Institute of Veterinary Biochemistry
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Identification and quantification of regional expression of members of the NADPH oxidase (NOX) enzyme family during the estrous cycles in the bovine oviduct

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Dedication

I dedicate this thesis to my dear wife Asma who has supported my research and long hours of study over the years, in spite of my strange obsession with working. I also dedicate this thesis to my mother and father for their love and support over the years and especially for their encouragement that one can do anything that one wants to if you approach each task with enough optimism. I also dedicate this thesis to my brothers and sister Ibitihal who have always shown support and interest in my work, even though they were far removed from the details. Finally, special dedication to the soul of my best friend, Waleed Mohi-Eldeen Surkety, a man with scarce qualities of self-denial, for instilling in me qualities that I admire.

Abbreviations

AA	Arachidonic acid	H_2O_2	Hydrogen peroxide
Acc. No.	Accession number	FAD	Flavin adenine dinucleotid
AIJ	ampullary-isthmic junction	fCyt-$_{b558}$	Flavocytochrome-$_{b558}$
AR	Acrosomal reaction	FSH	Follicle stimulating hormone
At	Annealing temperature	I-A	Ipsi-lateral ampulla
BPB	Bromphenol blue	I-I	Ipsi-lateral isthmus
bp	Base pair	kDa	Kilo Dalton
C-A	Contra-lateral ampulla	LH	Luteinizing hormone
Ca^{2+}	Calcium ion	LPS	Lipopolysaccharide
cDNA	Complementary DNA	M	Marker
C-I	Contra-lateral isthmus	MMuLV	Moloney Murine Leukemia Virus
CL	Corpus luteum	MOPS	3-N-Morpholino propanesulfonic acid
$cPLA_2$	Cytosolic phospholipase A_2	MOX1	Mitogenic oxidase 1
C_t	Threshold cycle	MPO	Myeloperoxidase
CYBA	Cytochrome-$_{b558}$-alpha	mRNA	Messenger RNA
CYBB	Cytochrome-$_{b558}$-beta	Mt	Melting temperature
ddNTPs	Dideoxy nucleotide triphosphates	NADPH	Nicotinamide adenine dinucleotide phosphate
DEPC	Diethylpyrocarbonate	NOH-1	NOX homologe-1
dH_2O	Distilled water	NOX	NADPH oxidase
DNA	Deoxy Ribo Nucleic Acid	NOXA	NOX activator
dNTP	Deoxy nucleotide triphosphates	NOXO	NOX organizer
DUOX	Dual oxidase	$•O_2$-	Superoxide
E2	Estradiol	OS	Oxidative stress
EDTA	Ethylene diamine tetra acetic acid	ORF	Open reading frame
GnRH	Gonadotropin releasing hormone	P4	Progesterone
GPO	Glutathione peroxidase	PBS	Phosphate buffer saline
GTPase	Guanine triphosphatase	PCR	Polymerase chain reaction

PG	Prostaglandins	SOD	Superoxide dismutase
PGE_2	Prostaglandin E_2	TAE	Tris-acetate-EDTA
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$	ThOX	Thyroid oxidase
PKC	Protein kinase C	TPR	Tetratricopeptide repeat
PPR	Prolin rich region	VSMCs	Vascular smooth muscle cells
ROS	Reactive oxygen species	ZP	Zona pellucida

1 Introduction and objectives

The oviduct as part of the bovine reproductive system possesses significant roles in transport of gametes, in successful fertilization and embryo transport. The oviduct mucosal cells synthesize and secrete oviductal fluid that enhances reproductive function of the oviduct. Additionally, oviductal mucosa expressed a vast variety of genes that is involved in reproductive events occurring in the bovine oviduct. The reproductive and productive features of female have remarkable values in animal production. Accordingly, there is justification for studies on their physiological events that occur in oviductal micro-environment and the factors which influence their reproductive performance.

One of the main objectives of reproductive technique research is to improve fertility in farm animals. Pregnancy wastage (embryonic and fetal mortality) is one of the most important problems leading to reproductive failure. Recently, reactive oxygen species (ROS) have been reported as one of the causes of infertility of unknown etiology (Burton et al., 2003; Agarwal and Allamaneni, 2004a). However, these free radicals also can be one of the factors which may interact with successful fertilization and thereby laid to establishment of pregnancy. Furthermore, other investigators indicated that ROS may play important roles in gametes maturation and fusion, fertilization, and early embryo development (Quinn and Harlow, 1978; Aitken and Brindle, 1993; Blondin et al., 1997; Guerin et al., 2001). NADPH oxidase (NOX) enzymes are trans-membrane proteins that transfer electrons across biological cell membranes and are involved in the production of ROS. The basic form of NADPH oxidase enzymes was first described and characterized in phagocytes and it was originally thought that this system was restricted only to phagocytes and used solely in host defense (Roos et al., 2003; El Benna et al., 2005). However, recent studies in the last decade indicate that similar NADPH oxidase systems are present in a wide variety of non-phagocytic cells as have been reviewed recently by Bedard and Krause (2007). The physiology of the NADPH oxidase is one of the factors that may have implications in the fertility and infertility in domestic animals.

The molecular basis of early reproductive events and etiology of recurrent pregnancy loss remain unclear and they are of a scientific challenge, so far the physiological and pathological roles of ROS produced by NOX in reproductive physiology are still under investigation. The balance between the benefits and risks from ROS and antioxidants both *in vivo* and *in vitro* appears to be necessary for the survival and normal reproductive functioning (Blondin et al., 1997). So better understanding of the molecular basis of NADPH oxidase system in reproductive processes of mammals is needed to be clarified. Although, as members of the

NOX family have been reported to play a critical role in the reproductive function, however, up to date no studies have reported NOX activity as well as their presence in mammalian oviducts. Furthermore, almost nothing is known on NOX expression and distribution in the different segments of the bovine oviduct. Furthermore, the factors controlling the expression of the different NOX isoforms in the oviduct are not clearly established.

Investigation of the expression pattern of these enzymes throughout estrous cycle gives a better understanding for the fertilization processes and early embryonic development as well as occurrence of pathological conditions.

Objectives

The study described in this thesis was designed and conducted with the following objectives:

a) To investigate the presence of selected NOX enzyme mRNAs in bovine oviductal cells.

b) To investigate the estrous cycle-dependant and regional-dependant changes in the expression levels of NOX enzyme components in the bovine oviduct using real-time RT-PCR.

c) To examine the effects of E2, P4, AA, or PGE_2 on the expression of NOX enzyme components in a primary oviductal cell culture.

2 Literature review

2.1 Functional anatomy and histological aspects of the bovine oviduct

The bovine oviducts are connecting the ovaries with the uterus through the utero-tubal junction and infundibulum. They are pair wise elongated convoluted tubes of 20-30cm in length in the cow. According to their anatomical and histological structures, each oviduct consists of three main parts: infundibulum, ampulla, and isthmus. Each of the three parts supports oviductal function in different aspects. The finger-like projections of the infundibulum serve to direct the ovulated egg as cumulus-oocyte-complex into the oviductal lumen. The ampulla, the place of final oocyte maturation, has predominantly active secretory cells that provide an optimal environment by synthesis of oviductal fluid components. The junction between the isthmus and ampulla, ampullary-isthmic junction (AIJ), is the site at which fertilization occurs (Hawk, 1987). Furthermore, the caudal isthmus has been shown to play an important role in sperm transport and reconstitutes the sperm reservoir (Yániz et al., 2000; Suarez, 2002, 2007).

The general histological structure of the oviduct wall in the different parts is similar. The wall is composed of three basic layers (from outside to inside): tunica serosa, tunica muscularis, and tunica mucosa. The luminal mucosal surface of the oviduct is differentiated throughout its length into primary and secondary folds. The former are longitudinal while the latter extend deep into the lumen and branch in different directions. The degree of folding is most pronounced in the infundibulum and is progressively lower and less complex toward the isthmus and uterus (Wrobel et al. 1993).

The mucosa is composed of a single surface layer of epithelium and a lamina propria (Lombard et al., 1950) and generally characterized by fold formation. Two epithelial cell types have been recognized in the bovine oviduct: secretory and non-secretory cells (Abe and Hoshi, 1997). Marked variations were observed in the oviductal epithelium depending on the oviductal segment (infundibulum, ampulla, and isthmus), basal or apical areas of the mucosal folds and phase of the estrous cycle (Abe and Oikawa, 1993; Yániz et al., 2000). It was also reported that the number of ciliated cells significantly decreased in the infundibulum at the luteal phase, but not in the other regions, where the height of ciliated cells decreased dramatically. The dominant cell type in the ampulla are non-ciliated cells (secretory cells), while ciliated epithelial cells dominate in the infundibulum and isthmus region and supporting the gamete transport. Studies by Abe and Oikawa (1993) demonstrated that the mean percentage and height of ciliated cells significantly decrease in ampulla during the luteal

phase compared to the phase around ovulation time. The isthmus of the bovine oviduct showed little changes in cell types between post-ovulatory and the luteal phase.

The oviductal movement has an important role in early reproductive events. Oviductal movements should be in the two opposite directions (from the ovary to the uterus and vise versa) to achieve transport of gametes, zygote, and early embryo (Bennett et al., 1988; Kotwica et al., 2003a). Variations of smooth muscles contractility and cilia movement among oviductal segments during the estrous cycle permits oviductal transport of gametes and embryo on proper time (Yániz et al., 2000).

Sperm and oocyte in the bovine oviduct were found to be affected by both the oviductal epithelium and fluid (Chian and Sirard, 1995; Way et al., 1997). Oviductal fluid has an influence on the sperm ability and availability to fertilization by enhancing sperm capacitation and acrosomal reaction (AR) (King et al., 1994), sperm-egg binding (Wegner and Killian, 1991) and thereby improving the fertilization rate. The oviductal fluid is generated by transudation from the blood into the oviductal lumen as well as by secretion of substances synthesized in the secretory epithelial cells (Ellington, 1991; Menezo and Guerin, 1997). Oviductal fluid is participating in maturation of ovulated eggs prior to fertilization and with the zygote once fertilization occurred (Kapur and Johnson, 1985; Leveille et al., 1987).

2.2 Bovine oviductal changes during estrous cycle

The bovine estrous cycle is regulated by the hypothalamic-pituitary-gonadal axis, which produces hormones that dictate reproductive events.

According to hormonal profiles in cattle, circulating P4 level starts to decrease when the corpus luteum (CL) starts regressing from the previous cycle (Echternkamp and Hansel, 1973) in turn allows concentration of follicle stimulating hormone (FSH) to increase. This stimulates the maturation of the follicle and stimulates the production of estradiol (E2) by the Graafian follicle. Elevation of E2 level has a positive feedback mechanism on hypothalamic gonadotropin releasing hormone (GnRH) secretion that in turn increases the pulsatile secretion of luteinizing hormone (LH) (Kesner et al., 1981; Walters et al., 1984). High level of LH before estrus onset and at the estrus time helps in the final maturation of ovulatory follicle along with FSH. On the day of standing estrus, physiological levels of circulating E2 acts synergistically with low P4 concentrations to induce the pre-ovulatory LH surge and ovulation takes place. Thereafter, the functional CL develops and peripheral P4 starts increasing gradually which exerts a negative feedback on LH and FSH levels subsequent to estrus. A

fifth hormone important in female reproduction is prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). This hormone is secreted by the endometrium and also affects structures on the ovary. In the absence of an embryo, the uterus begins to release pulses of $PGF_{2\alpha}$ into its venous drainage to the ovaries (Silvia et al., 1991; McCracken et al., 1999). $PGF_{2\alpha}$ causes the regression of the luteal tissue of the CL. Thereafter, circulating progesterone decline rapidly and removing progesterone's negative feedback on the anterior pituitary.

There were changes in local distribution in sex steroid hormones in the bovine oviduct during the estrous cycle. The oviduct ipsi-lateral to the CL bearing ovary showed significant higher concentrations of P4, E2, prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ than that at the contra-lateral side (Wijayagunawardane et al., 1998). It was noted that P4 level is higher during luteal phase, while E2, PGE_2 and $PGF_{2\alpha}$ are increased during the post-ovulatory phase. Therefore, hormonal control of gene expression at the level of transcription may occur in the mammalian oviduct throughout the estrous cycle.

The reproductive tissues of the cow are under the influence of P4 for the most days of the estrous cycle (14-16 days) with shorter period exposure to E2 of about one day without any P4 to counter its effects. Earlier studies have shown that ovarian hormones influenced proliferation, ciliation and secretory processes of the oviduct epithelium in the mammals (McDaniel et al., 1968; Brenner et al., 1974; Bajpai et al., 1977; Odor et al., 1980). E2 induced hypertrophy, maturation and increases in cell height of non-ciliated secretory epithelial cells. In contrast, P4 caused atrophy and diminished secretory activity. Consistent with morphological changes, a transcriptome study identified extensive estrous cycle-dependent changes in gene expression patterns in bovine oviductal epithelial cells (Bauersachs et al., 2004). During bovine estrus cycle, genes involved in the regulation of protein secretion and mRNAs of secreted proteins were up-regulated around the ovulation time, whereas transcripts of genes involved in transcription regulation showed a slight up-regulation during the luteal phase.

2.3 Some aspects of oviductal immunity

The immune response in the oviduct requires most of the same cellular and chemical strategies as do other mucosal surfaces. The immune system has evolved to be crucially regulated by the ovarian hormones E2 and P4 (Wira and Rossoll, 1995; Kaushic et al., 1998) which prepare the reproductive tract for successful fertilization, implantation and pregnancy. E2 specifically has stimulatory effects on the secretory immune system (Sullivan et al., 1983),

while P4 acts as counter-influence to E2 in immune protective responses of the reproductive tract. E2 has been shown to stimulate the accumulation of both IgA and IgG in uterine secretions while P4 administration prevents the estrogen-induced increase in IgA and IgG in the uterine lumen. Furthermore, it has been described that E2 and P4 influences some functions of phagocytes, such as chemotaxis, phagocytosis and oxidative burst activity in the cow (Roth et al., 1983).

Different types of leukocytes have been reported in the different bovine reproductive tissues throughout the estrous cycle (Cobb and Watson, 1995). In the bovine oviduct, lymphocytes, monocytes and granulocytes were found to be dispersed among epithelial cells and mainly localized at the lamina propria (DuBois et al., 1980; Eriksen et al., 1994). It has been shown that leukocytes in the different parts of the bovine oviduct are distributed significantly depending on the estrous cycle phase. The number of mast cells and lymphocytes was significantly higher in the isthmus than in the ampulla of the oviduct during the estrous cycle (DuBois et al., 1980). Lymphocyte numbers increased significantly in the ampulla when compared to their number in the isthmus during the luteal phase.

Functional immune cells in the oviduct recognize the antigen and signal other immune cells through secretion of mediators, chemokines, and cytokines (Fazeli et al., 2005). Among the elements of the immune response against the antigen, phagocytes have a prominent role in reproductive immunity (Anderson et al., 1985). Phagocytized micro-organisms can be killed by oxygen dependent (respiratory burst) and oxygen independent mechanisms (initiate immune system: lysozymes and proteolytic enzymes). Although neutrophils produce and release a variety of toxic agents directed toward microbial killing, the system that depend on reactive oxygen species is especially potent (Roos et al., 2003). Concomitant with the start of phagocytosis during the reproductive tract inflammation, the oxygen metabolism of leukocytes accelerates and is connected with the production and release of large amounts of superoxide and H_2O_2 (Kovalski et al., 1992; Wang et al., 1997). The superoxide generating via neutrophils NADPH oxidase (NOX) enzymes serves as continued source of a vast array of reactive oxygen species (ROS). These oxidants can also cause collateral damage to nearby tissue. Therefore, their production level should be tightly regulated to ensure that they are only generated when and where required.

2.4 Reactive oxygen species (ROS)

2.4.1 General definition

ROS are oxygen-derived small molecules which are present as free radicals implicated in many physiological processes. They are defined as molecules that contain one or more unpaired electrons (**Fig. 1**) (Halliwell and Gutteridge, 1984). They are formed either by loss or gain of a single electron from non-radicals during normal cell metabolism by certain enzymatic (NADPH oxidase) or non-enzymatic mechanisms (Grisham and Granger, 1988; Thannickal and Fanburg, 2000).

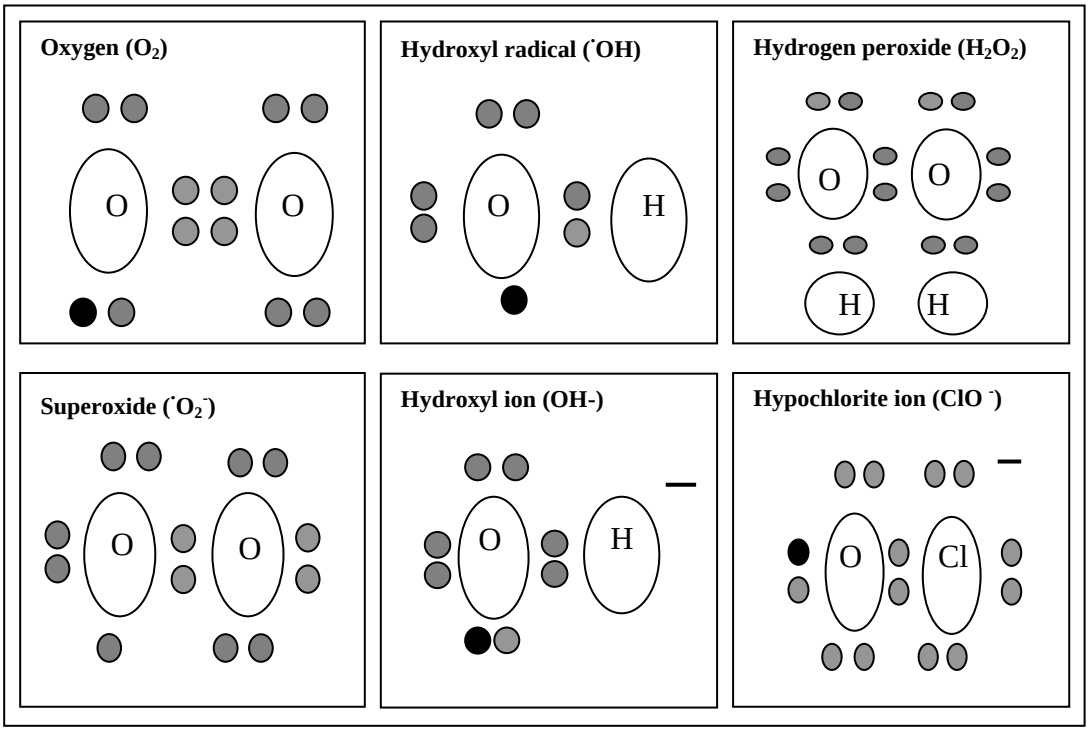

Fig. 1: Reactive oxygen species (ROS). The black circle indicates the unpaired electron that can be lossed or acquired by the atom, where gray circle indicates the stable electrons of the atom.

ROS were first discovered in host defense mechanisms (Sbarra and Karnovsky, 1959; Iyer et al., 1961). During respiratory burst of phagocytosis, the membrane NADPH oxidase enzyme is responsible for electron transfer from biological substrates to oxygen molecules generating first superoxide anions. The H_2O_2 generated by the oxidase serves as a co-substrate for peroxidases, either myeloperoxidase (MPO) in neutrophils and monocytes or eosinophil peroxidase in eosinophils, in the oxidation of halides to generate hypochlorous acids, a potent antimicrobial agent. The produced ROS are contributing to kill the invading microorganisms (Segal, 1989) together with proteases liberated from the granules.

2.4.2 ROS function in reproductive events

Gametes reside in a metabolically active environment of free radicals. Therefore, ROS existence in the environment surrounding the gametes and early embryo may implicate in fertilization process and subsequent embryo development. Spermatozoa and oocytes were found to release superoxide during their maturation and fusion in hamster and human (Miesel et al, 1993; Sikka et al., 1995) which supported the presence of NOX enzyme system in sperm and oocyte. H_2O_2 was found to be involved in the process of capacitation and AR in hamster, human and bovine (Miesel et al, 1993; de Lamirande et al., 1997; Blondin et al., 1997). Developmental competence of bovine oocytes can be improved when ROS production system was added to the culture medium (Blondin et al., 1997). An increase in the percentage of *in vitro* embryo production correlated to the superoxide production rather than H_2O_2. Reducing the O_2 concentration in *in vitro* environment can increase embryonic development (Tervit et al, 1972). Several studies revealed that the binding process of sperm to the zona pellucida (ZP), gamete fusion and AR that associated with fertilization are promoted by low levels of ROS and blocked by NOX inhibitors (de Lamirande et al., 1997; Lapointe et al., 1998; El Mouatassim et al., 2000; O'Flaherty et al., 2003; Aitken et al., 2004). Fertilization process involved egg-sperm fusion has been found to be blocked with antioxidant factors and high concentrations of ROS (El Mouatassim et al., 2000; O'Flaherty et al., 2003; Aitken et al., 2004).

In contrast, ROS generating-NOX have been reported to cause damage of cell membrane lipids, nucleic acids and proteins (Halliwell and Gutteridge, 1984). Spermatozoa are very susceptible to damage by higher ROS level (Chen et al., 1997). Oxidative stress (OS) due to increased ROS level also appears to contribute to reduced development of oocytes and embryos *in vitro* (Guerin et al., 2001). Increased ROS have been hypothesized to play a causative role in the etiology of defective spermatozoa function through peroxidation of the unsaturated fatty acids within the sperm plasma membrane (Aitken and Brindle, 1993). The plasma membrane of sperm loses the integrity and fluidity necessary for acrosome reaction and fusion with the oocyte membrane. While Luvoni et al. (1996) reported that *in vitro* maturation and fertilization of bovine oocytes cultured with ROS scavengers (GPO; glutathione peroxidase) resulted in significant improved proportions of oocytes undergoing cleavage and morula/blastocyst development.

2.5 NADPH oxidase (NOX) family

2.5.1 Definition and general structure

NOX family members are trans-membrane glycoproteins that transport electrons across biological membranes from NADPH to molecular oxygen to produce ROS (Ellis et al. 1989; Rotrosen et al., 1992). Seven members of the NOX enzyme family are (**Table 1)** homologs to $gp91^{phox}$ (phagocytic oxidase or NOX2) and 6 cytoplasmic and membrane subunits are required for NOX enzymatic activities. Additionally after the identification of NOX1 (Suh et al., 1999), subsequent studies by Dupuy et al. (1999) and Cheng et al. (2001) showed the cloning and tissue expression of four additional homologs of phagocytic NOX2, NOX3, NOX4, NOX5 and DUOX.

NOX member	Alternative name	Class	Required subunit
NOX1	NOH-1, MOX-1	Membrane enzyme	All cytosolic subunits
NOX2	$gp91^{phox}$, CYBB	Membrane enzyme	All cytosolic subunits
NOX3	-	Membrane enzyme	All cytosolic subunits
NOX4	Renox	Membrane enzyme	$p22^{phox}$
NOX5	-	Membrane enzyme	Cytosolic Ca^{2+}
DUOX1 and 2	ThOX1 and 2	Membrane enzyme	Ca^{2+}, DUOXA1 and 2
Cytosolic subunit			
$p22^{phox}$	CYBA	Cytosolic subunit	-
$p67^{phox}$	NOXA1, NCF2	Cytosolic subunit	$p47^{phox}$, $p40^{phox}$
$p47^{phox}$	NOXO1, NCF1	Cytosolic subunit	$p40^{phox}$
$p40^{phox}$	NOXO2, NCF4	Cytosolic subunit	-
RabA1	-	Membrane subunit	-
Rac 1 and 2	-	Cytosolic subunit	-

Table 1: Members of the NADPH oxidase (NOX) family and their activation subunits.

