteolysis. These apoptotic action is further enhanced by the activation of the extrinsic pathway. Luteal cells stimulated by TNFα and INFγ express Fas on their cell surface when the progesterone level declines. Cytotoxic T cells, express FasL and promote luteal cell death by activation the extrinsic pathway. This happens only when the progesterone level is decreased, which is known to happen at the end of the functional luteolysis.

The results of these study show that ECM degrading proteases have two different roles in the life span of the CL: first they enable the sprouting of capillaries during angiogenesis in the developing CL, but during induced luteolysis their role seems to be to degrade ECM components of the CL stroma, which detaches endothelial cells from vessel basement membranes. These leads together with the infiltration of macrophages and cytotoxic T cells to apoptosis of endothelial and luteal cells.

8 Zusammenfassung

Untersuchungen zu extrazellulären Matrix Proteasen sowie apoptotischen und anti-apoptotischen Faktoren im bovinen Corpus Luteum

Die Arbeit wurde in zwei Teile unterteilt, wobei sich der erste Teil mit der Entwicklung einer Methode zur Gewinnung von boviner Uterus Milch *in vivo* während der Prä-Implantationsphase für die Erforschung regulativer Faktoren beschäftigt. Im zweiten Teil wurden zwei verschiedene Proteasesysteme zum Vergleich in Follikeln 20 Stunden nach GnRH (Gonadotropin releasing hormon) Applikation (kurz vor Ovulation) als auch während des Östrus Zyklus und der induzierten Luteolyse im bovinen Corpus Luteum (CL) untersucht. Desweiteren wurde die Expression von apototischen als auch anti-apoptotischen Faktoren während des Östrus Zyklus und der induzierten Luteolyse im bovinen CL evaluiert.

Für die Entwicklung einer Methode zur Gewinnung von Uterus Milch *in vivo* während der ersten 24 Graviditätstage im Rind wurden 9 Kalbinnen mit Hilfe des Ovsynch Verfahrens synchronisiert und künstlich besamt. Eine Epiduralanästhesie wurde vor Beginn der Uterusspülung gegeben und alle zwei Uterus Hörner wurden mit 13ml 0,9% NaCl gespült, wobei ein Embryokatheter mit Ballon verwendet wurde. Die Spülungen erfolgten bei allen Kalbinnen am 5., 7., 12., 17. und 24. Graviditätstag. Der Katheter wurde in beiden Hörnern 1cm cranial der Bifurcatio uteri placiert. Es konnten zwischen 3ml und 13ml Spülflüssigkeit zurückgewonnen werden. Die Uterus Milch des ipsilateralen Hornes wurde nach einem Embryo durchsucht und ein EDTA-Stabilisator wurde zu der Uterus Milch beider Hörner gegeben. Eine Infektion des Uterus trat bei drei Kalbinnen nach der zweiten Spülung und bei fünf Kalbinnen nach der dritten Spülung auf. Eine Kalbin zeigte keine Anzeichen einer Infektion. Zwischen dem 17. und 24. Trächtigkeitstag zeigten alle neuen Kalbinnen deutlich Anzeichen einer Brunst. Es war möglich Progesteron, Östradiol-17β, PGF2α und VEGF mittels Enzym Immunassay (EIA) und Radio Immunassay (RIA) in der Uterus Milch nachzuweisen. Wegen der Infektion des Uterus konnte keine statistische Auswertung vorgenommen werden. Es konnte jedoch festgestellt werden, daß sich das Progesteron Level zwischen <0,1 und 0,4ng/ml bewegte und keine Korrelation zur Entzündung zeigte. Durch den Verdünnungseffekt der Spülflüssigkeit sollten die Werte der nativen Uterus Milch um das ca. 10-fache höher liegen. Die Werte der Östradiol-17β Expression schwankten sehr stark, was daran zu erkennen war, daß die Werte der Kalbinnen ohne Infektion bis zum 12. Trächtigkeitstag nicht miteinander in Beziehung gesetzt werden konnten. Es konnte in einer Testreihe gezeigt werden, das in mit EDTA-Stabilisator versetztes 0,9% NaCl hohe Östradiol-17β Werte mittels EIA im Vergleich zu 0,9% NaCl ohne Stabilisator messbar sind. Das Level von VEGF lag zwischen 0,08 und 2,58ng/ml, wobei eine höhere Expression während der Infektion gemessen wurde. Der Verlauf der PGF2α Expression zeigte eine deutliche Korrelation zur Infektion. Allerdings zeigten sich auch Unterschiede zwischen einzelnen Kalbinnen und auch zwischen beiden Uterus Hörnern einer