All NOX are predicted to encode proteins of around 65kDa and like NOX2, consist of 5-6 conserved predicted trans-membrane α-helices containing two putative iron-containing-hemes conjugated to domains III and V. **Fig. 2** shows the core structure of NOX enzymes that are associated with the cell membrane. The cytoplasmic COOH terminus contains a conserved flavin adenine dinucleotide (FAD) and a NADPH binding domain. In addition to the general

motive of the NOX family, NOX5 and DUOX contain an additional calcium-binding domain (EF-hand motifs) in its cytoplasmic N-terminus. Furthermore, DUOX protein also contains an additional trans-membrane peroxidase-like domain.

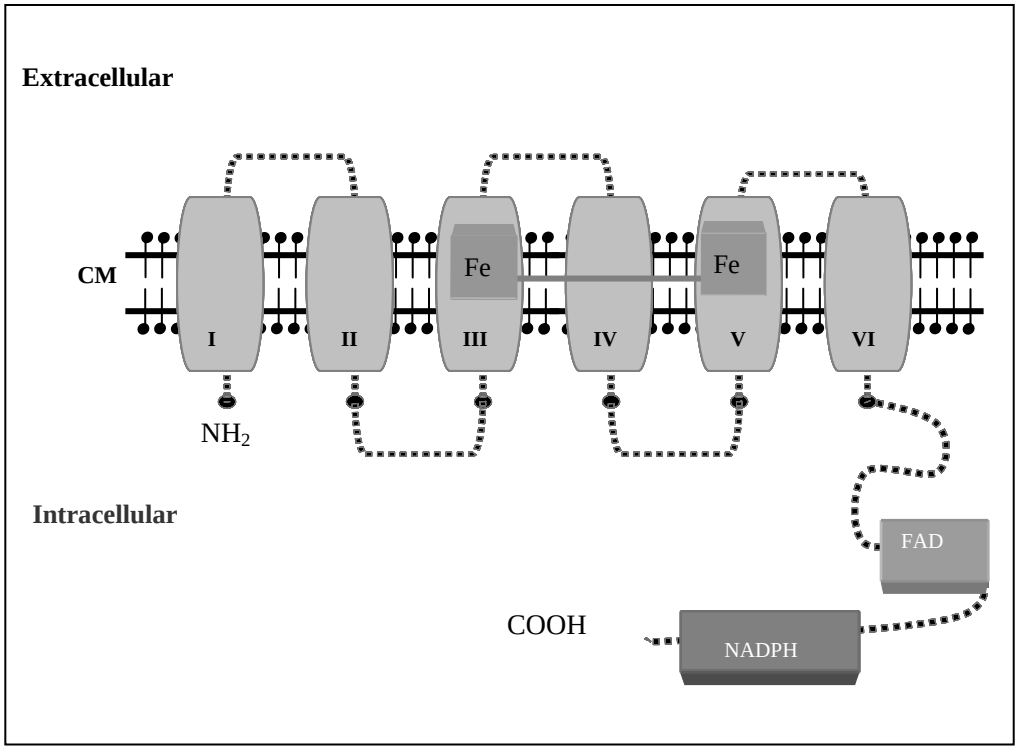

Fig. 2: General structure of NOX members. The grey tubes represent the α-helix transmembrane domain I-VI. CM: cell membrane. (Modified from Geiszt, 2006).

2.5.2 NOX enzyme activation

The mechanism of activation is almost similar for most members of the NOX family (NOX1-4), but different for NOX5 and DUOX1 and -2. The latter require intercellular Ca^{2+} for their enzymatic activities. Depending on the state of cell activity, the NOX enzyme is present in two states: active and inactive (**Fig. 3**).

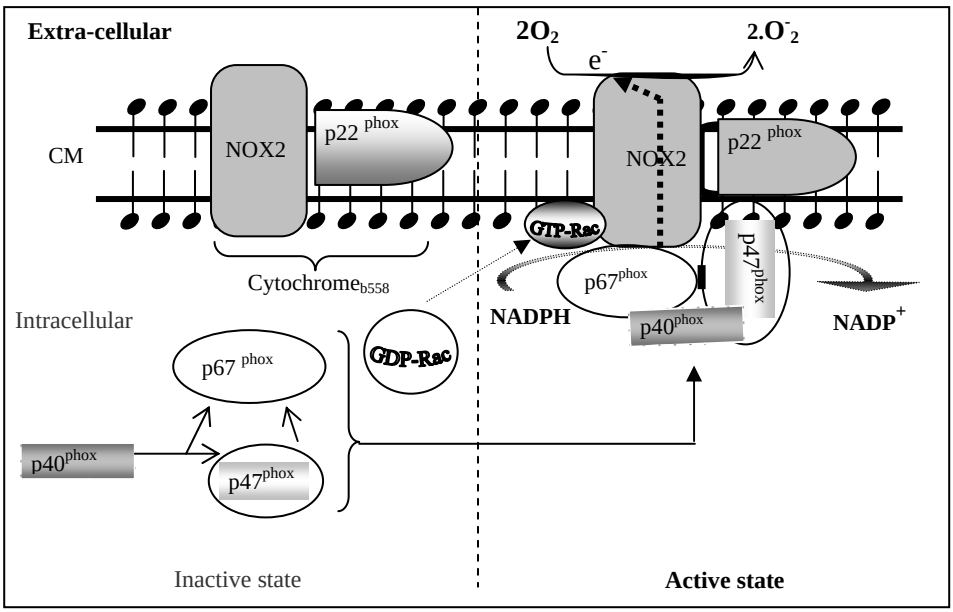

Fig. 3: Schematic diagram representing active and inactive state of NOX2 enzyme in the cell. CM: cell membrane, e^-: electron (Modified from Sumimoto et al., 2004).

Early attempts using cell-free system have been done to reconstitute NADPH-oxidase activity *in vitro*. This system demonstrated that proteins in the membrane as well as the cytoplasm are required for their activity (Babior, 1994). Under normal cell resting conditions, these enzymes are not active. The NOX and $p22^{phox}$ combine together to form flavocytochrom$_{b558}$ (fCyt-$_{b558}$) at the cell membrane. Activation of NOX enzymes seemed to require the five cytosolic subunit proteins because neutrophils are not able to produce ROS when two cytosolic factors were absent (Volpp et al., 1988). The cytosolic subunits are involved in protein-protein interaction responsible for the activation of this system leading to ROS production. The general activation mechanism is similar for the first three members NOX1, NOX2, NOX3, but NOX4 needs only $p22^{phox}$ for its full enzymatic activity.

Two cytosolic factors, $p47^{phox}$ and $p67^{phox}$, were demonstrated to be important for NOX activity based on impairment of ROS generation by the cell deficient in cytosolic subunits, in which this component was missing or mutated (Lomax et al., 1989). A third cytosolic protein known as $p40^{phox}$, which is constitutively associated with $p67^{phox}$ in phagocytes, is also

involved in the NOX enzyme complex upon cell activation (Wientjes et al., 1993). According to the current model of NADPH oxidase assembly, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ translocate as complex to associate with NOX-$p22^{phox}$ complex at the cell membrane during cell activation (Clark et al., 1990; Wientjes et al., 1993; Someya et al., 1993).

Generally, when the cells were stimulated by microorganism or chemicals NOX system initiate the production of ROS (Ago et al., 1999; Groemping et al., 2003). The details of the mechanism of NOX enzyme activation has been well documented by Babior (1994) and De Leo and Quinn (1996). The key event is the phosophorylation dependant conformation change of the $p47^{phox}$ protein. This change induces translocation of $p47^{phox}$ (**Fig. 4,** step **1**) to the cell membrane (Clark et al., 1990) and binds to the $p22^{phox}$ prolin rich region (PRR) of the fCyt-$_{b558}$, where $p47^{phox}$ serves as connector for the cytosolic subunits to assemble at the membrane.

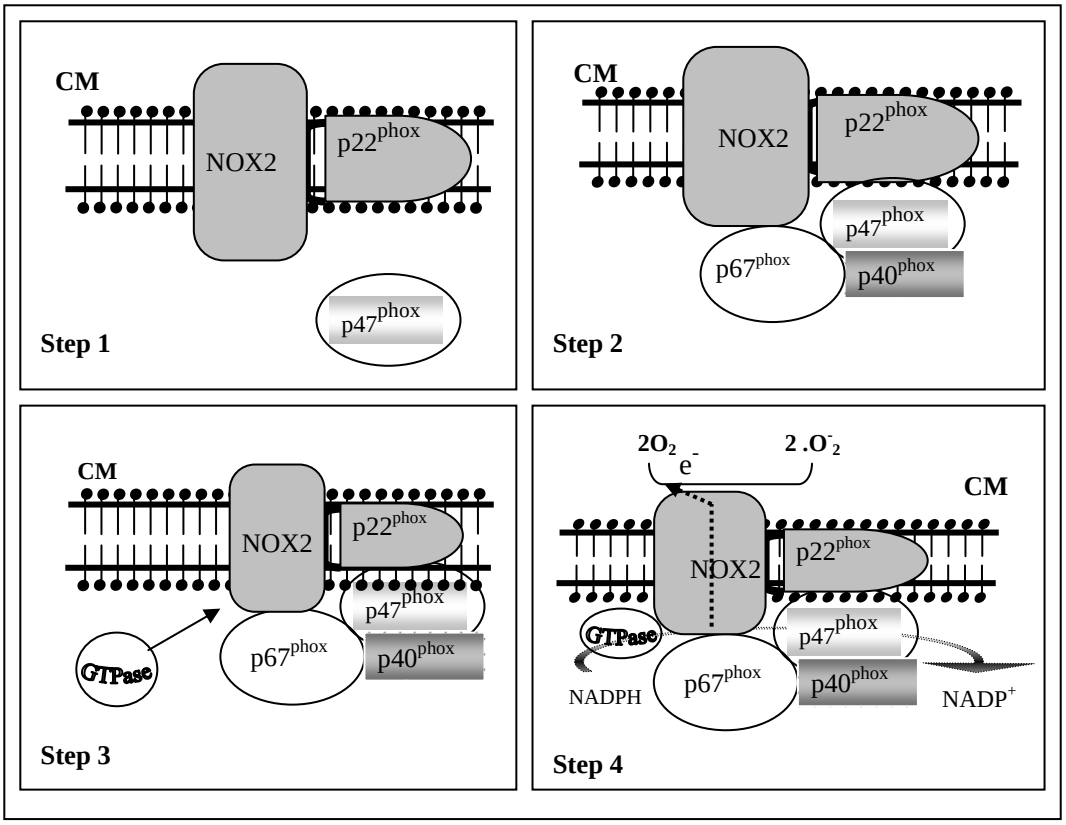

Fig. 4: Steps of activation mechanism of NOX2 enzyme. CM: cell membrane, e^- : electron.

Subsequently, $p67^{phox}$ became active (**Fig. 4,** step **2**) via its C-terminus SH3 domain binding to the active form of $p47^{phox}$ at the cell membrane. The formation of $p67^{phox}$/ $p47^{phox}$ complex requires the presence of active $p40^{phox}$, which acts as central component of the superoxide production. The two subunits, $p47^{phox}$ and $p67^{phox}$, exist as a heterotrimeric complex with 1:1:1 stoichiometry in the cytosol of resting cells in association with $p40^{phox}$. The $p40^{phox}$

serves as bridge between $p47^{phox}$ and $p67^{phox}$ at the membrane, where the $p40^{phox}$ binds to $p67^{phox}$ (step **2**).

The translocation of this activated cytosolic complex leads to the activation of the membrane associated protein fCyt-$_{b558}$, which contains a heme compound and thereby leads to the assembly of the electron transferring system. The $p47^{phox}$ and $p67^{phox}$ subunits, both are essential for NOX activation, possess distinct roles in the regulation of electron flow in fcyt-$_{b558}$. $p67^{phox}$ facilitates electron flow from NADPH to the flavin center resulting in the regulation of FAD, while $p47^{phox}$ is required for electron flow to proceed beyond the FAD center to the heme groups in fCyt-$_{b558}$ and then to molecular oxygen (Cross and Curnutte, 1995). In addition, interaction between $p67^{phox}$ and small GTPase is required for the completion of electron transfer to fCyt-$_{b558}$ (**Fig**. **4**, step **3**).

Complete assembling of all NOX subunits at the cell membrane in complex with fCyt-$_{b558}$ (**Fig. 4**, step **4**) promotes the electron transfer to two molecules of oxygen and generates two molecules of superoxide ($^{\cdot}O_2^-$) that serves as start substance for the production of other ROS (Billavite et al 1983; Ellis et al. 1989) according to the following equation:

$$2O_2 + NADPH \xrightarrow{NOX} 2^{\cdot}O_2^- + NADP^+ + H^+$$

2.5.3 NOX enzymes in detail

2.5.3.1 NOX2

The $gp91^{phox}$, the first originally identified protein of this family, has been renamed NOX2. NOX2 is encoded by a gene called cytochrome-$_b$ β-subunit (CYBB) (Orkin, 1987). The molecular mass of human glycosylated NOX2 protein is approximately 91kDa and consists of 570 amino acids (Harper et al., 1985). The bovine NOX2 sequence contains 1778 nucleotides and encodes a protein of around 91kDa which is identical in length to the human (92% gene homology) and porcine (94% gene homology) homologs (Davis et al., 1998). Furthermore, both the human and bovine sequences contain five potential glycosylation sites located in similar regions, suggesting that both may exhibit similar patterns of glycosylation.

NOX2 mRNA was described in a large number of tissues, including the intestine, pancreas, ovary, placenta, prostate (Cheng et al. 2005) as well as in mucosal (Sallesa et al., 2005) and endothelial cells (Jones et al., 1996). The structure and regulation mechanism of NOX2 followed the general features of NOX enzymes (see section **2.5.2**). The microbial lipopolysaccharide (LPS) and cytokines increased the level of NOX2 mRNA expression and modulated superoxide generation in phagocytes (Newburger et al., 1991).

NOX2 was firstly reported to participate in host defense mainly through killing microorganism by the direct action of generating ROS (Orkin, 1987). In fact, NOX2 plays a critical role in conferring leukocytes the ability to function as specialized phagocytes adapted to process antigens and kill pathogens (Savina et al., 2006). NOX2 may be also involved in cell arrest and apoptosis. Up-regulation of NOX2 mRNA expression in gastric mucosal cells was associated with cell aging and apoptosis (Sallesa et al., 2005).

2.5.3.2 NOX1

After the identification of NOX2 ($gp91^{phox}$) in non-phagocytic cells, NOX1 has been cloned by Suh et al. (1999). Based on the protein sequencing information, NOX1 protein revealed 56% identity to NOX2, with a molecular mass ranged between 55 and 60kDa containing 564 amino acids (Suh et al., 1999; Cui et al., 2006).

The activation of this enzyme followed the general pattern of NOX enzymes activation. NOX1 is also regulated based on the high structural homology between NOX1 and NOX2 by cytosolic proteins. The cytosolic components of the NOX enzymes increase the full enzymatic NOX1 activity and ROS production (Banfi et al., 2003; Geiszt et al., 2003a).

NOX1 mRNA was mainly high expressed in colon epithelial cells. However, it was also expressed in different cell types including prostate, uterus, and vascular smooth muscle cells (VSMCs) (Suh et al., 1999; Banfi et al., 2001). The primary function of NOX1 enzyme is production of ROS. Therefore, NOX expression is linked to a number of biological responses including host defense, cellular proliferation, and angiogenesis. Induction of NOX1 expression by bacterial lipopolysaccharide (LPS) can protect gastric mucosal cells because resulted in 10-fold increase of superoxide production to protect mucosal cells (Kawahara et al., 2001). Analyses of NOX1 mRNA and protein expression showed that NOX1 is involved in mucosal innate immunity and inflammation (Geiszt et al., 2003a). NOX1 expression level increased as regional colon epithelial activities increased and Szanto et al. (2005) found a correlation between expression of host defense proteins and bacteria density in the colon.

2.5.3.3 NOX4

NOX4, similar to other NOX enzymes, contains the electron transfer centres that are required to pass electrons from NADPH to molecular oxygen to form superoxide. Geiszt et al. (2000) found that the sequence of NOX4 encode for a protein of 578 amino acids with a predicted molecular weight of about 67 kDa. Furthermore, Shiose et al. (2001) detected NOX4 protein

at 75kDa and 66kDa. Because NOX4 contains four putative N-glycosylation sites they suggest that the larger protein is the glycosylated form of this oxidase. NOX4 protein showed only a 39% and 35% homology to NOX2 and NOX1, respectively. Hwang et al. (2003) cloned a 1.5kb NOX4 from bovine endothelial cells and found that the bovine NOX4 has a high degree of conservation compared to human NOX4 and revealed 91% nucleotide and 94% amino acid homology.

NOX4 has been reported to be constitutively active and might not require subunits for further activation (Geiszt et al., 2000). ROS production by this enzyme might be dependant on the expression level or the direct post-translatory modification. Neither co-expression of $p47^{phox}$ nor $p67^{phox}$ has any effects on the NOX4-dependent ROS production (Kawahara et al, 2005, Martyn et al., 2006), suggesting that these regulatory proteins are not involved in oxidase enzymatic activity. NOX4 appears to be intrinsically active and requires only $p22^{phox}$ for its activity (Martyn et al., 2006). This may be due to up-regulation of the catalytic subunit $p22^{phox}$.

NOX4 was initially identified as an NADPH oxidase highly expressed in the adult and fetal kidney particularly in the tubular system (Shiose et al., 2001; Cheng et al., 2001). Additionally, NOX4 was detected in several adult tissues including pancreas, placenta, ovary, testis and skeletal muscle (Cheng et al., 2001). It is known that NOX4 is also expressed in a variety of cells including airway epithelial cells (Kim et al, 2008), renal epithelial cells (Geiszt et al., 2000) and the cardiovascular system (Ago et al., 2004).

The physiological function of NOX4 is currently unknown. High expression level in the kidney suggests that NOX4 dependant ROS generation in renal epithelial cells may play a role in inflammatory processes in the kidney. Studies of Park et al. (2004) indicated that NOX4 is coupled to Toll-like receptor 4 mediated inflammation in response to bacterial LPS.

2.5.3.4 NOX5

NOX5 was originally identified by Cheng et al. (2001), who described a cDNA of 2199bp length encoding a predicted protein of 565 amino acids with a molecular mass of 65kDa and 27% and 29% protein homology to human NOX2 and NOX1, respectively. Furthermore, Western blot analysis revealed that the molecular mass of NOX5 protein is 60-75kDa (BelAiba et al., 2007). Among the NOX family, NOX5 has a unique structure when compared to other NOX members. NOX5 shows an extended cytoplasmic N-terminal containing EF hand calcium-binding domain (De Deken et al., 2000).

In contrast to the members of NOX family, the cytosolic subunits are not required for the NOX5 activation (Banfi et al., 2004). This attributed to the intracellular Ca^{2+} levels which induce a conformation change of NOX5 when binding to EF hands Ca^{2+}-binding domains in the NOX5 N-terminus (Dupuy et al., 2000; Banfi et al., 2001). Fu et al. (2006) indicated that decreasing intracellular pH associated with Ca^{2+} elevations induced NOX5 mRNA expression. Furthermore, the sensitivity of NOX5 to low intercellular Ca^{2+} levels was found to be increased when NOX5 molecules were exposed to specific phosphorylation as well as enhanced enzyme activation (Jagnandan et al., 2007).

The expression of NOX5 was found mainly in phagocytic cells. In particular NOX5 mRNA was expressed with higher levels in lymphocytes of the spleen and lymph node (Banfi et al., 2001). In addition, NOX5 was detected in human uterus, ovary, and placenta (Cheng et al., 2001).

In spermatogenesis, a role of NOX5-generating ROS has been proposed at different stages: from inducing apoptosis in early spermatogensis to capacitation and sperm-egg fusion because NOX5 was expressed in the spermatozoa (Banfi et al., 2001). ROS-dependent signaling and regulation of transcription factors is the most likely explanation in the function of NOX5 in testis. The generation of ROS is physiologically important to the spermatozoa in regulating every aspect of sperm function including their fertilization abilities (de Lamirande et al., 1997). Due to decreased sperm ATP level, the sperm progressive motility was inhibited as ROS production increased (Armstrong et al., 1999). Furthermore, Breitbart et al. (1992) reported ROS as key elements in the regulation of AR and capacitation by regulating the sperm-exocytose via modulating the activity of protein kinase C (PKC).

2.5.3.5 DUOX

Dupuy et al. (1999) and De Deken et al. (2000) cloned a large molecular weight homolog of NOX2 which is expressed in porcine and human thyroid glands and encodes a 1207 and 1210 amino acids protein, respectively. They were referred to as dual oxidase-1 (DUOX1 or ThOX1) and dual oxidase-2 (DUOX2 or ThOX2), because they have both a peroxidase homology domain and a NOX2 domain beside a thyroperoxidase domain. These homologs have a large molecular mass of 175-180 kDa due to the presences of the additional trans-membrane peroxidase domain. DUOX1 and DUOX2 displayed 53 and 47% protein similarity with NOX2. Furthermore, the two human DUOX genes are arranged head-to-head, separated by 15kb, where the DUOX1 and DUOX2 expression are regulated in both thyroid and non-

thyroid cells by a promoter region within 150bp of the first exon of DUOX1 and within 250bp of DUOX2 (Pachucki et al., 2004). As reported by De Deken et al, (2000), the human DUOX1 and DUOX2 revealed 77% identical at the amino acid level.

In contrast to the NOX family general structure and like NOX5, DUOX show an extended cytoplasmic N-terminal containing EF-hand motif that may account for its calcium-dependent activity (De Deken et al., 2000; Banfi et al., 2001). Additionally, DUOX have a unique trans-membrane peroxidase-like domain. Due to its unique structure, DUOX enzymes are needing $Ca2^{+}$ for their enzymatic activities (Dupuy et al., 2000; Banfi et al., 2001).

DUOX mRNA and protein were detected in other non-thyroid tissue. Human DUOX1 was highly expressed in the lung, pancreas, placenta, prostate, testis, and salivary gland (Edens et al., 2001), while human DUOX2 was expressed with higher level in the trachea, stomach, colon, rectum, prostate, and testis (Edens et al., 2001; Geiszt et al., 2003b). In all of these tissues, epithelial cells are the primary sites of DUOX expression.

Recently, two cytosolic DUOX maturation factors, DUOXA-1 and DUOXA-2, were identified and required for normal translocation of DUOX1 and DUOX2 protein, respectively. The two factors were translocated to the plasma membrane during the activation of the enzymes (Grasberger and Refetoff, 2006). It was suggested that DUOX1 and -2 are novel H_2O_2 sources that can support lactoperoxidase-mediated antimicrobial defense mechanisms on mucosal surfaces. Furthermore, there is increasing evidence that DUOX1 has additional functions in the cell signaling. Low concentrations of H_2O_2 can function as an intracellular signal and activate signaling cascades involved in growth and differentiation of many cell types (Rhee, 1999).

2.5.4 NOX activating subunits

2.5.4.1 $p22^{phox}$

One subunit of the NOX enzyme complex is the membrane protein $p22^{phox}$. The $p22^{phox}$, also known as $cytochrome_{b558}$ alpha chain (CYBA), is a 22kDa α-subunit associated with the membrane. Human $p22^{phox}$ is a membrane glycoprotein and is predicted to contain trans-membrane regions (Taylor et al., 2004) to form a stabilizing hetero-complex with NOX2 as well as NOX1 and NOX4 (Kawahara et al, 2005). The bovine $p22^{phox}$ mRNA contains 576 nucleotides and encodes a protein of 191 amino acids with a molecular weight of 21kDa, 4 amino acids shorter than the human homolog.

$p22^{phox}$ is responsible for the presence of functional NOX enzyme complexes (Groemping et al., 2003), as it is comprised a complex with NOX, the active enzyme form $fCyto_{b588}$. The functional importance of $p22^{phox}$ was demonstrated by co-expression of NOX2 with $p22^{phox}$ that is required for the efficient assembly of the mature enzyme, catalytically active $fCyt\text{-}_{b558}$ (Parkos et al., 1989). The highly conserved prolin rich region (PRR) in the cytoplasmic domain of $p22^{phox}$ is involved in interaction with the cytoplasmic NOX subunit $p47^{phox}$ (Sumimoto et al., 1996). The PRR is described as a connector between NOX enzymes and the cytosolic subunits (Dahan et al., 2002; De Mendez et al., 1997), where it acts as an anchor for both $p47^{phox}$ and $p67^{phox}$. Therefore, $p22^{phox}$ mediates the regulatory mechanism of NOX-dependant ROS.

In ovariectomized mice, circulatory E2 deficiency resulted in $p22^{phox}$ mRNA up-regulation in the VSMC and thereby increased superoxide production through NOX enzyme activation (Wassmann et al., 2005). Furthermore, E2 replacement was found to correct this effect, while P4 treatment induced a significant overexpression of $p22^{phox}$. Furthermore, $p22^{phox}$ has been shown to be involved in cellular signaling through stimulation of NOX-dependant ROS.

2.5.4.2 $p67^{phox}$

The subunit $p67^{phox}$ is a multidomain protein implicated in essential NADPH oxidase protein-protein interactions (Dusi et al., 1993). It was found that $p67^{phox}$ acts as activation subunit for the assembling of NOX enzyme complexes and their subunits (Cross and Curnutte, 1995). Subsequently, $p67^{phox}$ has been identified in cytosol from bovine neutrophils (Jouan et al., 1993) by using Western blotting, and functional comparisons have documented the conserved nature of these proteins between species. The bovine $p67^{phox}$ sequence contains a predicted opening reading frame (ORF) of 1581 nucleotides and encodes a protein of 527 amino acids, which is 1 amino acid longer than the human one. The amino acid sequencing of the human and bovine homologs revealed 88% homology.

Phosphorylation of $p67^{phox}$ is a prerequisite for its translocation from the cytosol to the membrane. Based in the functional structure of $p67^{phox}$, the protein contains four tetratrico-peptide repeat (TPR) domains in the N-terminals, which interacts with the Rac subunit (Hiroaki et al., 2001). There is a highly conserved activation domain that facilitates binding between NOX and $p67^{phox}$ following assembling of active enzyme (Takeya et al., 2003). Interestingly, $p67^{phox}$ has additional two SH-3 domains on the N-terminus side, which can bind to the PRR domain of $p47^{phox}$ in resting and activated cells (Heyworth et al., 1991).

$p67^{phox}$ contains a catalytic NADPH binding site for electron transfer to the FAD in the cytochrome-$_{b558}$ complex (Cross and Curnutte, 1995).

Among different cell types, $p67^{phox}$ was found to be expressed mainly in the cells of the immune system (Leto et al., 1990; Jones, 1994). Lymphocytes and lymphoid cell lines in addition to phagocytic cells abundantly express $p67^{phox}$ mRNA and protein (Kobayashi et al., 1995).

2.5.4.3 $p47^{phox}$

Activation of the $\cdot O_2^-$-generating NADPH-oxidase, NOX1 and NOX2, in the cells requires the assembly of a membrane-bound fCy-$_{b558}$ and the cytosolic factor $p67^{phox}$ under the control of $p47^{phox}$. As a novel cytosolic subunit of NOX2-complexes, $p47^{phox}$ was first identified by Volpp et al. (1989). Due to its unique structure, $p47^{phox}$ subunit appears to have a key role in translocation of the other cytosolic subunits required for the assembling of NOX2 enzyme complexes. Studies by Heyworth et al. (1991) using leukocytes deficient in $p47^{phox}$ indicate that this subunit is required for translocation of $p40^{phox}$ and $p67^{phox}$. Upon cell stimulation, $p47^{phox}$ undergoes conformational changes rendering the protein in a state accessible to $p22^{phox}$, thereby leading to activation of the oxidase (El Benna et al., 1994, Leto et al., 1994). Volpp et al. (1989) showed that the single ORF encodes for 390 amino acids representing a 41kDa protein. Concerning bovine $p47^{phox}$ homology, the ORF was predicted to contain 1176 nucleotides and encodes a protein of 392 amino acids, which is 2 amino acids longer than the human homolog (390 amino acids) (Bunger et al., 2000). However, at the amino acid sequence and nucleotide levels, the bovine $p47^{phox}$ show 87.4% and 85.2% identity to human homologs, respectively.

The $p47^{phox}$ mRNA is highly expressed in myeloid cells (Volpp et al., 1988), although it has also been detected in other tissues including the colon and colon epithelial cell line (Gu et al., 2003).

2.5.4.4 $p40^{phox}$

The role of $p40^{phox}$ in the phagocyte NADPH oxidase, NOX1 and NOX2, has been enigmatic since it was discovered more than a 15 years ago as a polypeptide that was co-purified in a 250kDa complex with $p67^{phox}$ and $p47^{phox}$ (Someya et al., 1993). In order to convert the normally inactive oxidase into the active state $p40^{phox}$ has been implicated in oxidase activity. Wientjes et al. (1993) showed that cDNA cloning and amino acid analysis revealed that

$p40^{phox}$ has significant sequence homology to $p47^{phox}$ and $p67^{phox}$, where the $p40^{phox}$ protein sequence showed a 70% identity to $p47^{phox}$ protein and has a SH3 domain with higher similarity to the SH3 of $p67^{phox}$. Human $p40^{phox}$ cDNA sequence shows an ORF of 1017 nucleotides and predicted to encode a protein of around 40 kDa with 339aa in length.

Indeed, the $p40^{phox}$ protein enhances membrane translocation of the cytosolic activators $p67^{phox}$ and $p47^{phox}$ and subsequent activation of the NOX2. Thus $p40^{phox}$ participates in activation by regulating membrane recruitment via interaction with $p67^{phox}$ as $p67^{phox}$ is constitutively associated with $p47^{phox}$ via tail-to-tail interaction (Ito et al., 2001).

Although $p40^{phox}$ mRNA is expressed mainly in myeloid cells such as neutrophils (Wientjes et al., 1993), but only few information are available for tissue and cells distribution and expression of $p40^{phox}$.

3 Material and methods

3.1 Materials

3.1.1 Chemicals

Chemicals	Company	Location
Agarose ultra-pure multipurpose	Carl Roth	Karlsruhe
Bromophenol blue (BPB)	Merck	Darmstadt
Diethylpyrocarbonate (DEPC)	Sigma	Deisenhofen
DNA size marker (0.5mg/ml, size 100-1000bp)	Fermentas	St. Leon-Rot
dNTPs set (100mM each)	Amersham Bioscience	Freiburg
Ethylene diamine tetra-acetic acid (EDTA)	Merck	Darmstadt
Ethanol 99.8% (Molecular biology grade)	Carl Roth	Karlsruhe
Ethidum bromide (10mg/ml)	Carl Roth	Karlsruhe
Formaldehyde (36.5%)	Sigma	Deisenhofen
Formamide (pure)	Sigma	Deisenhofen
Glycerin (water free)	Merck	Darmstadt
Hexamer ($pd(N)_6$ random hexamer)	Amersham Biosciences	Freiburg
β-Mercaptoethanol	Merck	Darmstadt
Mineral oil	Sigma	Deisenhofen
Morpholino propanesulfonic acid (MOPS)	Carl Roth	Karlsruhe
Phosphate buffered saline (PBS)	PAA Laboratory	Cölbe
Sodium acetate	Merck	Darmstadt
Sodium hydroxide (NaOH) (0.1M)	Merck	Darmstadt
TAE buffer (10X)	Carl Roth	Karlsruhe

3.1.2 Equipment

Description	Equipment	Company	Location
RNA agarose gel electrophoresis system	Gel electrophoresis GNA-100	Amersham Bioscience	Freiburg
DNA agarose gel electrophoresis system	Mini-sub cell GT	Bio-Rad	München
Digital camera	Gene Genius	Syngene	Cambridge, England
DNA electrophoresis power supply	Power pack P25	Biometra	Göttingen
Micropipettes	Volume: 2.5µl, 10µl, 20µl, 100µl, 200µl, 1000µl	Eppendorf	Hamburg
Microwave	Microstar MD 6459	MTC-Medion Technology Center	Mülheim/Ruhr
Mini vortex mixer	MS2 Mini-shaker, IKA Lab. Technology	Janke and Kunkel	Staufen
PCR thermo cycler	Master cycler gradient	Eppendorf	Hamburg
Real-time PCR cycler	Rotor-Gene RG-3000	Corbett Research	Mortlake, Australia
Sensitive balance	Sartorius MARB 200	Sartorius AG	Göttingen
pH-meter	Digital-pH-meter 646	Knick Electronic Equipments	Berlin
Spectrophotometer	Nanodrop model ND-100	PeqLab biotechnology	Erlangen
Thermo micro-centrifuge	Biofuge-Fresco	Heraeus	Hanau
Mixer	Thermomixer compact	Eppendorf	Hamburg

3.1.3 Consumable materials

Product	Description	Company	Location
Cell scraper	Disposable sterile scraper	Greiner Bio-One	Frickenhausen
Tubes (2ml) with cap	Reaction tubes	Eppendorf	Hamburg
Micropipette tip with filter	Volume : 2.5µl, 10µl, 20µl, 100µl, 200µl, 1000µl	Eppendorf	Hamburg
Reaction tubes (0.2ml)	PCR tubes	Eppendorf	Hamburg
Real-time PCR tubes (10µl)	Stripe tubes	LTF-Labortechnik	Wasserburg
Pipettes (10ml)	Serological plastic pipettes	Carl Roth	Karlsruhe
Sample tube (50ml)	Conical tubes with screwed caps	Biozym Scientific	Hess. Oldendorf
Scalpels	B. Braun Cutfix	Aesculap	Tuttlingen

3.1.4 Commercial Kits

Kit	Description	Company	Location
Invisorb Spin DNA extraction kit	DNA Extraction	Invitek	Berlin
ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing kit	DNA sequencing	Applied Biosystems	Darmstadt
NucleoSpin RNA/Protein extraction kit	RNA/Protein extraction	Macherey-Nagel	Düren

3.1.5 Enzyme

Description	Enzyme	Company	Location
Immolase (5U/µl)	For PCR	Bio-Line	Luckenwalde
M-MuLV Reverse Transcriptase (200U/µl)	Reverse transcriptase	Fermentas	St. Leon-Rot
RQ1 RNase-free DNase (1U/µl)	DNase for DNA digestion	Promega	Mannheim
2X Sensi-Mix (dt)	Real-time PCR	Quantace	Berlin

3.1.6 Stock solutions and buffers

Buffers and solutions were prepared using autoclaved distilled water or DEPC-H_2O as indicated, otherwise distilled water (dH_2O) was used.

Buffers and solutions	Chemical composition and preparation
10X MOPS stock solution	0.2M MOPS 50mM Sodium acetate 10mM EDTA (pH 8.0) The mixture was autoclaved for 20 min at 120ºC.
1X MOPS	50ml 10X MOPS stock solution 450ml DEPC-H_2O
1X TAE	25ml TAE buffer stock solution (10X) 225ml H_2O
BPB saturated aqueous solution	BPB in access dissolved in DEPC-H_2O. The saturated supernatant was used after spin down of the solids.
BPB/Glycerin solution	15µl BPB saturated aqueous solution 85µl DEPC-H_2O 100µl Glycerin
2% (w/v) DNA-agarose gel stained with ethidium bromide	0.6g Agarose 300ml 1X TAE Buffer Mixed well and boiled in microwave for 3min 5µl ethidium bromide (10mg/ml) was added and mixed.
Ethanol for RNA extraction 70% (v/v)	70ml Ethanol, HLPC Gradient grade 30ml DEPC-H_2O

Buffers and solutions	**Chemical composition and preparation**
DEPC-H_2O	1L H_2O 1ml DEPC Autoclaved at 121ºC for 20 min.
DNA electrophoresis loading buffer (6X)	25mg BPB 5ml Glycerine 40µl EDTA (0.5M; pH 8.0) 1ml TAE buffer (10X) 3.96ml H_2O
dNTPs working solution (10 mM each)	10µl 100mM dATP 10µl 100mM dCTP 10µl 100mM dGTP 10µl 100mM dTTP Diluted with 60µl PCR-H_2O.
PCR-H_2O	100ml H_2O, autoclaved at 121°C for 20min.
PCR mixture per sample	2.5µl Immolase buffer (10X) 0.75µl MgCl (50mM) 0.5µl dNTPs (10mM each) 0.5µl Primer for. (20µM) 0.5µl Primer rev. (20µM) 17.25µl PCR-H_2O 0.1µl Immolase enzyme (5U/µl)
Primer (20µmol) working solution	The lyophilized primer was resolved in PCR-H_2O to obtain a concentration of 200µmol and stored at -20ºC. A 20µmol working solution was prepared by diluting the stock solution 1:10 with PCR-H_2O.
Real-time PCR mixture per sample	0.2µl Primer for. (20µM) 0.2µl Primer rev. (20µM) 0.2µl SYBR green solution (50X) 3.4µl PCR-H_2O 5.0µl 2X SensiMix (dt)
RNA loading buffer	75µl Formamide 25µl Formaldehyde (37%) 10µl MOPS buffer (10X) 1.0µl Ethidium bromide solution (10mg/ml) 6.0µl BPB/Glycerin solution 2.0µl BPB saturated solution
Random hexamer (50µM) working solution	7.14µl Hexamer ($pd(N)_6$ stock solution 92.0µl H_2O

Buffers and solutions	Chemical composition and preparation
Denaturing RNA agarose gel (1%)	0.4g Agarose 4ml 10X MOPS buffer 30ml DEPC-H_2O Mixed well and boiled in microwave for 3min. After cooling to 60ºC, 6.4ml formaldehyde was added and the volume was completed to 40ml by DEPC-H_2O.
RT reaction mixture per sample	8µl M-MuLV RT-buffer (5X) 4µl dNTPs (10mM each) 3µl random hexamer (50µM) 24µl PCR-H_2O 1µl M-MuLV Reverse Transcriptase (200U/µl)

3.1.7 Computer software

Program	Software name
DNA quantification	Rotor-Gene **v.6.0** software
DNA/Protein homology search	BLAST search (National Center of Biotecnology, USA, URL: http://www.ncbi.nlm.nih.gov)
DNA sequence analysis	DNASIS Sequencing software
Primer designing	HUSAR Primer Online-software
RNA quantification	ND100 **v 3.1.0**
Statistical analysis	SPSS **v 12.0.** for Windows
Graphics drawing	Excel for Windows, Microsoft office 2003

3.2 Methods

3.2.1 Collection of oviducts

Oviduct samples were collected at the slaughterhouse after 15-20 minutes of death. The reproductive tracts with abnormal morphological changes (ovarian hypoplasia, uterine and oviducts adhesion), signs of inflammation (endometritis, cervicitis, mucometra), and cystic ovaries were excluded. The classification of collected oviducts was based on the stage of the estrous cycle after visual inspection of the reproductive tract (corpus luteum, follicles, cervix, and mucus) according to Irland et al. (1980) and Arosh et al. (2002). Therefore, the collected oviducts were classified into one of the following four groups:

1. Post-ovulatory phase (day 1-5)
2. Early-to-mid luteal phase (day 6-12)
3. Late luteal phase (day 13-18)
4. Pre-ovulatory phase (day 19-21)

At the same time, ipsi-lateral and contra-lateral oviducts (**Fig. 5**) were identified for each cow. The ipsi-lateral oviduct was identified according to the site at which ovulation had occurred or will occur (corpus luteum or Graaf follicle at the corresponding ovary). Thereafter, the obtained oviducts were kept on ice and transported to the laboratory within 2 hours.

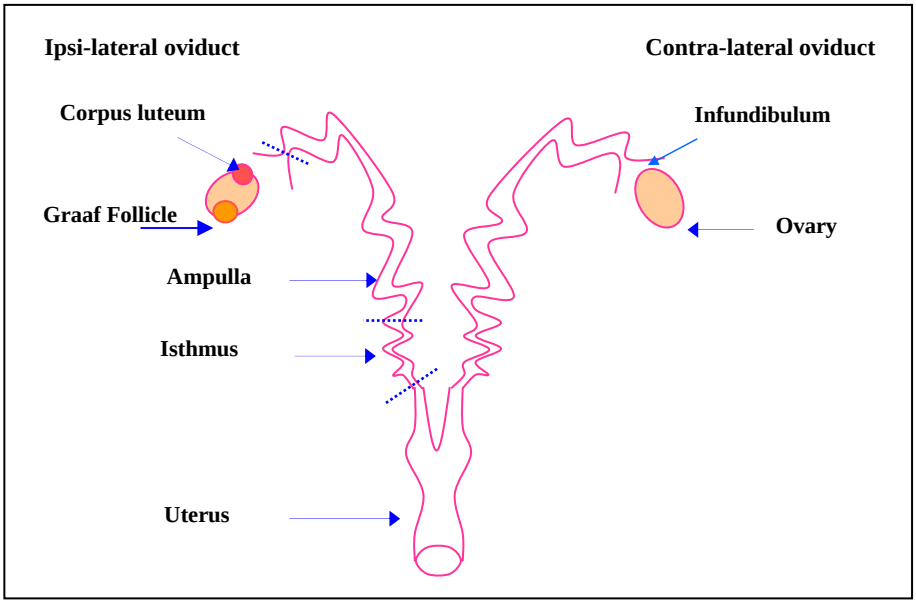

Fig. 5: Diagram showing the bovine reproductive tract. Ipsi-lateral oviduct to the ovulation site (corpus luteum or Graaf follicle) was indicated.

3.2.2 Harvesting and preparation of oviductal cells

The oviducts from the ipsi- and contra-lateral site were further subdivided into two separate regions, ampulla and isthmus. In the laboratory, the oviducts were trimmed from the surrounding serosal tissue. In order to obtain oviductal cells, oviducts were opened along the longitudinal axis using a scissor. Oviductal mucosal cells from each part were carefully scraped with aid of a cell scraper and transferred with PBS (pH 7.5) solution in a 2ml tube. To get the oviductal cell pellet, the scraped oviductal cells were centrifuged for 5min at 3000Xg and 4ºC. Thereafter, PBS was removed and the cell pellet was kept at -80ºC for further analysis. Finally, the following samples were obtained for each cow: ipsi-lateral ampulla (I-A), ipsi-lateral isthmus (I-I), contra-lateral ampulla (C-A), and contra-lateral isthmus (C-I).

3.2.3 mRNA analysis

3.2.3.1 Total RNA extraction

All extraction processes were done under a hood to avoid harmful effects of β-mercaptoethanol. RNA is sensitive to RNase enzyme degradation. Therefore, it is necessary to avoid RNase contamination when working with RNA. RNase can come from the skin or microorganism in the laboratory environment. Wearing gloves, using DNase/RNase free material and using autoclaved solutions is important during working with RNA. Furthermore, pipettes, forceps and the hood bench were cleaned with ethanol (70%).

Total RNA was extracted from oviductal mucosal cells using NucleoSpin RNA/Protein kit following the manufacturer's instructions. Cell lysis buffer (per sample) was prepared by mixing 700µl of supplied buffer (**RP1**) with 7µl β-mercaptoethanol. Directly to the cell pellet (30-50mg), 700µl of prepared lysis buffer were added, mixed by pipetting up and down to ensure the complete lysis and homogeneity of the sample. The genomic DNA contamination was reduced by using DNA column filters. 700µl of the lysate mixture was transferred to the DNA filter placed in a 2ml collecting tube and centrifuged for 1min at 11000Xg. Thereafter, the filter column with bound DNA was discarded and an equal volume of 70% ethanol was added to the lysate to adjust the RNA binding conditions. A RNA-binding filter was placed into a new collecting tube. Then 700µl of the lysate mixture were transferred directly onto the RNA binding filter, followed by centrifugation for 1min at 11000Xg. For volumes more than 700µl, centrifugation was repeated with the remaining lysate mixture. The flow-through was discarded. Thereafter, the filter unit was placed in a new collecting tube. The ethanol residues and salt contaminations were eliminated from the filter by adding 350µl of supplied membrane desalting buffer (**MDB**). The RNA filter was centrifuged for 1min at 11000Xg. To eliminate completely genomic DNA, the filter was incubated for 15min with freshly prepared 100µl DNase reaction mixture (prepared for one sample by mixing of 10µl RNase-free DNase with 90µl supplied reaction buffer). After the incubation, the filter column was washed with 200µl of supplied wash buffer (**RA2**), centrifuged for 1min at 11000Xg, the filtrate was discarded. Then the filter column was placed in a new collecting tube and additional two serial washing steps were done using 600µl and 250µl of supplied washing buffer (**RA3**) with centrifugation for 1min at 11000Xg in each step, respectively. In a new 1.5ml collecting tube, total RNA was obtained by elution of the filter column by incubating with elution buffer (DEPC-H_2O) for 5min, followed by centrifugation for 1min at 11000Xg.

3.2.3.2 Quantification of extracted total RNA

The principle of the RNA quantification in a sample is depending on the extinction of RNA (degree of UV light absorption). For soluble RNA, the measured optical density (OD) of 1 unit corresponds to 40µg/ml for RNA at the wavelength of 260nm. In the present study, the concentration of isolated total RNA from oviductal cells was quantified in a 2µl sample volume using the NanoDrop spectrophotometer. However, RNA samples can contain contaminating proteins. Proteins have their own absorbance properties for UV light at a different wavelength (280nm). Therefore, the ratio of absorbance at 260nm and 280nm was used to determine the RNA sample purity. The ratio of ≥1.8 indicates good purity of RNA. After quantification, the extracted total RNA was kept at -80ºC until further analysis.

3.2.3.3 Denaturing gel electrophoresis of RNA

The quality and integrity of isolated RNA can be determined after denaturing agarose gel stained with ethidium bromide staining to visualize intact 28S and 18S rRNA bands. The staining for the 28S rRNA band should be approximately twice as intense as the 18S rRNA band (**Fig. 6**). Furthermore, most RNA forms secondary structures via intra-molecular base pairing that may changes the RNA size which prevents it from migrating according to its real size. Therefore, denaturing gel electrophoresis was used in the present study to determine the quality of extracted RNA.