Kalbin während der ersten Spülung. Dies könnte durch die unterschiedliche Massagestärke oder Sensitivität der Uterus Hörner während der Spülung verursacht sein, die eine Freisetzung von PGF2α verursachen kann. Folgende Änderungen sollten bei der Methode zur Gewinnung von Uterus Milch *in vivo* vorgenommen werden: um eine Endometritis zu verhindern, sollte nur eine Spülung per Gravidität durchgeführt werden, wobei auch der Embryo gewonnen werden sollte. Es sollten wieder alle zwei Hörner gespült werden, wobei für jedes Horn ein Katheter verwendet werden und sich die Spülung in zwei Abschnitte teilen sollte: zuerst sollte eine Spülung zu Gewinnung von Uterusmilch mit 13ml 0,9% NaCl durchgeführt werden, danach sollte der Embryo mit 5x50ml sterilem PBS Puffer ausgespült werden. Der gewonnene Embryo kann dann in RNA-Later bei -80° eingeforen werden. Die gewonnene Uterus Milch sollte in zwei Aliquots unterteilt werden, wobei das Aliquot für die Östradiol-17β Untersuchung keinen EDTA-Stabilisator enthalten darf. Außerdem sollte eine Zentrifugation erfolgen, um die zellulären Bestandteile der Uterus Milch abzutrennen. Die verbleibende Flüssigkeit kann bei –20° aufbewahrt werden. Ein Teil der gewonnen Zellen kann zur Zellidentifikation herangezogen werden, der Rest sollte bei –80° für weitere molekular-biologische Untersuchungen aufgewahrt werden. Für die Untersuchung des Prostaglandin Gehaltes in der Uterus Milch, sollte eine mögliche Behandlung der Kalbinnen mit einem Prostaglandin Synthese Hemmer kurz vor der Spülung erwogen werden.

Für die Untersuchung von verschiedenen extrazellulären Matrix Proteasen and apoptotischen als auch anti-apoptotischen Faktoren im bovinen Follikel und CL wurden RNA Proben eines vorhergehenden Experimentes verwendet. In diesem Experiment wurden 26 Kühe mit einer luteolytischen Dosis eines PGF2α-Analogs behandelt und die CLs wurden mittels transvaginaler Ovariektomie zu folgenden Zeitpunkten entnommen: 0, 2, 4, 12, 24, 48 und 64 Stunden (h) nach Induktion der Luteolyse. Zusätzlich zu diesem Experiment wurden noch weitere fünf Kühe 0,5h nach PGF2α Applikation ovariektomiert. Für die Untersuchungen während des Östrus Zyklus wurden CLs vom Schlachthof gesammelt. Aus Vergleichsgründen wurden Follikel 20 Stunden nach GnRH Injektion im Rahmen einer Superovulation entnommen. Die mRNA Qualität wurde mittels Bioanalyzer untersucht und zeigte eine gute bis sehr gute Qualität aller Proben. Die mRNA Expressions Level wurden mittels quantitativer real time PCR (Rotor-Gene mit SYBR Green I) untersucht. Die erhaltenen Expressionsdaten wurden mit dem Bestkeeper Index aus vier verschiedenen Housekeeping Genen normalisiert, indem die $\Delta\Delta$ CP Methode verwendet wurde. Es wurden folgende Faktoren im Follikel 20h nach GnRH Injektion und im bovinen CL während des Östrus Zyklus und der induzierter Luteolyse untersucht: Matrixmetalloproteinasen (MMP-1, MMP-2, MMP-9, MMP-14, MMP-19), deren Gewebe Inhibitoren (TIMP-1, TIMP-2), das Plasminogen Aktivator (PA) System (Tissue–PA (tPA), Urokinase-PA (uPA), Urokinase-PA-Rezeptor (uPAR), PA-Inhibitor–1(PAI-1), PA-Inhibitor-2 (PAI-2)), welche in der Lage sind extrazelluläre Matrix (ECM) abzubauen. Desweiteren wurden folgende Faktoren im CL während des Östrus Zyklus und der induzierten Luteolyse evaluiert: Monocyte chemoattractant protein-1 (MCP-1), Faktoren des extrinsischen apoptotischen Weges (Tumor necrosis factor alpha (TNFα), TNF-Rezeptor-1 (TNFR1), TNF-Rezeptor-2 (TNFR2), Fas, Fas-Ligand (FasL)) und des intrinsischen Weges (p53,