In order to run the denaturing gel electrophoresis, the gel tank with gel supported plate and comb was first filled with 0.1M NaOH for 30min. Then they were rinsed 3 times with DEPC-H_2O. Thereafter, a 1% denaturing RNA agarose gel was freshly prepared as described in section **3.1.6**. The gel was cooled down to 60°C, immediately poured into the supporting plate and the comb placed into the gel. After an hour, the solidified gel was covered with 1X MOPS electrophoresis buffer. In the meantime of the gel solidification, the RNA sample was prepared. 2µg of total RNA was diluted in a total volume of 10µl DEPC-H_2O. The diluted RNA sample was mixed with 10µl of RNA loading buffer. The sample-loading buffer mixture was incubated for 10min at 70°C and immediately placed on ice. Through this approach, the RNA secondary structures were destroyed. After 5min incubation on ice, the sample was centrifuged in a cold centrifuge (4°C) at 11000Xg. Thereafter, 17µl of the sample mixture were transferred into each gel well. Electrophoresis was performed for 2h under constant voltage of 87V. The gel image was obtained using a digital camera system (Gene Genius Bio-imaging system). The RNA quality was determined by observing intact 28S and 18S rRNA bands (**Fig. 6**).

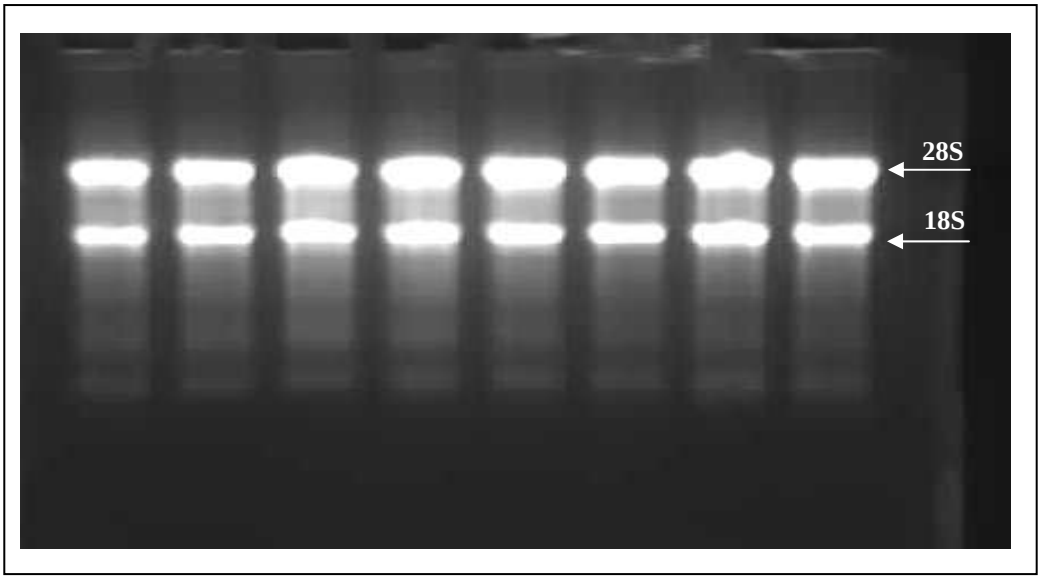

Fig. 6: The formaldehyde gel electrophoresis image depicts the quality of the isolated total RNA as indicated by 28S and 18S rRNA bands. No visible genomic DNA was observed.

3.2.3.4 Reverse Transcription (RT)

RNA can not serve as template for the application by polymerase chain reactions (PCR). Therefore, the RNA must be converted into cDNA. The conversion of RNA into cDNA can be achieved by a simple enzymatic reaction (RT) with the Moloney Murine Leukemia virus (MMuLV) reverse transcriptase.

The RT was performed as a two step reaction. First, any remaining genomic DNA content in the samples was eliminated using DNase. The reaction mixture (20µl volume) for one sample contained: 10µl total RNA sample (0.5µg/10µl PCR-H_2O), 5µl PCR-H_2O, 4µl of 5X RT-supplied buffer, and 1µl of DNase enzyme (RNase-free). Thereafter, the reaction mixture was thoroughly mixed and centrifuged for 30sec at 13000Xg. The mixture was incubated in the block cycler for 30min at 37ºC and then for 5min at 70ºC to stop DNase activity. After DNA digestion, the sample was incubated immediately for 5min on ice.

In the second step, the first stranded cDNA was synthesized from RNA templates. The RT reaction was performed in a total reaction volume of 60µl containing 40µl reaction mixture and 20µl RNA sample from the first step. The reaction mixture contained (see section **3.1.6**): 5X MMuLV buffer, MMuLV enzyme, dNTPs, and random hexamers. Samples without RT enzyme served as a control of the DNase efficiency. The sample reaction mixture was incubated in a block cycler with the temperature profile as follow: 25ºC for 10min, 42ºC for 60min, and 90ºC for 2min. Thereafter, the cDNA sample was mixed and centrifuged for 30sec at 13000Xg and aliquots were stored at -20ºC for quantification subsequently by real-time PCR.

3.2.4 Primers design and examination

3.2.4.1 Primer design

The primers used in this study were designed by online computer software and were synthesized by MWG-Biotech AG (Ebersberg, Germany). **Table 2** shows all the information regarding the used primers.

Target gene	Designed sequences 5′ → 3′	Product size (bp)	Acc. No. and position of amplicon
18S rRNA	For. GAG AAA CGG CTA CCA CAT CCA A Rev. GAC ACT CAG CTA AGA GCA TCG A	317	AF176811 (75-391) (Odau et al., 2006)
NOX1	For. ACT GGA TCT GCA GGG AGA TG Rev. CTG TCA GGA TGT CAT TGG CC	186	EF380359.1 (423-608)
NOX2	For. TGG TGT CCA AGC TGG AGT G Rev. TGA ACA CGT CCT CAC TGG C	207	NM_174035.3 (1063-1269)
NOX4	For. CTT CAC AAC TGT TCC TGG CC Rev. TGA TAC TTC AGC AGC CCT CC	183	AY354499.1 (438-620)
NOX5	For. GCT GTG CTC ATT GGT GCA GG Rev. GTG CAG CTC CAG AAA ACG GC	273	XR-027980 (1696-1968)
DUOX1	For. AGA ACA GCA AGA ATG GGC TG Rev. AAA TTT CTC CTC CGG AGC TG	322	XM-587550.3 (1568-1889)
DUOX2	For. GCT CAC AAC CGG AGA CCT GC Rev. TGG ATA GCT CCT CTC CCG GG	265	XM-001253634.1 (1719-1983)
$p22^{phox}$	For. GTT CAC CCA GTG GTA CCT GG Rev. AGC AGG AAG CCA GCA GGT AC	119	NM_174034.2 (121-342)
$p67^{phox}$	For. TGG AAG ACC CCA GTT GTC AC Rev. TGT TCT CAC ACC ACA GCG TC	311	NM_174120.2 (1069-1379)
$p47^{phox}$	For. ACC CTC AAG GAG ACC TTC CC Rev. AGC ATA GGC TCC AGG TCC AG	242	XM-594385.3 (1030-1271)
$p40^{phox}$	For. CGA GGT AAA GAC GAA AGG GG Rev. AGG AGG TGC TTC ATG TGA GC	222	NM_001045983 (235-437)

Table 2: The table shows the primer used for the investigation of NADPH oxidase family transcripts. Gene name, primer sequences, expected product size, **EMBL** accession number (**Acc. No**.) and amplicon position are indicated.

3.2.4.2 Polymerase chain reaction (PCR)

PCR was performed using designed primers for each specific gene. The target genes, primer sequences and expected sizes of amplicons are described in **Table 2**. The PCR was performed in a total volume of 25µl including 2.5µl cDNA (see section **3.1.6**). The PCR protocol of 40 cycles was performed using hot-start PCR enzyme Immolase and therefore initiated by a step of 10 min at 94°C to activate the enzyme. Follow by 38 cycle amplification including denaturing at 94ºC for 30sec, annealing at specific primer annealing temperature. Additionally, extra elongation step of 72°C for 2min was added to finalize the amplification. For verification of the PCR, all PCR products were visualized under UV light after gel electrophoresis on 2% agarose gel containing ethidium bromide. For the DNA gel protocol see section **3.1.6**.

3.2.4.3 Gradient PCR for estimation of optimal primer annealing temperature

The optimal primer annealing temperature allows the primer to select and bind specifically to their complementary position on the DNA template. To determine the optimal annealing temperature, gradient PCR was carried out using a gradient thermocycler (Eppendorf Master Cycler gradient) with a gradual increasing temperature as presented in **Table 3**. The following PCR protocol was performed for the used primer pairs.

To perform the reaction, a total volume of 60µl cDNA products were mixed thoroughly with the PCR reaction mixture (see section **3.1.6**) and centrifuged for 30sec at 13000Xg. Following the preparation of the sample reaction mixture, the mixture was divided into the 12 reaction tubes as each tube contained 25µl. The applied PCR protocol was as follow: the sample reaction mixture was heated at the first step for the activation of Immolase to 94ºC for 10min, followed by 38 cycles of amplification including denaturing at 94ºC for 30sec and a gradient annealing temperature during PCR reaction of 52.0-68.5ºC (**Table 3**). An additional extension step was performed at the end at 72ºC for 2min to complete the PCR reaction.

Well No.	1	2	3	4	5	6	7	8	9	10	11	12
Temp. ºC	52.0	52.2	53.1	54.6	56.4	58.5	60.7	62.9	64.9	66.6	67.8	68.5

Table 3: The temperature gradient setting in the PCR thermocycler block for the determination of optimal annealing temperature of the used primers.

3.2.5 DNA analysis

3.2.5.1 DNA agarose gel electrophoresis

The validity of the PCR products was checked with DNA gel electrophoresis. DNA fragments were separated in the gel by their sizes, facilitating the determination of suspected product size by using a suitable DNA ladder marker.

The preparation of the 2% agarose gel stained with ethidium bromide is described in section **3.1.6**. A volume of 50ml gel was poured into the gel supporting plate and the comb was placed into the gel. After one hour solidification, the gel was covered with 200ml of 1X TAE electrophoresis buffer and the comb was removed. 25µl of each PCR product was mixed with 5µl of 6X DNA loading buffer. Thereafter, the mixture was loaded into the gel wells. To determine the size of the PCR products, DNA ladder marker was loaded into a separate well. The electrophoresis was run with a constant voltage of 87V for one hour. The stained DNA bands were visualized using UV light and the photo of the gel was recorded using the digital camera system (Gene Genius Bio-imaging system). According to the arrangements of the samples in the thermocycler block, the optimal annealing temperature of the primer can be determined by observing the intensity of the specific DNA bands (**Fig. 7.**).

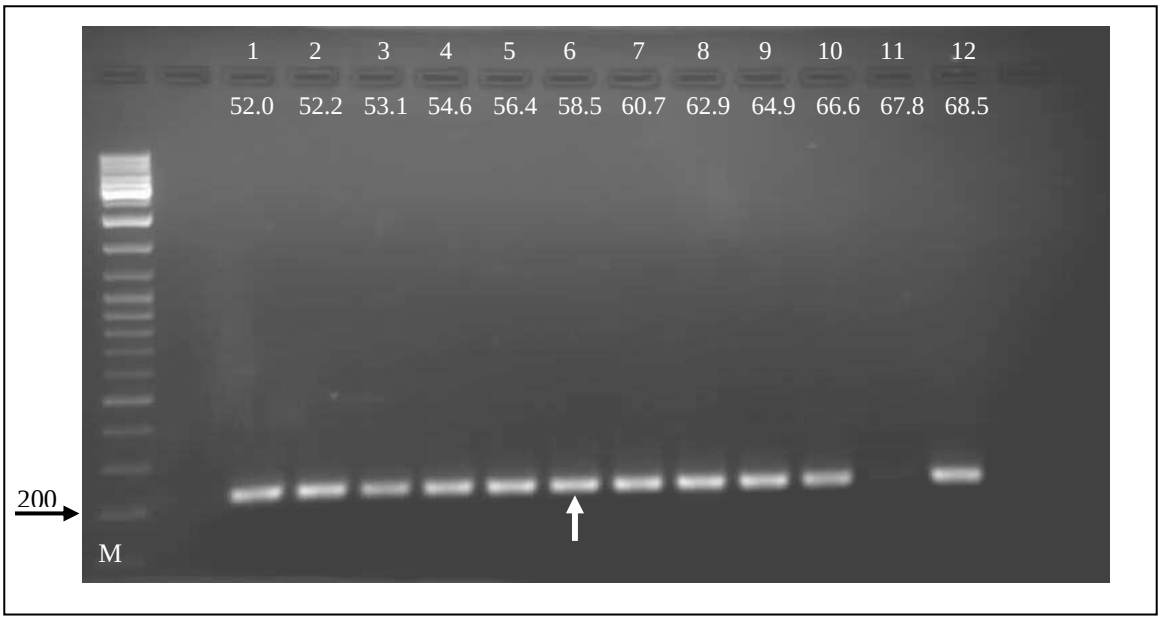

Fig. 7: Image of the DNA gel stained with ethidium bromide showing PCR products (e.g. for NOX2, 207bp) and the most bright strong band was used to determine the optimal annealing temperature (vertical arrow). The product size was determined by comparing the DNA products to the corresponding size of DNA marker bands (horizontal arrow). M: DNA marker (range 100-1000bp).

3.2.5.2 Extraction and purification of PCR products

The PCR products were extracted from the gel using the Invisorb Spin DNA extraction kit following the manufacturer's instructions. The specific PCR products were carefully excised under UV light from the agarose gel with a sharp scalpel. The weighed gel slices were placed into a 2.0ml reaction tube and 500µl of gel solubilizer (**S**) were added. Thereafter, the mixture was incubated in an Eppendorf thermomixer at 50°C for 10min. After gel slices were completely dissolved, a volume of 250µl of Binding Enhancer solution was added to the suspension mixture and vortexed. Approximately 800µl of the solution were transferred onto a new Spin filter which was placed into 2.0ml collecting tube. Then the filter was centrifuged at 12000xg for 1min. For volumes more than 800µl, the residual liquid was loaded onto the filter and the centrifugation was repeated. Subsequently, the Spin filter was washed at 12000xg for 30sec with 500µl of supplied washing buffer. The filtrate was discarded. A further washing step was repeated and the filtrate was discarded. An extra centrifugation step was done at 13000xg for 4min in order to remove all residual wash buffer. The Spin filter was transferred into a new 1.5ml supplied receiver tube. 30µl of the supplied elution buffer was transferred into the center of the filter and incubated for 5min. Thereafter, the Spin filter was centrifuged for 1min at 12000xg. Finally, the yield of PCR products (ng/µl) was measured at 260nm using the Nanodrop spectrophotometer. The samples were kept at 4ºC.

3.2.5.3 DNA sequencing

DNA sequencing was performed to determine the homology of PCR products to known bovine sequences published in the gene bank.

The DNA sequencing was done by the chain-termination sequencing approach according to the method described by Sanger et al. (1977). The principle based on the incorporation of dideoxyribonucleotides (ddNTPs). The ddNTPs have no terminal 3′-OH group that is needed for DNA amplification. When ddNTPs are involved in the polymerase chain reaction, the reaction will be terminated at the specific ddNTP (either A, C, T, or G) which was incorporated in the DNA strand. The fragments of the sequencing reaction were separated by capillary gel electrophoresis technique. The labeling of ddNTPs with four different fluorescent dyes facilitates the differentiation between DNA fragments to obtain the DNA sequence.

Sequencing reaction was carried out using a commercial DNA sequencing kit. In each reaction tube, 10µl reaction volume was prepared by mixing 6µl PCR product (50-100ng/µl),

2µl Terminator Ready Reaction Mix (contained DNA polymerase, dNTPs, ddNTPs, buffer), and 2µl of forward or reverse primer (3.2µmol). The reaction mixture was centrifuged for 30sec at 13000Xg. In order to avoid sample evaporation, the sample mixture was covered with 20µl mineral oil. Thereafter, the tube was placed in the block cycler for 25 cycles with the following temperature profile: 96ºC for 10sec, 50ºC for 5sec, 60ºC for 4 min after complete cycling, and the sample was cooled down to 4ºC.

The analysis of sequencing was done commercially on a capillary electrophoresis DNA Analyzer, ABI 3730XL (GATC Biotech, Germany). The resulted sequences were evaluated with the DNASIS software program and related to known sequences in the **EMBL** database.

3.2.5.4 Quantitative Real-Time PCR

In the present study, the amount of mRNA in the samples was quantified by real-time RT-PCR using sequence specific primers for each gene. Quantification of amplified products based on a fluorescence dye binding specifically to all double-stranded DNA during the PCR. In the real-time PCR machine, fluorescence of the dye was activated at 480nm and the DNA-bound dye emitted light of 520nm wavelength. The signal intensity is similar to the measured amount of amplified product (fluorescence density) at each cycle during PCR. The software of the Real-time cycler constructs the curve of gene amplification by plotting the increase in the fluorescence intensity versus cycle number. The curves have three segments (**Fig. 8**): an early background phase (no signal was detected), the exploration phase (detection of PCR products accumulate). During this phase, the threshold was set as the first point of fluorescence detection was above the base line. The threshold cycle (C_t) was defined as the cycle number at which the fluorescence of the dye exceeds the fixed threshold. The last segment is the plateau phase, where the amount of product will not change because some reagents become limiting and the enzyme activity decreased. C_t always was located within the exploration phase, where is a linear relation between logarithm (**log**) of the changes in fluorescence intensity, the cycle number and the reaction components are not limiting. Therefore, the standard curve can be constructed from the logC_t versus the log standard concentrations values.

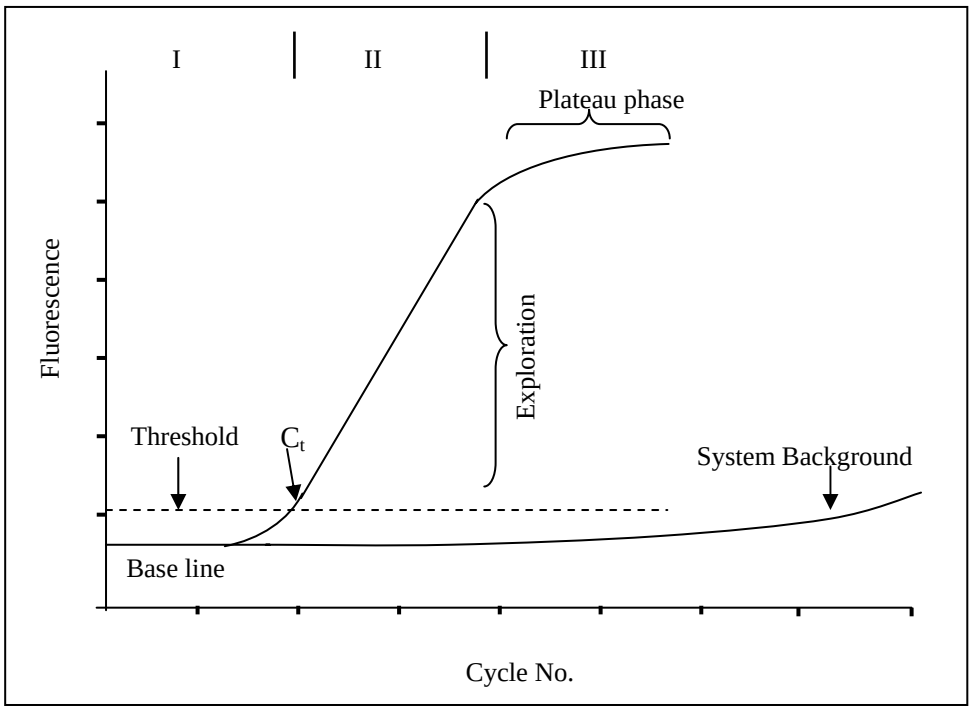

Fig. 8: Representative scheme of the sample amplification curve. The diagram shows the three phases of the curve (I, II, III). C_t: cycle threshold.

The SYBR green fluorescent dye was used in the present study as DNA-binding dye. Real-time PCR was carried out using the SensiMix (dt) kit. The protocol was performed in 10µl volume containing 9µl reaction mixture (contained primer pair, SYBR green solution, SensiMix (dt); see section **3.1.6**) and 1µl cDNA sample. PCR-H_2O or products of reverse transcription without enzyme were used as negative controls. The reaction was run in the Rotor Gene RG-3000 cycler with temperature profile as follows: for 10min at 95ºC (enzyme activation), followed by cycling program as 95ºC for 15sec (denaturing), at specific primer annealing temperature for 20sec, 72ºC for 30sec (elongation). Depending on special conditions for each primer pairs the protocol was adapted individually (annealing temperature, number of cycles, reaction efficiency, see **Table 4**). In order to determine the specific gene product, melting of the PCR products was started after complete cycling by gradually heating from 50ºC to 95ºC by 0.5ºC/3sec.

PCR products were quantified using the standard curve. A standard curve was obtained from the serial dilutions of the PCR product of the same target gene under investigation with known concentration. The dilution series was made using 2µl of each PCR products and PCR-H_2O in ratio of 1:10 to give 10 serial dilutions from 1:10 up to $1:10^{10}$. The first 3 highest standard concentrations were excluded. The amount for the unknown gene sample was calculated from the standard curve of the specific gene. The relative quantification relies on the comparison between expression of a target gene versus a reference gene. Therefore, 18S

rRNA (housekeeping gene) data measured in the samples were utilized to normalize the real-time data of the target genes.

Gene	At	No. of cycles	Mt	R E (%)
18S rRNA	61ºC	25	84.4ºC	-
NOX1	63ºC	45	82.3ºC	1.00
NOX2	59ºC	40	84.3ºC	0.73
NOX4	63ºC	45	78.8ºC	0.96
NOX5	65ºC	40	84.8ºC	1.03
DUOX1	63ºC	45	86.8ºC	1.00
DUOX2	63ºC	40	87.5ºC	0.94
$p22^{phox}$	60ºC	45	85.8ºC	0.91
$p67^{phox}$	60ºC	45	84.8ºC	0.96
$p47^{phox}$	67ºC	40	90.3ºC	1.02
$p40^{phox}$	57ºC	45	85.5ºC	1.00

Table 4: The table shows the specific reaction conditions for each gene examined including the optimal annealing temperature (At), cycle number, melting temperature (Mt) and reaction efficiency (RE).

3.2.6 Primary cell culture

Cell culture experiments using primary bovine epithelial cells were done to study the influences of ovarian steroid hormones, arachidonic acid (AA) and prostaglandin E_2 (PGE_2) on the gene expression of NOX enzymes in bovine oviductal cells. Stimulated primary oviductal cell cultures were performed by Simone Odau and all details are described by Odau (Thesis, Berlin 2006). Briefly, harvested mucosal oviductal cells were washed twice and suspended in Earle salt buffered M199 to obtain approximately 1-2 $x10^5$cells/ml. Thereafter, 5 ml of the cell suspension were seeded in 6-well plates. The cell culture was incubated for three days at 39°C and 5% CO_2 in a humidified atmosphere to allow the cell confluence and adherence. Subsequently 12h after medium change, the confluent cells were stimulated for 6h with physiological concentration of 17β-estradiol (E2; 10pg/ml), progesterone (P4; 10ng/ml), AA (10µM) or PGE_2 (0.25 µM). The sample collection for RNA isolation was done at time points 0h, 2h, 4h, or 6h of treatment, respectively. Cell cultures without hormonal treatment

were used in parallel as control for each of the time points. Viability of the cultured oviductal cells at day 4 of culturing was determined by trypan blue exclusion and was >95%.

3.2.7 Statistical analysis

Statistical analysis were performed using statistic program software SPSS (**v12.0**) for Windows package (SPSS Inc. Chicago, IL, USA, 2003). The histograms were drawn using Microsoft Excel software for Windows (Microsoft office, 2003). The results were presented as mean ±SEM. The values of the sample mRNA quantification data were normalized with 18S rRNA data. The corrected values for mRNA were expressed as ratio multiplied by factor 10^6.

The General Linear Model (Univariat Analysis of Variance) was performed for *in vivo* study of each mRNA quantified by real-time RT-PCR. Univariat analysis test was done to investigate the effects of the estrous cycle on the mRNA expression of NOX enzymes or activation subunits. When the test indicate a significant difference ($p<0.05$), thereafter Levene's test of equal of variances was carried out to verify the homogeneity of variances. Accordingly, Post-hoc-test (Tukey or Dunnett-T3) was performed to determine the significant difference in mRNA expression between the different estrous cycle phases. It is important to note that each oviduct pair could not be related to the same animal. Therefore, the t-test for two independent variables was performed to determine the local expression differences in mRNA of NOX and activation subunits between the ampulla and isthmus of ipsi- and contra-lateral oviducts for each estrous cycle phase. A possible correlation between NOX1 and NOX2 with their activation subunits ($p22^{phox}$, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$) as well as between NOX4 and $p22^{phox}$ during different phases of the estrous cycle was also assessed using Pearson correlation test.

To reveal the differences in mRNA expression for *in vitro* study (cell culture experiment) a non-parametric test (Wilcoxon, Kruskal-Wallis) was performed after the distribution of standardized residuals was tested. Because the mRNA basic expression level in the cells was variable between the different animals, the mRNA expression at 0h for each animal was set to 100%. The values of gene quantification at time points 2h, 4h, or 6h were calculated as relative percentage to the corresponding corrected 0h values. As a percentage value, the normal distribution were not assumed, so Wilcoxon test for two dependent samples was used to compare between the control samples and their corresponding treated sample values at the same time points. The level of significance was set at $p < 0.05$.

4 Results

The presented results in this study were obtained from the *in vivo* and *in vitro* analysis of mRNA expression of NOX components (enzyme and activation subunits) in bovine oviductal cells. The expression level obtained for NOX components mRNA and 18S rRNA was expressed as fg specific mRNA/µl cDNA. The expression levels were normalized with measured 18S rRNA data in the same samples. The obtained results for normalized mRNA expression level were multiplied by factor 10^6 to obtain the corrected values. Furthermore, the absolute expression levels of each mRNA were calculated as fg/µg total RNA. Correlations were calculated between NOX enzyme (NOX1 and NOX2) and their activation subunit ($p22^{phox}$, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$) and between NOX4 and $p22^{phox}$ using Pearson Correlation.
The data obtained from *in vitro* analysis (cell culture experiments) were presented as percentage showing the relative variation. The data were collected from untreated and treated cells at time points 0h, 2h, 4h, or 6h of incubation, respectively. The measured mRNA expression level of the untreated cells at time point 0h was set to 100%. Accordingly, the mRNA expression of the NOX family members at the remaining time points was calculated as percentage in relation to the corresponding corrected 0h values. This calculation was done to reduce the variation differences between the different cell culture samples.

4.1 mRNA expression of NOX1

The data depicted in **Fig. 9** show that NOX1 mRNA was expressed in oviductal cells of ipsi- and contra-lateral oviducts during all phases of the estrous cycle. However, no differences were observed in the expression levels of NOX1 mRNA between different phases of the estrous cycle (**Fig. 9A**). The expression of NOX1 mRNA remained in similar levels in examined segments of the oviduct (**Fig. 9B**). The absolute expression level of NOX1 mRNA in the bovine oviduct was in the range of 1-3 fg/µg total RNA.

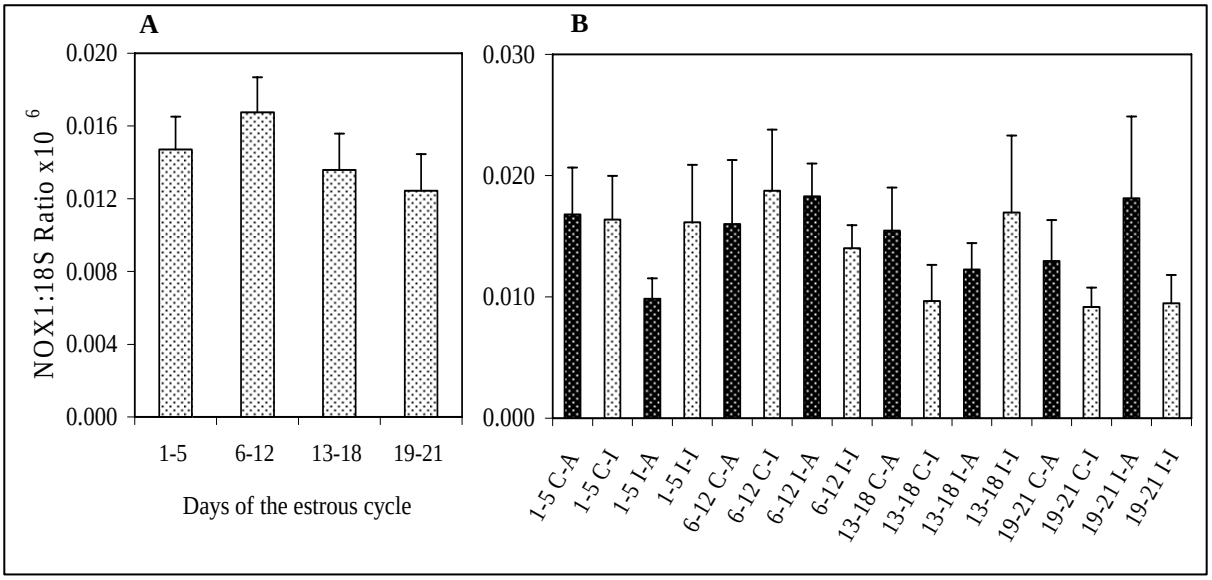

Fig. 9: Expression of NOX1 mRNA in oviductal cells during the estrous cycle (A) and in ampulla and isthmus of ipsi-lateral or contra-lateral oviducts (B). The results are presented as mean ±SEM (n = 7 animals). 1-5: post-ovulatory phase; 6-12: early-to-mid luteal phase; 13-18: late luteal phase; 19-21: pre-ovulatory phase. C-I: contra-lateral isthmus; C-A: contra-lateral ampulla; I-I: ipsi-lateral isthmus; I-A: ipsi-lateral ampulla.

Sequencing of the PCR products revealed a 100% homology to the predicted bovine sequence (EMBL-No.: XM 001789578.1).

The summarized results in **Fig. 10** display the levels of NOX1 mRNA in oviductal cell cultures treated with estrogen (E2) or progesterone (P4) as assessed by quantitative real-time PCR. Obviously, no significant differences were observed in NOX1 expression between treated and untreated cells during experimental period. Furthermore, it was noted that untreated and treated cell showed a marked decrease in NOX1 mRNA expression after 6h when compared to the initial control value at 0h. The general expression level of NOX1 mRNA in E2 or P4 treated cells was between 4 and 8 fg/µg total RNA.

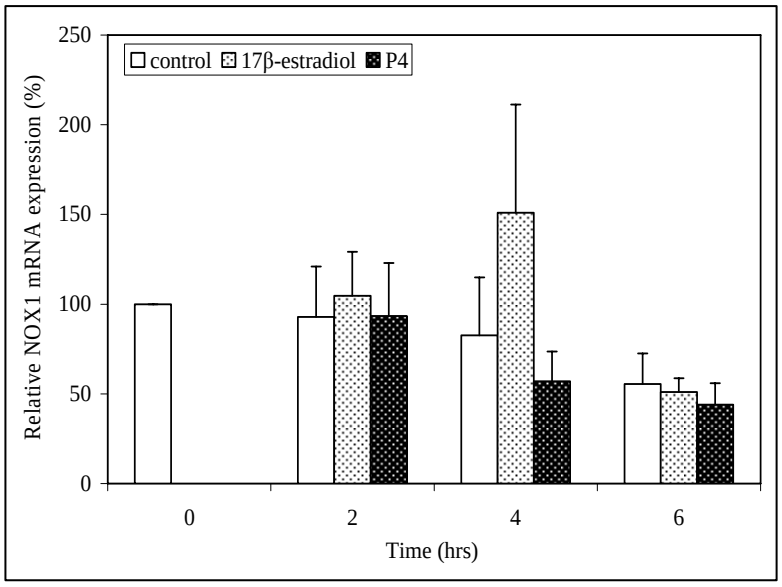

Fig. 10: Relative expression levels of NOX1 mRNA in cultured bovine oviductal cells stimulated with 10pg/ml E2 or 10ng/ml P4. The results are presented as mean ±SEM (n = 6 animals).

The results of the effects of arachidonic acid (AA) or prostaglandin E_2 (PGE_2) treatment on NOX1 mRNA expression in primary oviductal cell cultures are depicted in **Fig. 11**. NOX1 mRNA was detected without any significant differences in untreated and treated cells during the whole experimental period at the same time points (2h, 4h or 6h). NOX1 mRNA expression level in AA or PGE_2 treated cells was in the range of 5 to 7 fg/µg total RNA.

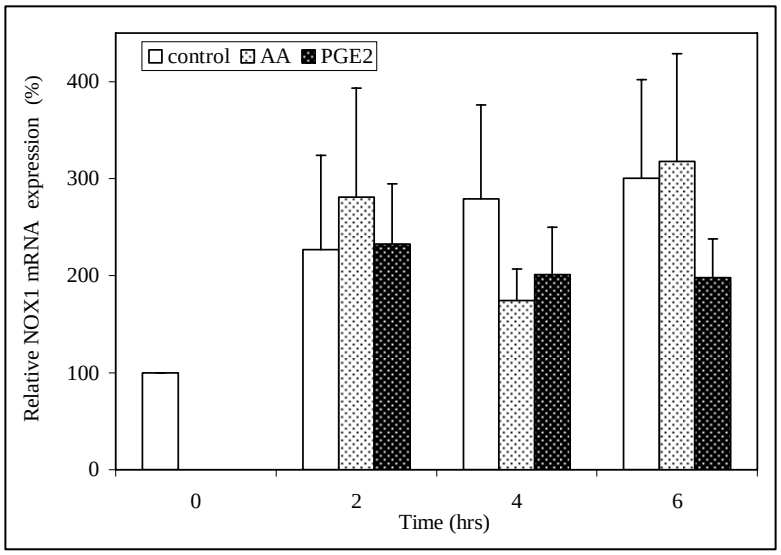

Fig. 11: Relative expression levels of NOX1 mRNA in cultured bovine oviductal cells stimulated with 10µM AA or 0.25 µM PGE_2. The results are presented as mean ±SEM (n = 6 animals).

4.2 mRNA expression of NOX2

No significant differences were observed for NOX2 mRNA expression between the phases of the estrous cycle (**Fig. 12A**). NOX2 mRNA content increased relatively during the pre-ovulatory phase compared to the post-ovulatory phase. But this increase did not reach the level of significance. No differences in NOX2 mRNA expression were observed between the oviducts neither from ipsi- or contra-lateral sites nor between ampulla and isthmus (**Fig. 12B**).

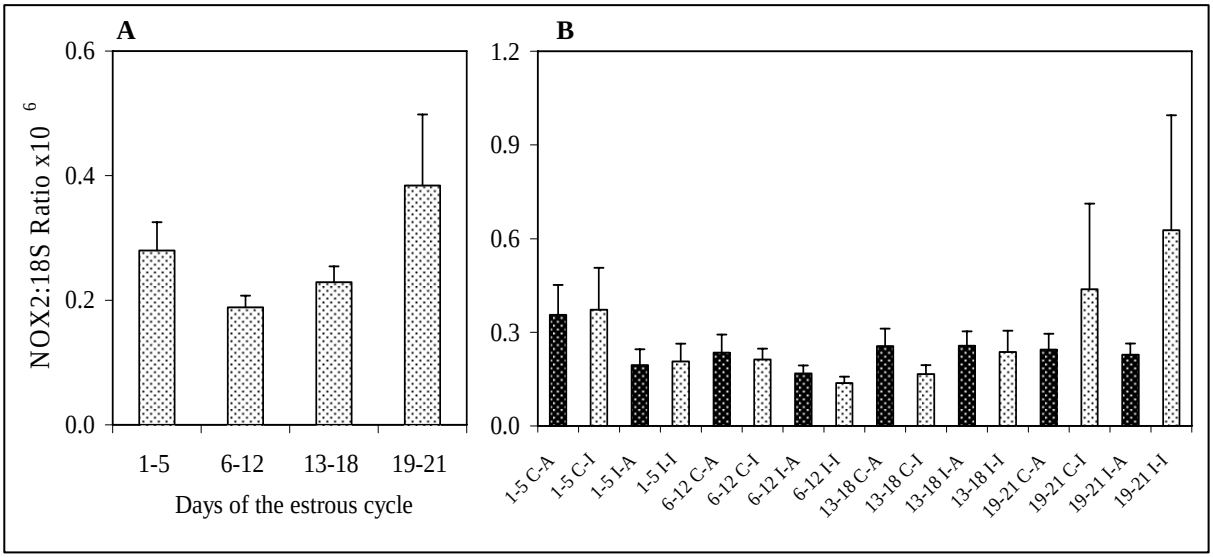

Fig. 12: Expression levels of NOX2 mRNA in bovine oviductal cells during the estrous cycle (A) and in ampulla and isthmus of ipsi-lateral or contra-lateral oviducts (B). The results are presented as mean ±SEM (n = 7 animals). 1-5: post-ovulatory phase; 6-12: early-to-mid luteal phase; 13-18: late luteal phase; 19-21: pre-ovulatory phase. C-I: contra-lateral isthmus; C-A: contra-lateral ampulla; I-I: ipsi-lateral isthmus; I-A: ipsi-lateral ampulla.

The range of the NOX2 mRNA expression in the bovine oviduct was between 20 to 80 fg/µg total RNA. Moreover, sequencing of NOX2 PCR products showed a 100 % homology to the known bovine sequence (EMBL-No.: NM_174035.3).

The expression levels of NOX2 mRNA in the oviductal cell cultures treated with E2 or P4 were depicted in **Fig. 13**. NOX2 mRNA was differentially expressed between untreated and treated cell cultures. A significant slight increase in NOX2 mRNA expression was noted in cells treated with E2 or P4 compared to the untreated ones after 2h treatment. There was a slight increase in NOX2 mRNA expression levels after 4h compared 0h but followed by a marked decrease of 75% after 6h incubation time of the untreated cells. As shown in **Fig. 13**, no significant difference was observed between the E2 treated and untreated cells after 4h and 6h of culturing.

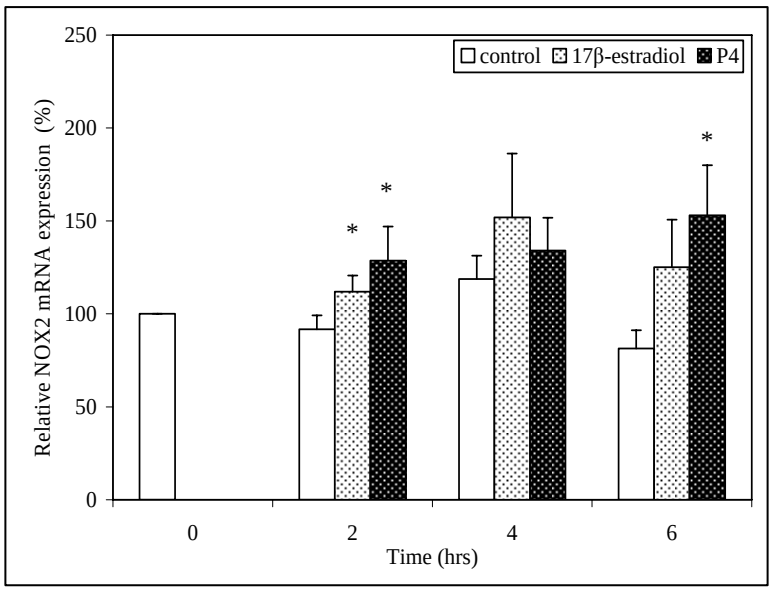

Fig. 13: Relative expression levels of NOX2 mRNA in cultured bovine oviductal cells treated with 10pg/ml E2 or 10ng/ml P4. The results are presented as mean ±SEM (n = 6 animals). The bar bearing (*) indicates a significant difference between the treated and untreated cells at the same time point ($P < 0.05$).

In contrast, application of P4 for 6h resulted in a significant increase of NOX2 mRNA where treated cells showed an increase of 72% in NOX2 mRNA level after the P4 treatment. The expression level of NOX2 mRNA in the cells treated with E2 or P4 was in the range between 6-10 fg/µg total RNA.

Fig. 14 shows no effects of AA or PGE_2 treatments on the expression levels of NOX2 mRNA in the oviductal cell culture for 6h incubation period.

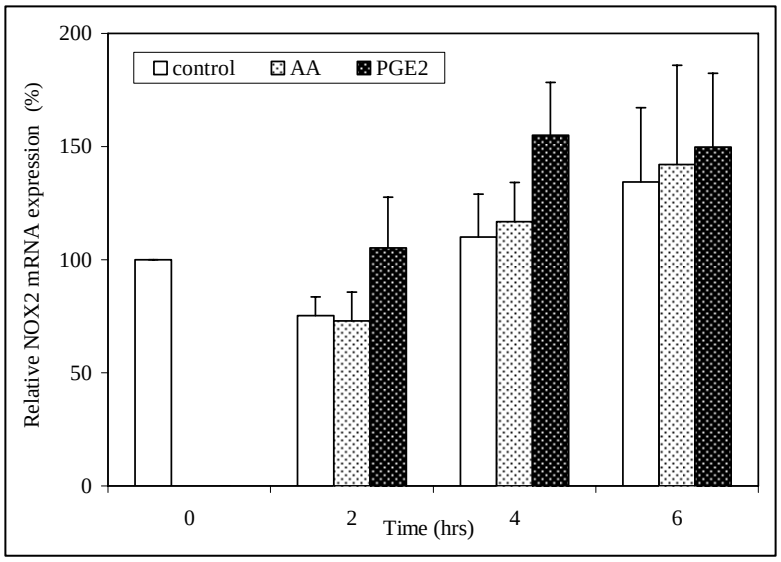

Fig. 14: Relative expression levels of NOX2 mRNA in cultured bovine oviductal cells stimulated with 10µM AA or 0.25 µM PGE_2. The results are presented as mean ±SEM (n = 6 animals).

Generally, the expression level of NOX2 mRNA in the untreated and treated cells tended to increase with advance of culturing time. The range of NOX2 expression level in the AA or PGE_2 treated cells was between 9 and 15 fg/µg total RNA.

4.3 mRNA expression of NOX4

The expression pattern of NOX4 mRNA revealed estrous cycle-dependant changes (**Fig. 15A)**. NOX4 mRNA expression showed a significant two fold increase during the post-ovulatory phase compared to the early-to-mid luteal phase. The pre-ovulatory phase revealed a two and three fold significant increase when compared to early-to-mid and late luteal phases, respectively. Despite the estrous cycle-dependant pattern of NOX4 expression, there were no significant changes in the expression level of NOX4 mRNA between the different regions of the oviduct during the estrous cycle (**Fig. 15B**). The expression level of NOX4 mRNA in the bovine oviduct was found to be in the range of 0.02-0.20 fg/µg total RNA. Sequencing of PCR products showed that the NOX4 PCR product has a 100% homology to the known bovine sequence (EMBL-No.: AY354499.1).

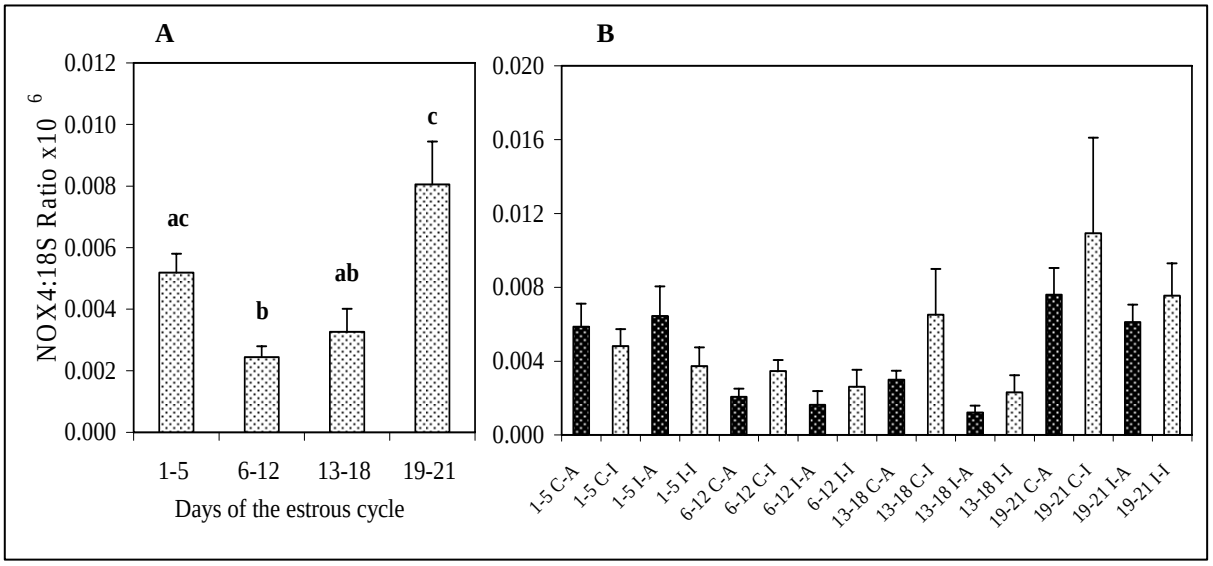

Fig. 15: Expression level of NOX4 mRNA in oviduct cells during the estrous cycle (A) and in ampulla and isthmus of ipsi-lateral or contra-lateral oviducts (B). The results are presented as mean ±SEM (n = 7 animals). The mean values with different later are significantly differences at $P < 0.05$. 1-5: post-ovulatory phase; 6-12: early-to-mid luteal phase; 13-18: late luteal phase; 19-21: pre-ovulatory phase. C-I: contra-lateral isthmus; C-A: contra-lateral ampulla; I-I: ipsi-lateral isthmus; I-A: ipsi-lateral ampulla.