Bax, Bcl-X_L, Smac, Survivin) als auch die Caspasen 3, -6 and 7. Während der ersten sieben Tage des Östrus Zyklus spielt die Angiogenese eine entscheidende Rolle in der Entwicklung des CL. Basal Membranen der Blutgefäße müssen durchbrochen werden, um Endothelzellen die Migration zu ermöglichen, welche für die Bildung neuer Kapillaren wichtig ist. MMP-1, MMP-2 und MMP-14 waren nicht reguliert, aber TIMP-2, MMP-9, MMP-19 und TIMP-1 zeigten eine zunehmende Exprimierung von 20h nach GnRH Applikation bis Tag 8-12 des Östrus Zyklus im CL. VEGF und bFGF können eine Stimulierung der Expression von MMP-1 und MMP-2 in lutealen Endothelzellen bewirken. Diese Proteasen ermöglichen es diesen Zellen während der Sprossung von neuen Kapillaren die vaskuläre Basalmembranen zu degradieren. MMP-19 wird auch von diesen Zellen exprimiert und hat die gleiche Wirkung auf Basalmembranen wie MMP-2. Die Wirkung von MMP-2 wird nicht nur von VEGF und bFGF reguliert, sondern auch von TIMP-2, welches während der Angiogenese im CL niedriger exprimiert wird. Dadurch könnte die MMP-2 Wirkung nicht inhibiert werden. TIMP-1 und TIMP-2 scheinen nicht nur für die Inhibierung der MMPs wichtig zu sein, sondern sie sind auch in der Lage die Proliferation von Endothelzellen während der Angiogenese zu stimulieren. Eine weitere Protease die von VEGF und bFGF während der Gefäßbildung stimuliert wird ist uPA. Dieser Faktor bildet zusammen mit uPAR und PAI-1 einen Komplex an der äußeren Zellmembran und ermöglicht es nun der Zelle sich von der vitronektin-reichen perivaskulären Region abzulösen und in das fibronektin-reiche Stroma des CL zu wandern, um neue Kapillaren zu bilden. Nicht nur die Angiogenese spielt eine kritische Rolle in der Formierung des CL, sondern auch die Inhibierung von apoptotischen Vorgängen während der Blutgefäßsprossung. Progesteron, bFGF und VEGF scheinen eine erhöhte Expression von apoptotischen Faktoren, wie p53 und Bax, zu inhibieren. Progesteron unterdrückt außerdem noch die Expression von Fas und Caspase3. Zusätzlich zur Supression von apoptotischen Faktoren findet eine Aufregulierung von anti-apoptotischen Faktoren, wie Bcl-X_L und Survivin, statt. TNFα wird während der Entwicklung des CL von einwandernden Makrophagen produziert und kann nicht nur als apoptotischer Faktor wirken, sondern erhöht auch die Produktion von PGE2 in Endothel- und Lutealzellen. PGE2 stimuliert zusammen mit TNFα die Proliferation von Endothelzellen während der lutealen Angiogenese.