The expression of NOX4 mRNA in the primary oviductal cell culture in response to E2 and P4 treatment was depicted in **Fig 16**. It is important to mention that NOX4 mRNA was detected only in three animals out of six. 2h and 6h after culture, NOX4 mRNA content

showed a decrease of about 50% in the control samples compared to the untreated cells at 0h, while it remained at the same level after 4h. Generally, there were no significant differences between untreated and treated cells after time points 2h, 4h or 6h. The NOX4 mRNA absolute expression level was in the range of 0.03 and 0.10 fg/µg total RNA for the E2 or P4 treated cells.

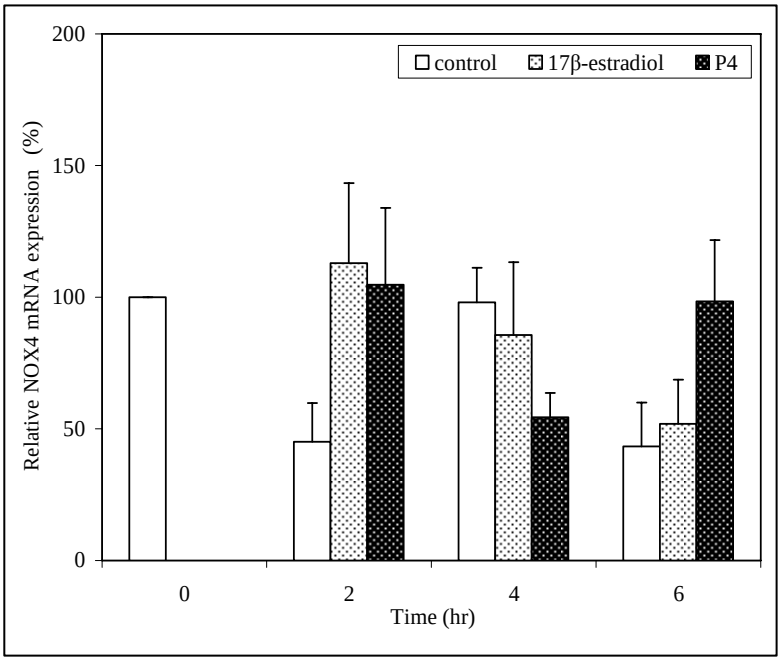

Fig. 16: Relative expression levels of NOX4 mRNA in cultured bovine oviductal cells treated with 10pg/ml E2 or 10ng/ml P4. The results are presented as mean ±SEM (n = 3 animals).

The expression of NOX4 in the oviductal cell culture treated with AA or PGE_2 for a time period of 6h was shown in (**Fig. 17**). No significant differences were observed between the untreated and treated cells for the expression of NOX4 mRNA. However, a 4h or 6h AA treatment resulted in decreased mean of expression level of NOX4 mRNA compared to the control at the same time points. It should be noted that 4h and 6h after culturing NOX4 mRNA was detected only in 3/4 animals for untreated cells compared to 4/5 animals for AA treated cells, respectively. However, the level of NOX4 mRNA detected in AA treated cells was still lower than that measured in untreated cells. Expression of NOX4 mRNA was induced in tendency by PGE_2 treatment, but without significant differences between the untreated and treated cells during the whole experimental period. NOX4 mRNA absolute expression levels were in the range of 0.02 and 0.04 fg/µg total RNA for the AA or PGE_2 treated cells.

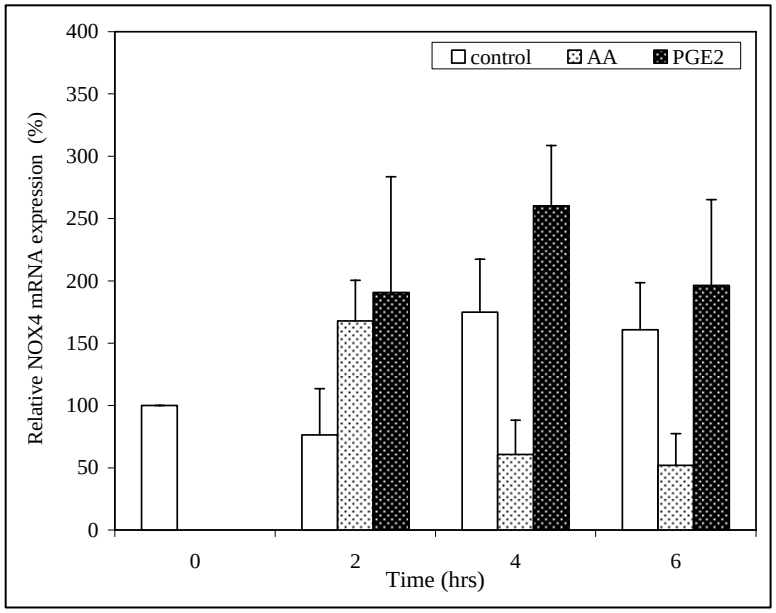

Fig. 17: Relative expression levels of NOX4 mRNA in cultured bovine oviductal cells stimulated with 10µM AA or 0.25 µM PGE_2. The results are presented as mean ±SEM (n = 6 animals). Note: after 4h and 6h, n = 3 and 4 animals in treated cells, respectively.

4.5 mRNA expression of NOX5

NOX5 mRNA was expressed in all regions of the bovine oviduct during all phases of the estrous cycle (**Fig. 18)**. The NOX5 mRNA expression revealed estrous cycle-dependant pattern (**Fig. 18A**). The results of statistical analysis showed that NOX5 mRNA was increased two fold during the post-ovulatory and early-to-mid luteal compared to the late luteal and pre-ovulatory phase of the cycle. There were significant differences in NOX5 mRNA expression between the isthmus and ampulla (**Fig. 18B)**. A two fold significant increase of NOX5 mRNA expression was detected in the isthmus of the ipsi-lateral oviduct during the post-ovulatory phase compared to the ampulla (**Fig. 18B)**. Furthermore, there was a significant increase in NOX5 mRNA expression in the isthmus and ampulla of ipsi- and contra-lateral oviducts compared to the contra-lateral ampulla during the pre-ovulatory phase.

The expression level of NOX5 mRNA in the bovine oviduct was found to be in the range of 0.02-0.50 fg/µg total RNA. NOX5 mRNA expressed in the bovine oviduct revealed 100% homology to the known bovine sequence (EMBL-No.: XR-027980).

For the *in vitro* study of NOX5 mRNA, the expression of this enzyme was tested in the primary oviductal cell culture treated with E2, P4, AA or PGE_2. On the basis of the quantitative real-time PCR results, NOX5 mRNA expression was detectable neither in the

untreated nor in the treated cells during the whole experimental period. Therefore, no relative expression measurements could be done for E2, P4, AA or PGE_2 treatments.

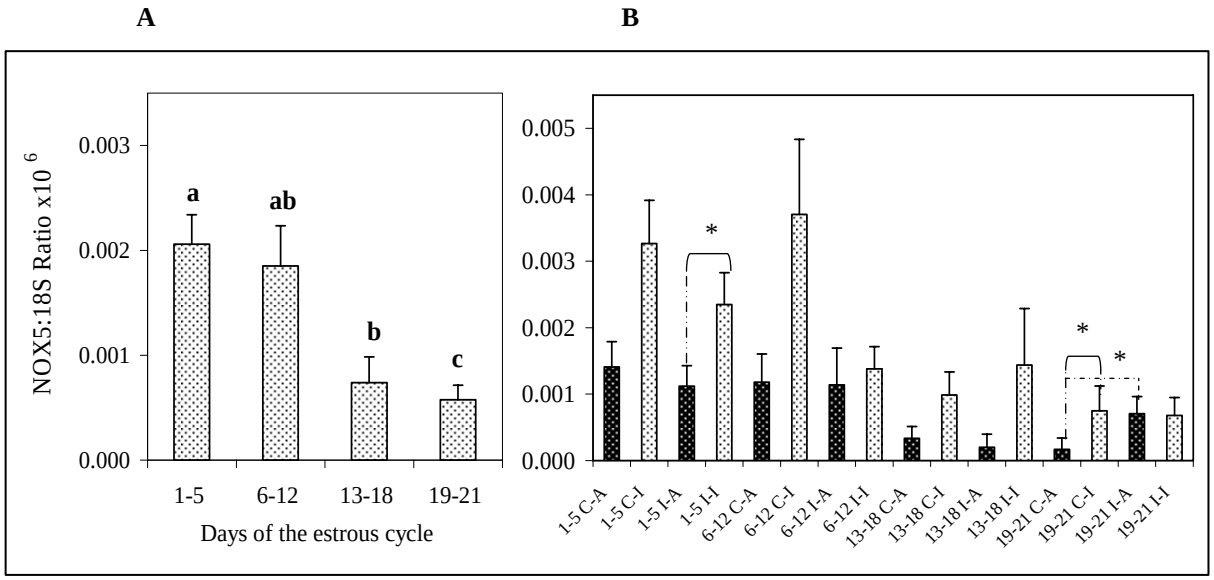

Fig. 18: Expression level of NOX5 mRNA in oviduct cells during the estrous cycle (A) and in ampulla and isthmus of ipsi-lateral and contra-lateral oviducts (B). The results are presented as mean ±SEM (n = 7 animals). The mean values with different letter are significantly differences at $P < 0.05$. The bar bearing (*) indicates a significant difference at $P < 0.05$. 1-5: post-ovulatory phase; 6-12: early-to-mid luteal phase; 13-18: late luteal phase; 19-21: pre-ovulatory phase. C-I: contra-lateral isthmus; C-A: contra-lateral ampulla; I-I: ipsi-lateral isthmus; I-A: ipsi-lateral ampulla.

4.6 mRNA expression of DUOX1

The data summarized in **Fig. 19** show that DUOX1 mRNA was expressed in bovine oviductal cells during the different estrous phases as well as in all oviductal regions. The analysis of variance (test between subject effects) indicated that expression of DUOX1 mRNA was significantly dependant on the estrous cycle. The result of statistical analysis (Post-hoc test) revealed that no differences were observed in DUOX1 mRNA expression in the bovine oviduct between the phases of the estrous cycle (**Fig. 19A**). DUOX1 mRNA was expressed with a two fold higher mean of expression level during the phase after ovulation compared to the phase before ovulation.

There were no significant differences observed between isthmus and ampulla of ipsi- and contra-lateral oviducts during all phases of the estrous cycle (**Fig. 19B)**. DUOX1 mRNA was found to be expressed with higher level in the bovine oviduct with an average range between 0.2-2.0 fg/µg total RNA. After PCR products were subjected to sequencing, 100% homology was found to the known bovine sequence (EMBL-No.: XM 587550.3).

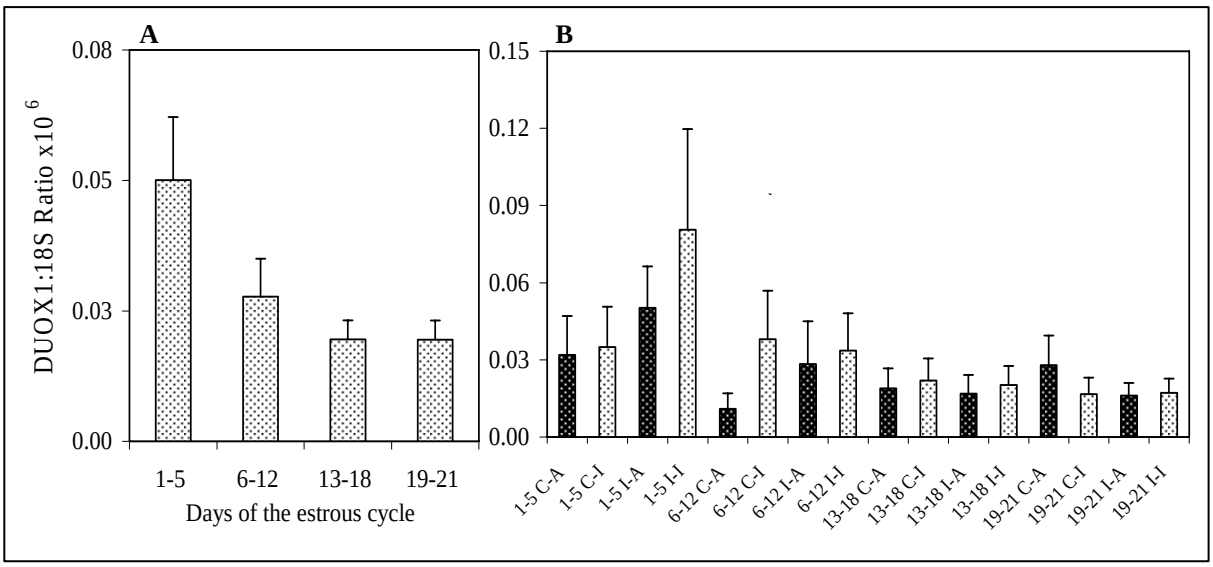

Fig. 19: Expression levels of DUOX1 mRNA in oviductal cells during the estrous cycle (A) and in ampulla and isthmus of ipsi-lateral or contra-lateral oviducts (B). The results are presented as mean ±SEM (n = 7 animals). 1-5: post-ovulatory phase; 6-12: early-to-mid luteal phase; 13-18: late luteal phase; 19-21: pre-ovulatory phase. C-I: contra-lateral isthmus; C-A: contra-lateral ampulla; I-I: ipsi-lateral isthmus; I-A: ipsi-lateral ampulla.

Fig. 20 shows the effects of E2 or P4 treatments on the expression of DUOX1 mRNA by oviductal cell culture during time course of 6h. The data showed that DUOX1 mRNA was detected in all cell culture samples during the experimental period. Differences were observed between the untreated and treated cells of the DUOX1 mRNA expression.

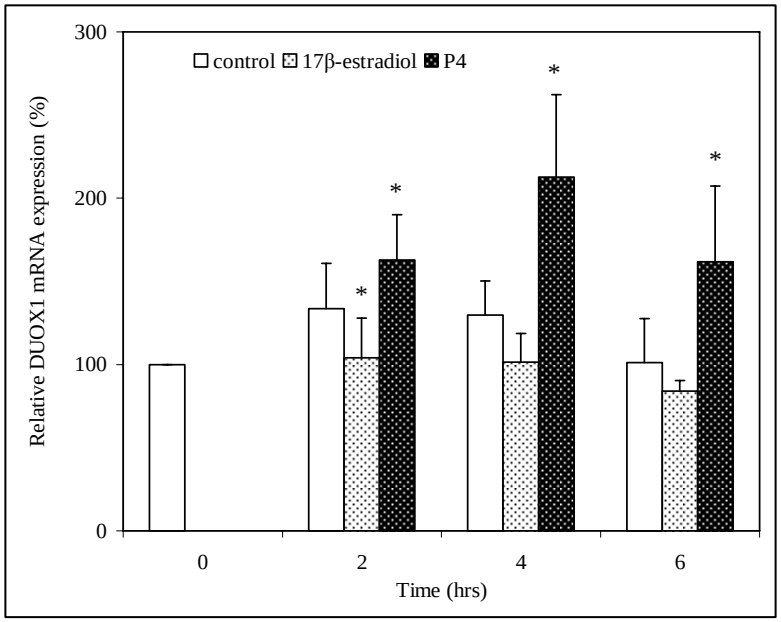

Fig. 20: Relative expression levels of DUOX1 mRNA in cultured bovine oviductal cells stimulated with 10pg/ml E2 or 10ng/ml P4. The results presented as mean ±SEM (n = 6 animals). The bar bearing (*) indicates significant difference between the treated and untreated cells at the same time point ($P < 0.05$).

During the experimental period, E2 treatment tended to decrease DUOX1 mRNA expression compared to the untreated cells at the same time points (2h, 4h, 6h), but the only significant decrease of 30% was observed 2h after treatment compared to the control group. In contrast to E2 treatment, application of P4 resulted in an increase of DUOX1 mRNA concentrations after 2h, 4h (83%), or 6h (61%) compared to untreated cells. The expression levels of DUOX1 mRNA in the cells treated with E2 or P4 were between 3-7 fg/µg total RNA.

The results summarized in **Fig. 21** revealed the effects of AA or PGE_2 in the expression level of DUOX1 mRNA in oviductal cell cultures. The expression of DUOX1 mRNA in the untreated and treated oviductal cell culture remained almost similar throughout the experimental period (**Fig. 21)** without significant differences. DUOX1 mRNA expression level was in the range of 5-8 fg/µg total RNA for the AA or PGE_2 treated cells.

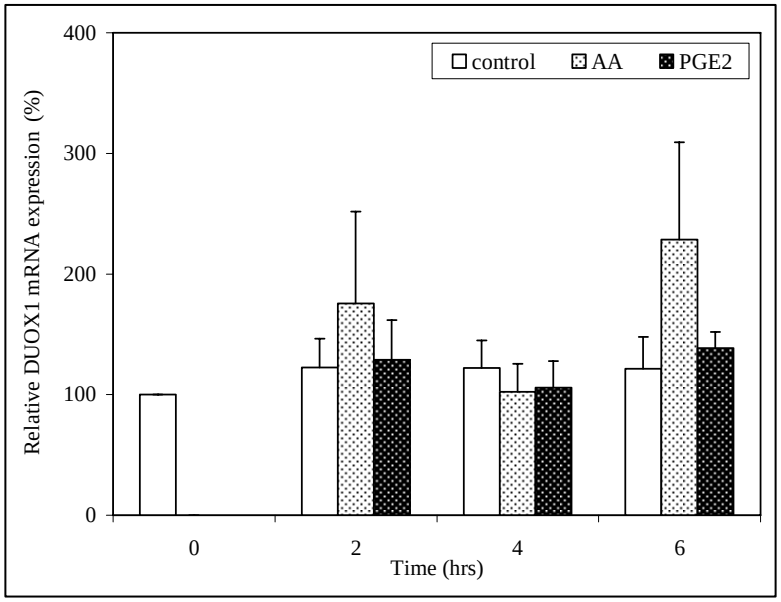

Fig. 21: Relative expression levels of DUOX1 mRNA in cultured bovine oviductal cells stimulated with 10µM AA or 0.25 µM PGE_2. The results presented as mean ±SEM (n = 6 animals).

4.7 mRNA expression of DUOX2

Preliminary data revealed that DUOX2 mRNA was not expressed in the bovine oviduct (**Fig. 22A**). But DUOX2 mRNA was detected in bovine endometrial cells (265bp) using RT-PCR (**Fig. 22B**).

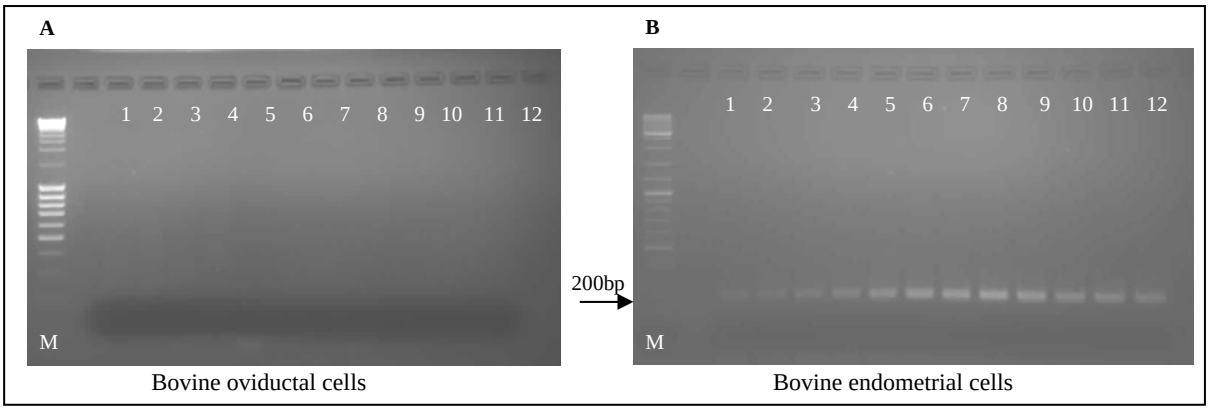

Fig. 22: Detection of DUOX2 mRNA by RT-PCR followed by amplicon separation using gel-electrophoresis in bovine oviductal and endometrial cells. Expected size of DUOX2 is 265bp. M: size marker (range 100-1000bp).

Further investigation by using real-time RT-PCR indicated that DUOX2 mRNA was specifically expressed in bovine endometrial cells, but DUOX2 transcripts were absent in the bovine oviduct. This was confirmed by the melting curve of the PCR products showing that DUOX2 was present in bovine endometrial cells. As indicated in **Fig. 23**, DUOX2 standard curve (red line) and amplification of the same gene in endometrial cells (blue line) showed the same melting point, which indicated one specific product. In contrast, no DUOX2 mRNA specific product was detected in oviductal cells (green line). Sequencing of the PCR products revealed that the amplified gene has a 100% homology to the predicted bovine sequence (EMBL-No.: XM 001253634.1).

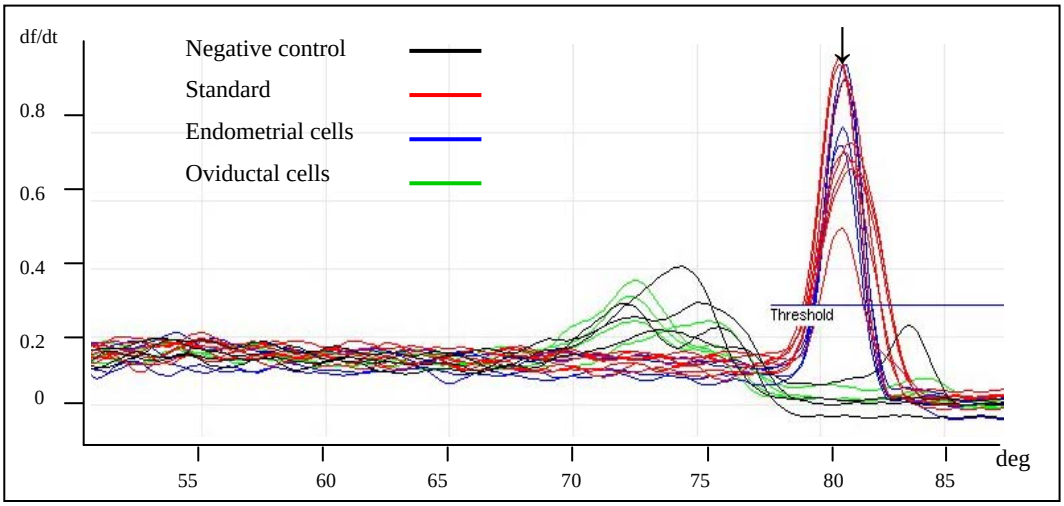

Fig. 23: Identification of DUOX2 mRNA in bovine oviductal and endometrial cells. The graph represents the melting curve of DUOX2 products generated by real-time PCR. The vertical arrow shows the same melting point for the standard and endometrial cells.

4.8 mRNA expression of p22^{phox}

The results presented in this study showed that p22^{phox} was expressed in all regions of ipsi-lateral and contra-lateral oviducts during all phases of the estrous cycle without any significant difference (**Fig. 24A and B**).

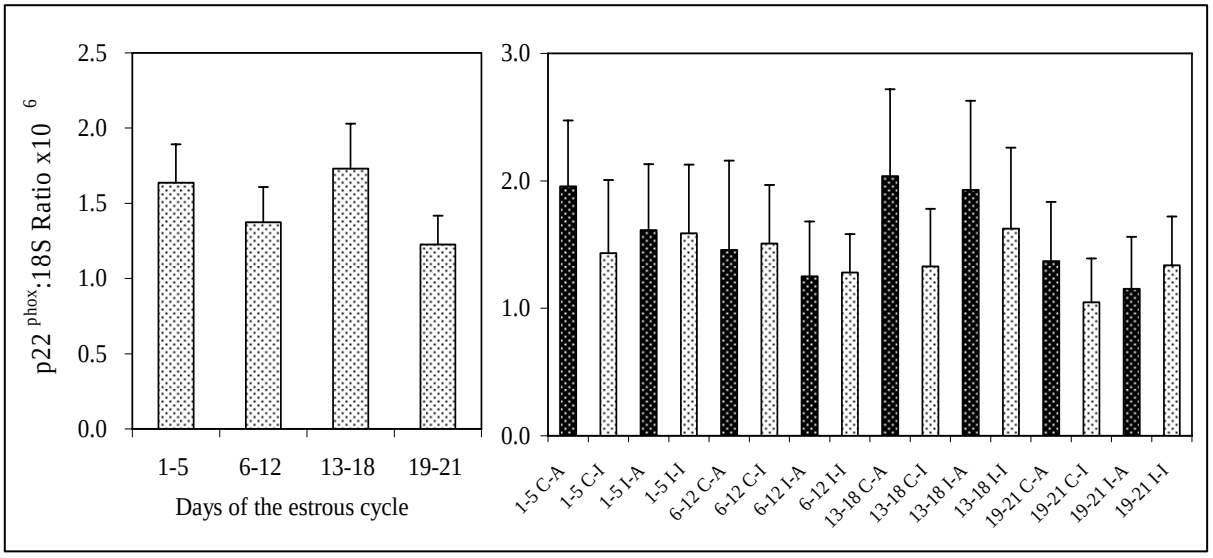

Fig. 24: Expression level of p22^{phox} mRNA in bovine oviductal cells during the estrous cycle (A) and in ampulla and isthmus of ipsi-lateral or contra-lateral oviducts (B). The results are presented as mean ±SEM (n = 7 animals). 1-5: post-ovulatory phase; 6-12: early-to-mid luteal phase; 13-18: late luteal phase; 19-21: pre-ovulatory phase. C-I: contra-lateral isthmus; C-A: contra-lateral ampulla; I-I: ipsi-lateral isthmus; I-A: ipsi-lateral ampulla.

p22^{phox} mRNA was found to be expressed in the bovine oviduct with an average range between 60-120 fg/µg total RNA. Furthermore, the results of the PCR product sequencing revealed that p22^{phox} has a 100 % homology to the known bovine sequence (EMBL-No.: NM_174034.2).

Fig. 25 shows the effects of E2 or P4 treatment on the expression of p22^{phox} mRNA in the oviductal cell culture. 2h after culturing, p22^{phox} mRNA in the untreated cells remains almost similar as its initial value at 0h. However, a significant increase in the mRNA expression of p22^{phox} was seen after 2h of E2 or P4 treatment compared to the untreated cells at the same time. 4h and 6h of E2 or P4 treatment have no significant effects on the expression of p22^{phox}. The expression levels of p22^{phox} mRNA in the cells treated with E2 and P4 was in the range between 60-80 fg/µg total RNA.

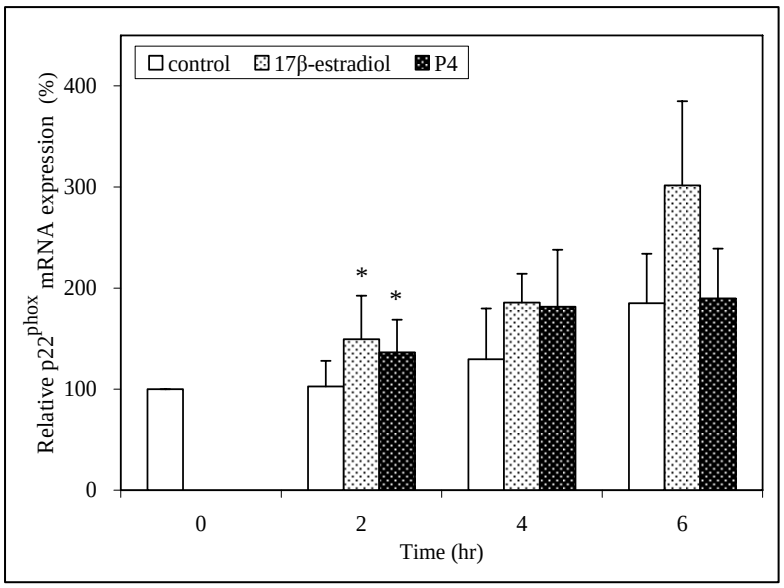

Fig. 25: Relative expression levels of p22^{phox} mRNA in cultured bovine oviductal cells stimulated with 10pg/ml E2 or 10ng/ml P4. The results presented as mean ±SEM (n = 6 animals). The bar bearing (*) indicates significant difference between the treated and untreated cells at the same time point ($P < 0.05$).

The effects of AA and PGE_2 on the expression of p22^{phox} of the oviductal cell culture were depicted on **Fig. 26**. No significant differences were observed between untreated and treated cells in the expression levels of p22^{phox} for the most time points. Only a significant increase in p22^{phox} mRNA expression after 6h PGE_2 treatment was observed. The range of p22^{phox} expression level in the AA and PGE_2 treated cells was between 25 and 40 fg/µg total RNA.

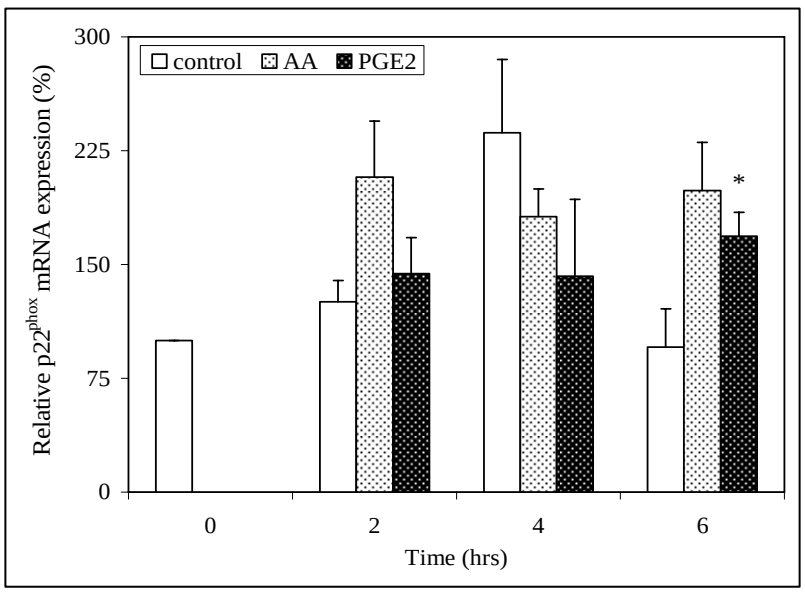

Fig. 26: Relative expression levels of p22^{phox} mRNA in cultured bovine oviductal cells stimulated with 10µM AA or 0.25 µM PGE_2. The results presented as mean ±SEM (n = 6 animals). The bar bearing (*) indicates a significant difference between the treated and untreated cells at the time point ($P < 0.05$).

4.9 mRNA expression of p67^{phox}

The data depicted in **Fig. 27** show that p67^{phox} mRNA was expressed in oviductal cells of ipsi- and contra-lateral oviducts during all phases of the estrous cycle. However, no differences were observed in the expression levels of p67^{phox} mRNA between different phases of the estrous cycle (**Fig. 27A**). The expression of p67^{phox} mRNA remained in similar levels between examined segments of the ipsi- and contra-lateral oviducts (**Fig. 27B**). The expression level of p67^{phox} mRNA in the bovine oviduct was in the range of 0.1-2.0 fg/µg total RNA. The sequencing of the PCR products revealed a 100% homology to the bovine sequence (EMBL-No.: NM_174120.2).

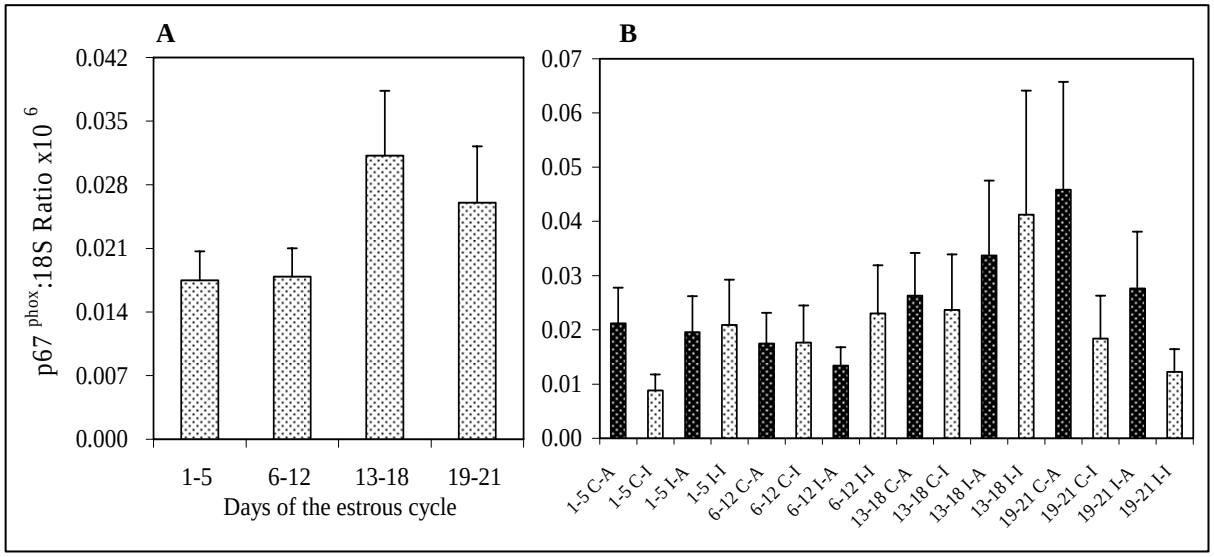

Fig. 27: Expression level of p67^{phox} mRNA in bovine oviductal cells during the estrous cycle (A) and in ampulla and isthmus of ipsi-lateral and contra-lateral oviducts (B). The results are presented as mean ±SEM (n = 7 animals). 1-5: post-ovulatory phase; 6-12: early-to-mid luteal phase; 13-18: late luteal phase; 19-21: pre-ovulatory phase. C-I: contra-lateral isthmus; C-A: contra-lateral ampulla; I-I: ipsi-lateral isthmus; I-A: ipsi-lateral ampulla.

The mRNA expression level of p67^{phox} was explored in the bovine oviductal cell culture in response to E2 or P4 treatment (**Fig. 28**). After 2h treatment, untreated and P4 treated cells showed similar level of p67^{phox} expression when compared to the initial value at 0h. Furthermore, P4 maintained the same level of p67^{phox} mRNA as for untreated cells after 6h. Although E2 increased p67^{phox} expression compared to untreated cells but significant difference was observed only after 4h of treatment. The general pattern of p67^{phox} in cell culture seemed to be increased with advancing of time. The absolute expression levels of p67^{phox} throughout the experimental period in the E2 or P4 treated cells was between 4 and 6 fg/µg total RNA.

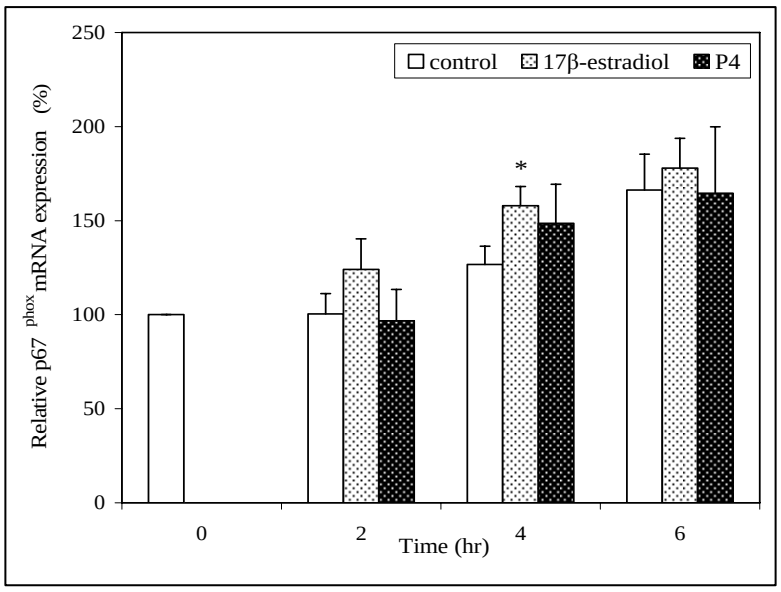

Fig. 28: Relative expression levels of p67^{phox} mRNA in cultured bovine oviductal cells stimulated with 10pg/ml E2 or 10ng/ml P4. The results presented as mean ±SEM (n = 6 animals). The bar bearing (*) indicates significant difference between the treated and untreated cells at the same time point (P < 0.05).

The results of the effects of AA or PGE_2 treatment on p67^{phox} mRNA expression in primary oviductal cell cultures are depicted in **Fig. 29**. The mRNA of p67^{phox} was detected without any significant differences in untreated and treated cells during the whole experimental period at the most time points (2h, 4h or 6h). However, the only significant increase was observed after 4h AA treatment compared to untreated cells. The absolute expression level of p67^{phox} mRNA in AA or PGE_2 treated cells was in the range of 1-2 fg/µg total RNA.

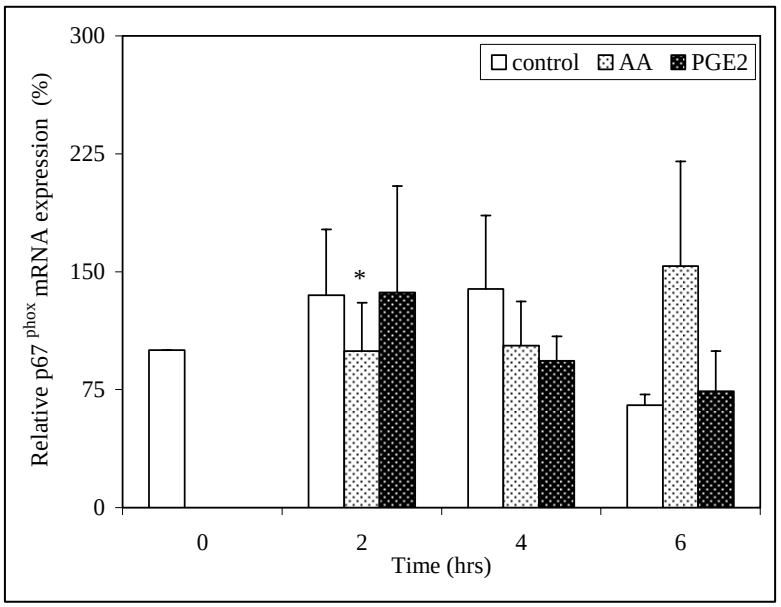

Fig. 29: Relative expression levels of p67^{phox} mRNA in cultured bovine oviductal cells stimulated with 10µM AA or 0.25 µM PGE_2. The results presented as mean ±SEM (n = 6 animals). The bar bearing (*) indicates a significant difference between the treated and untreated cells at the time point (P < 0.05).

4.10 mRNA expression of p47^{phox}

The expression of p47^{phox} was examined in the bovine oviduct during the estrous cycle to reveal its expression pattern. The data in **Fig. 30** shows the expression of p47^{phox} mRNA in the isthmus and ampulla of ipsi- and contra-lateral oviducts during all phases of the estrous cycle. Obviously, the estrous cycle and regions of oviduct has no significant effects on the expression of p47^{phox} (**Fig. 30A and B**).

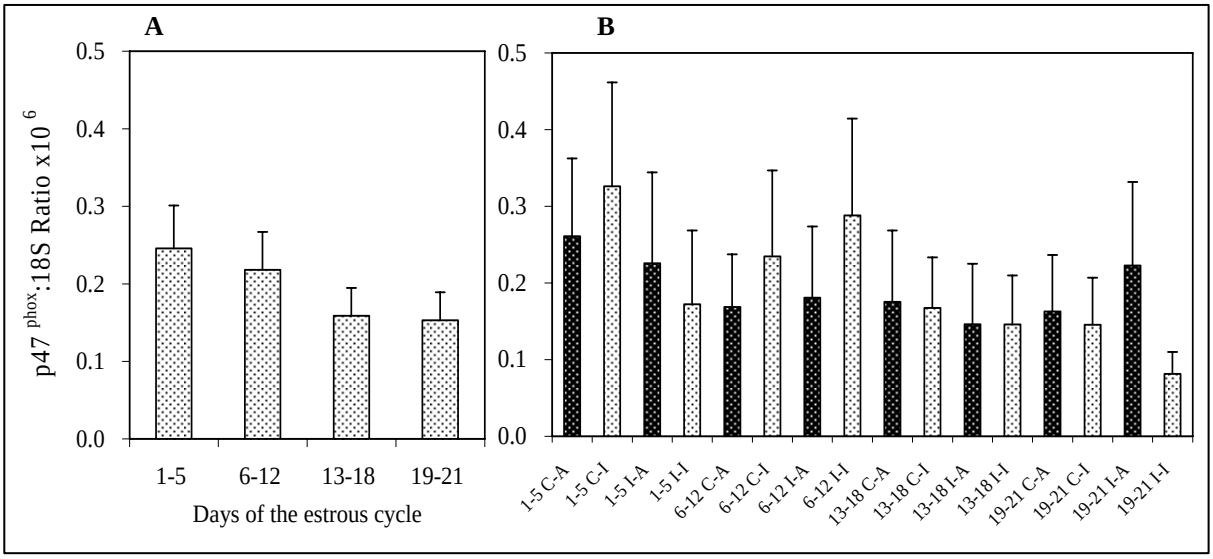

Fig. 30: Expression level of p47^{phox} mRNA in bovine oviductal cells during the estrous cycle (A) and in ampulla and isthmus of ipsi-lateral or contra-lateral oviducts (B). The results are presented as mean ±SEM (n = 7 animals). 1-5: post-ovulatory phase; 6-12: early-to-mid luteal phase; 13-18: late luteal phase; 19-21: pre-ovulatory phase. C-I: contra-lateral isthmus; C-A: contra-lateral ampulla; I-I: ipsi-lateral isthmus; I-A: ipsi-lateral ampulla.

p47^{phox} mRNA was expressed in bovine oviductal cells with higher level at range of 4-14 fg/µg total RNA compared to other NOX subunits. After PCR product sequencing, p47^{phox} showed a 100 % homology to the known bovine sequence (EMBL-No.: XM 594385.3).

In the present study, the effects of the ovarian steroid hormones on p47^{phox} expression level were examined *in vitro* in the bovine oviduct. The subunit p47^{phox} mRNA was detected in all examined cell cultures (**Fig. 31**). Generally, there were no differences in p47^{phox} mRNA contents between the untreated and treated cells after 2h or 4h treatment. The most important observation is that application of E2 or P4 increased significantly the expression of p47^{phox} mRNA by 36% and 64% after 6h treatment compared to the untreated cells, respectively. Throughout the experimental period, the p47^{phox} mRNA levels in the E2 or P4 treated cells were between 2 and 4 fg/µg total RNA.

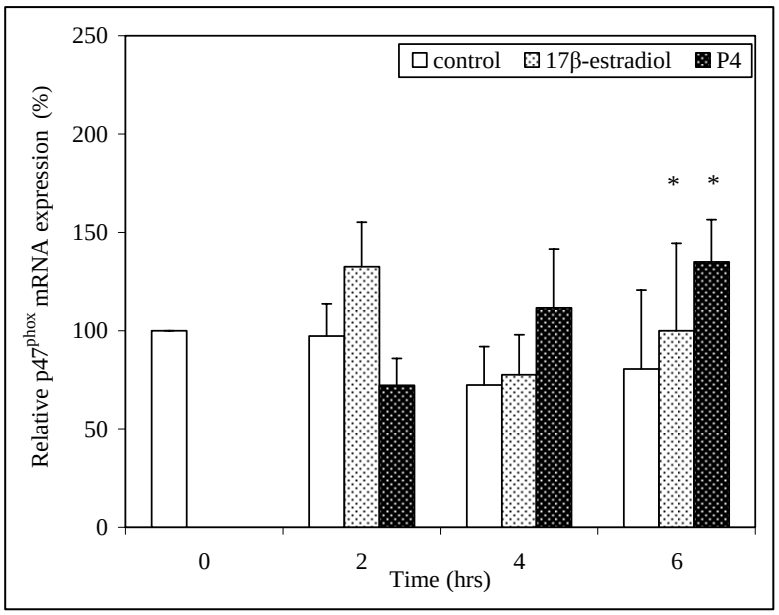

Fig. 31: Relative expression levels of $p47^{phox}$ mRNA in cultured bovine oviductal cells stimulated with 10pg/ml E2 or 10ng/ml P4. The results are presented as mean ±SEM (n = 5 animals). The bar bearing (*) indicates a significant difference between the treated and untreated cells at the same time point ($P < 0.05$).

The results obtained for the AA or PGE_2 treatment on oviductal cell culture demonstrated that application of AA or PGE_2 have a marked effect on the $p47^{phox}$ mRNA expression (**Fig. 32**). The subunit $p47^{phox}$ mRNA was expressed in all oviductal cell cultures during the experiments. 2h and 4h after treatment, the levels of $p47^{phox}$ mRNA expression remained almost at the same level in the untreated cells compared to the initial value at 0h.

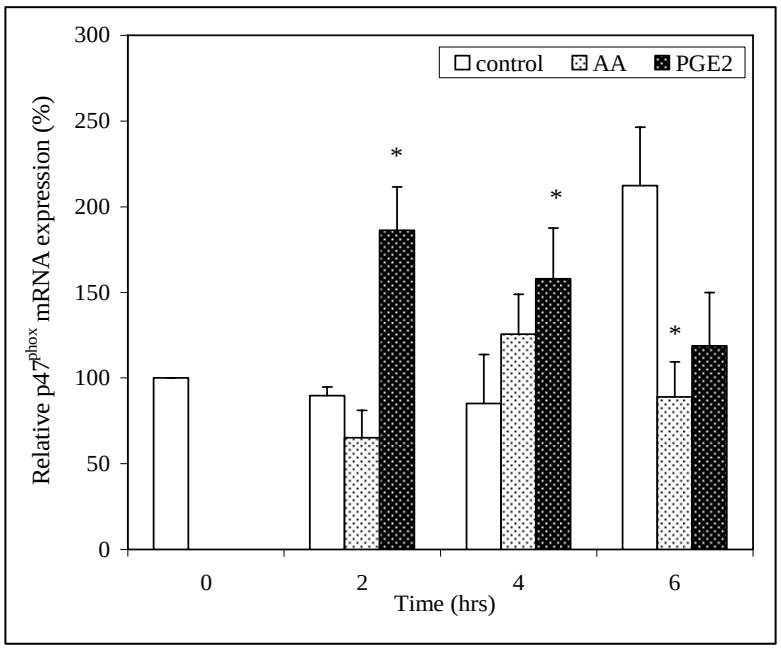

Fig. 32: Relative expression levels of $p47^{phox}$ mRNA in cultured bovine oviductal cells stimulated with 10µM AA or 0.25 µM PGE_2. The results presented as mean ±SEM (n = 5 animals). The bar bearing (*) indicates a significant difference between the treated and untreated cells at the time point ($P < 0.05$).