Während der induzierten Luteolyse zeigten alle untersuchten MMPs und TIMP-1 eine erhöhte Expression. Nur TIMP-2 wurde bis 64h nach PGF2α Injektion abreguliert. PGF2α wirkt als luteolytisches Agens und führt zur Expression von MCP-1 auf lutealen Endothelzellen. Daraufhin migrieren Monozyten und zytotoxische T-Zellen in den regressierenden CL und beginnen auch MCP-1 auf ihrer Oberfläche zu expremieren, welches zu weiteren Anlockung von mononukleären Zellen führt. Monozyten exprimieren uPA, uPAR und PAI-2, um in das Stroma des CL einwandern zu können. Makrophagen werden während der Luteolyse für die Phagozytose von apoptotischen Zellen und für die Degradierung von ECM Komponenten benötigt. Sie sind in der Lage verschiedene MMPs, wie MMP-1, MMP-9 und MMP-2, zu expremieren. Diese MMPs degradieren Kollagen I, welches den größten Teil der Strukturkollagene im CL Stroma bildet, um möglicherweise die Migrationsfähigkeit der Makrophagen zu erhöhen. MMP-2 wird auch durch von

Makrophagen produziertes TNFα in mikrovaskulären Endothelzellen aufreguliert. Zur selben Zeit findet eine Abregulierung von TIMP-2 statt, so dass es zu keiner Inhibierung von MMP-2 kommt und eine Ablösung der Endothelzellen von der Gefäßbasalmembran erfolgt. Dies führt zur Apoptose von diesen Zellen, welche während der Luteolyse scheinbar als erste der Apoptose anheimfallen. Der Zelltod scheint eine wichtige Rolle während der Luteolyse des CL zu übernehmen. Der extrinsische Weg, der durch TNFα, dessen beiden Rezeptoren, Fas und FasL vermittelt wird, scheint während der induzierten Luteolyse aufgrund der massiven Aufregulierung dieser Faktoren eine größere Rolle als der intrinsische Weg zu spielen. TNFα und FasL zeigen eine stark erhöhte Expression von Beginn der induzierten Luteolyse, welche man auf die Infiltration von T-Zellen und Makrophagen, die diese Faktoren expremieren, zurückführen kann. Endothelzellen exprimieren TNFR1 und TNFR2 auf ihrer Oberfläche während der Luteolyse. Dies führt durch die Bindung von TNFα zur Aktivierung des extrinsischen apoptotischen Weges. INFγ verstärkt diese apoptotische Wirkung auf Endothelzellen. Es scheint möglich zu sein, dass die Apoptose von Lutealzellen erst nach der Degradierung von Kapillaren, welche zu einer Unterversorgung mit Sauerstoff und der Bildung von ROS führt, stattfindet. Diese reaktiven Sauerstoff Radikale führen zu einer Aufregulierung von p53 und Bax als apoptotische Faktoren des intrinsischen Weges. Daraufhin folgt die Aktivierung der Caspasen und die Apoptose der Lutealzellen. Dieser apoptotische Prozess wird außerdem noch durch die Aktivierung des extrinsischen Weges verstärkt, wobei mit TNFα und INFγ stimulierte Lutealzellen Fas auf ihrer Oberfläche exprimieren sobald der Progesteronspiegel abfällt. Zytotoxische T-Zellen exprimieren FasL und fördern den Tod der Lutealzellen durch die Aktivierung des extrinsischen Weges. Dies findet allerdings nur nach Abfall des Progesteronlevels am Ende der funktionellen Luteolyse statt.

Die Ergebnisse dieser Studie zeigen, dass ECM degradierende Proteasen verschiedene Rollen während der Lebensspanne des CL übernehmen: zum einen ermöglichen sie die Sprossung von Kapillaren während der Angiogenese im sich formierenden CL, zum anderen scheinen sie eine wichtige Rolle während der induzierten Luteolyse durch Degradierung von ECM Komponenten zu spielen. Diese Degradierung führt zur Ablösung der Endothelzellen von den vaskulären Basalmembranen, welches zusammen mit der Einwanderung von Makrophagen und zytotoxischen T-Zellen zur Apoptose von Endothel- und Lutealzellen führt.

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11 Curriculum vitae

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12 Publications, Abstracts, Posters, Presentations

Publications

A.S.S. Abdoon, O.M. Kandil, B. Berisha, H. Kliem, D. Schams Morphology of dromedar camel oocytes and their ability to spontaneous and chemical parthenogenetic activation, Reprod. Dom. Animal (submitted)

H. Kliem, H. Welter, W.D. Kraetzl, H.H.D. Meyer, B. Berisha, D. Schams Expression of extracellular matrix degrading proteases and their inhibitors during oestrous cycle and induced luteolysis in the bovine CL (in progress)

Abstracts and Posters

H. Kliem, W.D. Kraetzl, B. Berisha, D. Schams 2005 mRNA expression of apoptotic and anti-apoptotic factors during PGF2α induced luteolysis in the bovine corpus luteum, Poster presentation at the second qPCR symposium, Freising-Weihenstephan, 05. - 09. 2005

B. Berisha, R. Fürst, H. Kliem, H.H.D. Meyer, D. Schams 2005 Expression of mRNA of apoptotic and anti-apoptotic factors during oestrous cycle and pregnancy in the bovine corpus luteum, Poster presentation at the second qPCR symposium, Freising-Weihenstephan, 05. - 09. 2005

B. Berisha, H. Welter, H. Kliem, H.H.D. Meyer, D. Schams 2006 Changes in mRNA expression of angiopoitin and Tie receptors in bovine follicles before and after GnRH application and after ovulation, Abstract for the 39th annual conference of Physiology and Pathology of Reproduction and 31th mutual conference on veterinary and human reproductive medicine, Hannover, 16.-17.02.2006

H.H.D. Meyer, H. Kliem, D. Schams, B. Berisha 2006 Monitoring of gene expression (transcriptome) during reproduction via qRT-PCR mRNA expression of apoptotic and anti-apoptotic factors during induced luteolysis, Abstract for the 16th annual meeting of the Indian society for the study of reproduction and fertility (ISSRF), Karnal (Haryana), India, 23.-25.02.2006

D. Schams, H.Kliem, H. Welter, H.H.D. Meyer, B. Berisha 2006 Extracellular matrix degrading proteases and their inhibitors in corpus luteum during estrous cycle and induced luteolysis in cow, Abstract for the 39th annual meeting of the Society for the Study

of Reproduction, Omaha, USA, 29.07. – 01.08.2006 (accepted)

A.S.S. Abdoon, H. Kliem, O.M. Kandil, D. Schams, B. Berisha 2006 Expression of steroid receptors in corpus luteum and endometrium during different stages of pregnancy in dromedary camel, Abstract for the 10th annual conference of the European Society for Domestic Animals Reproduction, Portoroz-Lipiza, Slovenia, 07. - 09.09.2006 (accepted)

D. Schams, H. Kliem, W.D. Kraetzl, B. Berisha 2006 Expression of extracellular matrix degrading proteases and their inhibitors in the corpus luteum during induced luteolysis in cow, Abstract for the 10th annual conference of the European Society for Domestic Animals Reproduction, Portoroz-Lipiza, Slovenia, 07. - 09.09.2006 (accepted)

Presentations

Expression of extracellular matrix proteases in the bovine CL during induced luteolysis, Montagsseminar 02/2005, Lehrstuhl für Physologie, Technische Universität München

Expression of apoptotic and anti-apoptotic factors in the bovine CL during induced luteolysis, Montagsseminar 12/2005, , Technische Universität München

Gene sequence

H. Kliem, H Welter, D. Schams and B. Berisha (2005) Bos taurus partial mRNA for second mitochondrial derived activator of caspases (SMAC gene), acc. no. of the EMBL database: AJ867847