Application of AA treatment has no significant effects on $p47^{phox}$ mRNA expression when compared to the control at the 2h and 4h time point. In contrast, cell culture treated with PGE_2, $p47^{phox}$ mRNA expression was significantly increased by 96% and 70% after 2h and 4h, respectively. Interestingly, a marked significant decrease of 123% in the $p47^{phox}$ mRNA expression was observed 6h after the cell cultures have been treated with AA compared to the control. Moreover, PGE_2 treatment induced no significant difference in $p47^{phox}$ mRNA expression compared to the control at the same time point but showed the same tendency. Generally, the levels of $p47^{phox}$ mRNA expression in AA or PGE_2 treated cells were between 3 and 9 fg/µg total RNA.

4.11 mRNA expression of p40^{phox}

The expression of cytosolic subunit p40^{phox} was also investigated in the bovine oviduct during the estrous cycle to explore its expression pattern. The data depicted on **Fig. 33** shows the expression of p40^{phox} mRNA in the isthmus and ampulla of ipsi- and contra-lateral oviducts during all phases of the estrous cycle. Interestingly, the estrous cycle and regions of the oviduct showed no significant differences in the expression of p40^{phox} (**Fig. 33A and B**).

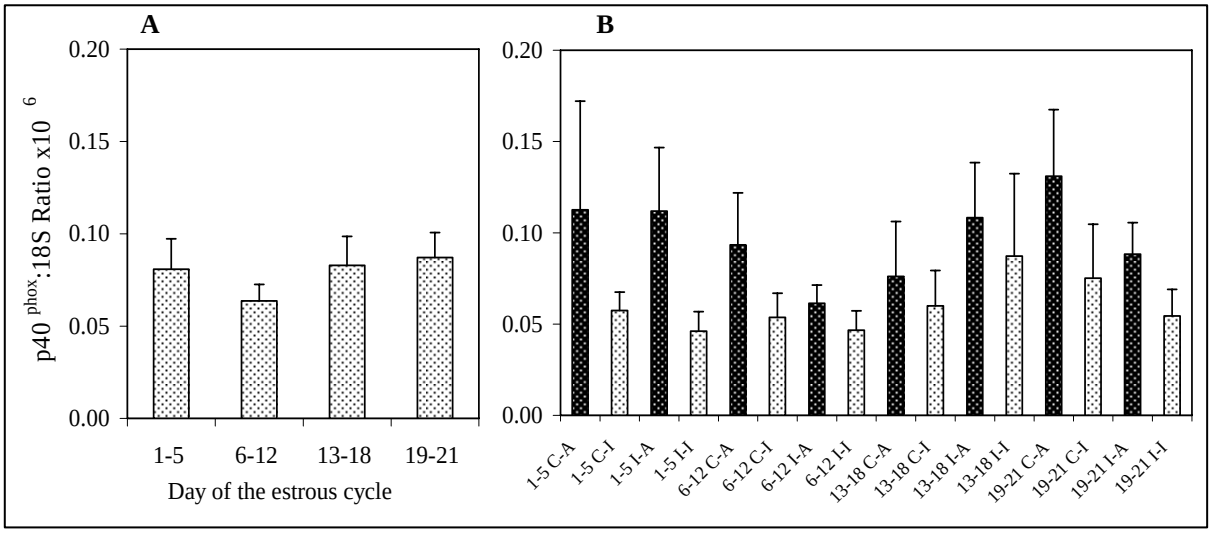

Fig. 33: Expression level of p40^{phox} mRNA in bovine oviductal cells during the estrous cycle (A) and in ampulla and isthmus of ipsi-lateral or contra-lateral oviducts (B). The results are presented as mean ±SEM (n = 7 animals). 1-5: post-ovulatory phase; 6-12: early-to-mid luteal phase; 13-18: late luteal phase; 19-21: pre-ovulatory phase. C-I: contra-lateral isthmus; C-A: contra-lateral ampulla; I-I: ipsi-lateral isthmus; I-A: ipsi-lateral ampulla.

Moreover, the absolute expression level of p40^{phox} mRNA was in the range between 0.5 and 2 fg/µg total RNA. The sequencing of PCR products showed that p40^{phox} expressed in bovine oviduct has a 100 % homology to the known bovine sequence (EMBL-No. NM_001045983).

Fig. 34 displays the effects of treatment of oviductal cell cultures with E2 or P4 for 6h. Generally, the expression of p40^{phox} levels in all cell cultures at the three time points was below the initial value at 0h. Furthermore, no significant differences were observed between the untreated and treated cells after time points 2h, 4h or 6h. The expression levels of p40^{phox} in the cells treated with E2 or P4 was in the range between 0.04 and 0.1 fg/µg total RNA.

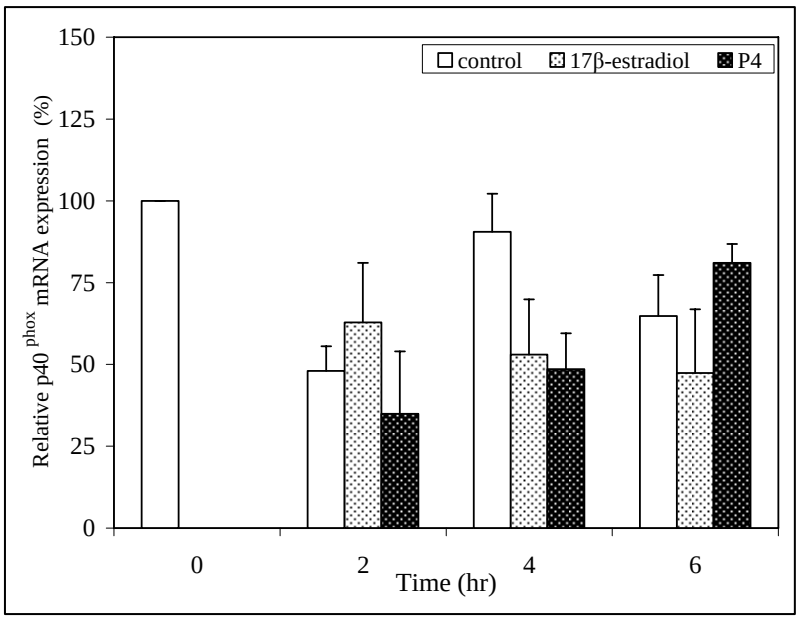

Fig. 34: Relative expression levels of p40^{phox} mRNA in cultured bovine oviductal cells stimulated with 10pg/ml E2 or 10ng/ml P4. The results presented as mean ±SEM (n = 6 animals). The bar bearing (*) indicates a significant difference between the treated and untreated cells at the time point ($P < 0.05$).

Although p40^{phox} was expressed in the oviductal cell culture treated with AA or PGE_2, but there was no significant difference between untreated and treated cells (**Fig. 35**). Interestingly, p40^{phox} was detected only in 2 and 3 animals after 4h and 6h of PGE_2 treatment. When the cells were treated with AA or PGE_2, p40^{phox} mRNA could be detected only in 2 animals after 4h and 6h. However, the general pattern of p40^{phox} expression seemed to be decreased with advanced culturing time and more pronounced in the PGE_2 treated cells. p40^{phox} mRNA expression level was in the range of 0.01-0.02 fg/µg total RNA.

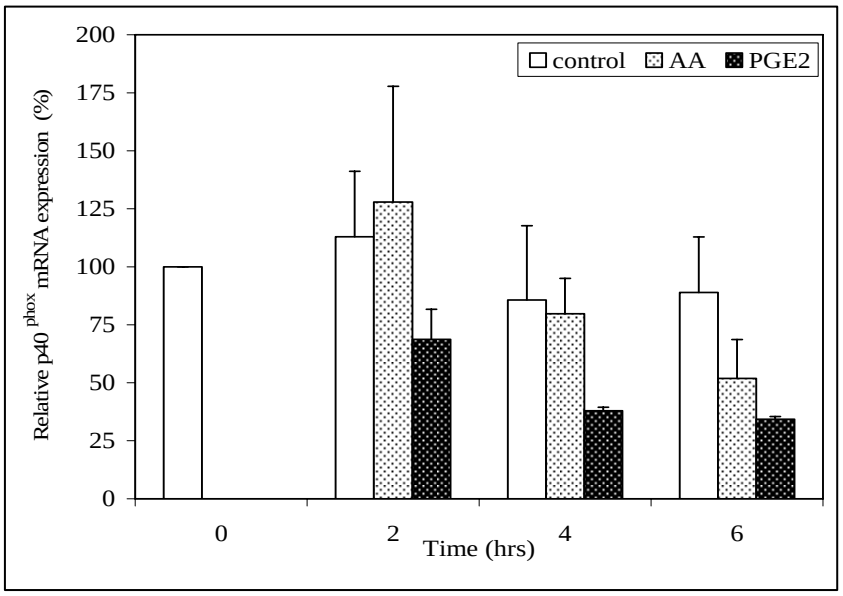

Fig. 35: Relative expression levels of p40^{phox} mRNA in cultured bovine oviductal cells stimulated with 10µM AA or 0.25 µM PGE_2. The results presented as mean ±SEM. Note: After 2h (n = 4 animals).

4.12 Relation between expression of some NOX members and NOX subunits

A correlation test was performed to reveal the expected relationship between NOX enzymes (NOX1, NOX2, and NOX4) and its activation subunits (**Table 5**). During the estrous cycle, Pearson correlation test confirmed the expected positive correlation between mRNA expression levels of NOX1 and $p22^{phox}$ (r^2 = .0339, P = 0.01), $p67^{phox}$ (r^2 = 0.253, P = 0.01), $p47^{phox}$ (r^2 = 0.243, P = 0.01) and $p40^{phox}$ (r^2 = 0.178, P = 0.01). No correlation was found between these subunits and NOX2 and between NOX4 and $p22^{phox}$ during the estrous cycle. However, these subunits correlated positively with NOX2 during certain phases of the cycle (**Table 5**).

Estrous phase	**NOX enzyme**	**Subunits**			
		$p22^{phox}$	$p67^{phox}$	$p47^{phox}$	$p40^{phox}$
Post- ovulatory	NOX1	++	++	+	.
	NOX2	.	.	.	.
	NOX4	+	NR	NR	NR
Early-to-mid luteal	NOX1	+++	.	.	++
	NOX2	+++	+	+	+++
	NOX4	.	NR	NR	NR
Late luteal	NOX1	++	+++	++	+++
	NOX2	+++	+	++	+++
	NOX4	.	NR	NR	NR
Pre-ovulatory	NOX1	++	++	+++	.
	NOX2	.	.	.	.
	NOX4	.	NR	NR	NR

Table 5: Details of the correlation between different NOX components in the bovine oviduct during the estrous cycle. The level of positive significant correlation was at P = 0.01 (+), at P = 0.001 (++) and at P = 0.0001 (+++), while (**.**) indicates no significant correlation. NR: not required.

5 Discussion

5.1 Validation of oviduct collection, cycle definition and mRNA expression analysis

Collection of bovine oviductal samples from the slaughterhouse is a technique with great potential for studying reproductive physiology in mammals (Ellington et al., 1998; Einspanier et al., 1999; Arosh et al., 2002; Gabler et al., 2002). The stage of the estrous cycle was defined by postmortem examination of the ovaries (follicle and corpus luteum), mucus in the cervix and the uterine horn lumen which is consistent with hormonal status of the animals (Ireland et al., 1980; Arosh et al., 2002). This method proved to be efficient as it is a convenient and economical source of animal tissues. Examination of health status of the cow reproductive tract was done by visual inspection, when there was any sign of inflammation or abnormal condition, the reproductive tract were excluded. Mucosal cells (epithelium, stroma cells) can be harvested from the oviducts by scraping techniques for in *in vivo* or *in vitro* studies. Generally, this procedure is simple, inexpensive, and has been found to be more reliable and resulted in the least amount of cellular damage. Due to hormonal changes between the different phases, the obtained oviducts were classified according to the estrous cycle phase: day 1-5: post-ovulation; day 6-12: early-to-mid lutesl; day 13-18: late luteal; day 19-21: pre-ovulatory. The post-ovulatory phase was associated with high level of E2 that progressively declines with time, while P4 concentration starts to increase to reach higher levels during early to-mid luteal phase (Hansel et al., 1973; Walters and Schallenberger, 1984). With the regression of the corpus luteum, concentration of progesterone in the circulation declines rapidly and is low throughout the follicular phase as compared with the luteal phase of the estrous cycle (Wettemann et al., 1972; Echternkamp and Hansel, 1973). Furthermore, concentrations of E2 remain relatively low during the mid- to late luteal phases of the estrous cycle, but fluctuate throughout the luteal phase (Wettemann et al., 1972; Chenault et al., 1975). High E2 levels are measured during the pre-ovulatory and at ovulation time (Echternkamp and Hansel, 1973; Walters et al., 1984). Since oviductal cells exposed to these hormonal fluctuations, there are many morphological and physiological changes occurring in oviductal environment during the estrous cycle. Therefore, the pattern and level of gene expression in the oviductal cells may significantly differ during the estrous cycle.

Quality and quantity of mRNA are critical for successful gene expression analysis. Postmortem tissue has been previously utilized as suitable source of good RNA quality from different tissues and cells (Heinrich et al., 2007). In the present study, preparation and

harvesting of oviductal cells were done within 2 to 3 hours. The storage of oviductal tissue under cooling condition was not harmful for RNA integrity. Total RNA evaluated by agarose gel electrophoresis appeared to yield highly intact RNA without significant degradation. This was supported by data obtained by Fitzpatrick et al. (2002) which indicated that a postmortem delay of up to 24h does not significantly affect the yield or quality of prepared RNA.

Isolation and culturing of bovine oviductal cells is a powerful tool to assess the functional role of oviductal cells. It should mimic the *in vivo* environment successfully such that the cells are capable of survival and response in a controlled manner. Therefore, to study expression of NOX components (NOX and NOX subunits) by primary oviductal cell culture in responses to different hormonal treatment provided a better understanding of the regulation of theses enzymes during the estrous cycle. The concentration of hormones used in this study to stimulate oviductal cell cultures was chosen based on physiological levels in the cow oviduct during the normal estrous cycle. The concentration of estrogen (E2: 10pg/ml) used for treatment of the cell culture was within the physiological concentration in serum before ovulation where it is at higher level, while the progesterone (P4) concentration (10ng/ml) was within the range during the luteal phase. PGE_2 concentration in the cow oviduct is highest during the pre-ovulatory period of the estrous cycle (Wijayagunawardane et al., 1998). Furthermore, the concentration of PGE_2 (0.25µM) and AA (10µM) was used in the present study depending on their level in the bovine species during the estrous cycle (Milvae and Hansel, 1983; Kotwica et al., 2003b; Childs et al., 2008).

New insights into the role of the oviduct in reproduction are commonly gained through the measurement of different mRNA levels by real-time PCR. Critical to the successful application of real-time PCR is to minimize the variation in the mRNA amount between the samples. Therefore, accurate normalization is required to correct the real-time data for differences in cellular input, RNA quality and enzymatic efficiency between the samples. 18S rRNA was found to be the best reference control gene used in the transcript quantification in bovine oviductal cells. As the relative amount of the 18S rRNA was found to be similar among the samples for all phases of the estrous cycle (Wijayagunawardane et al., 2003) and further the biological differences are probably not relevant in the 18S rRNA expression between the estrous phases (Odau, 2006).

5.2 NOX enzymes

NADPH oxidases (NOX) are enzymes that catalyze the production of reactive oxygen species (ROS) from oxygen and NADPH. For three NOX members (NOX1-3), the enzyme complex consists of two membrane-bound components and three components in the cytosol. However, these subunits are not required by the other members for their enzymatic activities. The oxygen species are involved in the physiological events in different animals' species including cattle (Blondin et al., 1997; O'Flaherty et al., 2003, 2005; Rizzo et al., 2007). However, up to date no information is available on the expression of NOX enzymes in the mammalian oviduct and its pattern of expression during the estrous cycle. In the present study, NOX isoforms and its activation subunits have been clearly shown to be expressed along the bovine oviduct throughout the estrous cycle. They may be suggested as another source of ROS in the oviduct. Cellular production of ROS can also occurs from non-enzymatic reaction such as the reaction which is catalyzed by iron and hydrogen peroxide and produces hydroxyl radical (Halliwell, 1978). These findings are consisting with results that NOX enzymes are essentially expressed in different mucosal surfaces in the body including some reproductive tissue (ovaries, uterus, placenta) (Suh et al., 1999; Geiszt et al., 2003a,b; Cheng et al., 2001). As shown in this work for the first time, expression of NOX enzymes components showed a distinct pattern during the different phase of the cycle (**Table 6**).

	Phase of the estrous cycle			
NOX components	Post-ovulatory	Early-to-mid luteal	Late luteal	Pre-ovulatory
NOX1	↔	↔	↔	↔
NOX2	↑	↓	↓	↑
NOX4	↑*	↓*	↓*	↑*
NOX5	↑*	↑*	↓*	↓*
DUOX1	↑	↓	↓	↓
DUOX2	Not expressed	Not expressed	Not expressed	Not expressed
$p22^{phox}$	↔	↔	↔	↔
$p67^{phox}$	↓	↓	↑	↓
$p47^{phox}$	↑	↑	↓	↓
$p40^{phox}$	↔	↔	↔	↔

Table 6: Expression of NOX enzyme components in the bovine oviductal cells during the estrous cycle. (*) indicated significant differences. The arrow indicates the level of expression between the estrous phase. ↑: up-regulated; ↓: down-regulated; ↔: indifferent.

Some NOX enzymes mRNAS are expressed differentially along the bovine oviduct with significantly higher levels around the time of ovulation. This could be related to an involvement of these enzymes in the post-maturation of oocyte and sperm function. The mRNA expression of NOX4, NOX5 and DUOX1 was found to be estrous cycle-dependant, while NOX1 mRNA has steady state level of expression throughout the cycle. NOX2 appeared to be up-regulated during the time of ovulation. However, depending on the obtained absolute value of expression level, NOX2 show highest expression levels (20-80 fg/µg total RNA) during the estrous cycle compared with other NOX enyzmes (0.02-14 fg/µg total RNA). This higher level of NOX2 expression may point to the importance of this NOX isoform to maintain basal level of ROS in the bovine oviduct required for normal cell function. The presence of other NOX in the bovine oviduct may act in concert with NOX2 to achieve precise regulation mechanism to control ROS levels along the bovine oviduct or may act as signal transduction leading to successful fertilization. For example, NOX5 was expressed with low absolute levels throughout the estrous, but its expression level was significantly increased after ovulation in the ipsi-lateral oviduct. Evidence has been reported on the role of reactive oxygen species in controlling a unique signal transduction cascade associated with sperm capacitation *in vitro* (Aitken et al., 1998a; de Lamirande and O'Flaherty, 2008). Moreover, the heterogeneous expression of NOX enzyme components found in the present investigation along the oviduct is a possible indication of providing balanced ROS levels. The mechanism for this heterogenous expression remains to be determined, but may be related to the heterogeneity of distribution of different cell types in the oviduct during the estrous cycle. This can be supported by earlier studies indicating that the mammalian oviduct is a steroid-responsive tissue. Hormonal changes that occur during the estrous cycle may influence not only the histology, physiology and secretory activities of ciliated and non-ciliated cells of the epithelium (Bajpai et al., 1977; Eriksen et al., 1994; Yániz et al., 2000; Ulbrich et al., 2006). That may indicate variation in expression of different genes by oviductal cells. The wide range of NOX expression levels (0.02-80 fg/µg total RNA) may reflect differences in enzyme activities during the different phase of the cycle. However, there was a relative abundance in the expression of these enzymes between the oviducts from both sides and different regions indicating different roles in oviductal function via regulation of ROS levels. Furthermore, a significant local expression difference was only noted for NOX5. Considerable differences in hormonal levels were found between the ipsi- and contra-lateral oviducts possibly due to ovarian follicle effects (Wijayagunawardane et al., 1998), which may reflect variations in the gene expression between the oviducts from both sides.

To complete this enzyme system in the oviduct, expression of activation subunits ($p22^{phox}$, $p67^{phox}$, $p47^{phox}$, and $p40^{phox}$) were also demonstrated during the estrous cycle, as these subunits are required by different members of NOX for the assembling of a complete NOX system. The present investigation indicated that the activation subunits were expressed with almost similar levels between the different phases of the estrous cycle. The present findings also showed a positive correlation between the mRNA expressions of NOX and their subunits during the different phases throughout the estrous cycle. This may indicate towards a precise mechanism to control NOX enzyme activity to produce ROS when and where it is required. NOX1 generates superoxide when co-expressed with the $p47^{phox}$ and $p67^{phox}$ subunits of NADPH oxidase, but not when expressed alone. Therefore, an equal distribution of these subunits between the isthmus and ampulla from ipsi- and contra-lateral oviducts may indicate to the presences of functional NOX enzymes in bovine oviduct. A key assumption in studying mRNA expression is that it is informative in the prediction of protein expression and indicating to active enzyme. However, expression of NOX components on the protein level was not yet assessed in the present study, but higher mRNA expression level along with presence of activation subunits may indicate for the peptide presence of functional NOX enzymes.

Although localization of NOX enzymes was not determined in the present study, this pattern of expression in the oviduct may represent a mechanism for providing controlled ROS production to serve different functions within oviductal lumen. Moreover, scraped oviductal cells were used in the present study and the obtained findings suggest NOX as a novel source of ROS at the oviductal mucosal surface. This suggestion is consistent with previous findings indicating that NOX mRNAS have been localized in mucosal cell membrane in different tissues. This agrees with the function of NOX as source of ROS for extra-cellular compartments (Geiszt et al., 2003a, b). Examination of NOX expression indicates that NOX mRNA is expressed in different mucosal surfaces at apical cell membranes from different tissues including urinary, respiratory, digestive tracts, and secretory glands (Suh et al., 1999; Dupuy et al., 1999; Geiszt et al., 2003a, b).

For normal oviductal function, ROS production and antioxidants should be remained in balance because when this balance is disrupted towards an overabundance of ROS, oxidative stress (OS) occurs. Moreover, balanced ROS levels have a benefit in multiple physiological processes from oocyte maturation to sperm function, fertilization and embryo development (Quinn and Harlow, 1978; Aitken and Brindle, 1993; Guerin et al., 2001). In addition, mRNA of all major antioxidant enzymes (GPO, SOD, catalase) has also been detected during the

estrous cycle in bovine oviductal tissues (Lapointe and Bilodeau, 2003) and suggested as different regulation mechanism against increased ROS production. The authors reported a higher expression level of GPO-3 mRNA in the isthmus throughout the estrous cycle. Also it was noted that increasing the amount of ROS sperm membranes are damaged through oxidation of unsaturated fatty acid (Jones and Mann, 1973). Maintenance of certain ROS levels in the oviductal environment appears to be necessary for gametes and embryos affecting their overall ability to achieve fertilization, pregnancy and immune defense. The benefit and the risk of ROS on maintenance of sperm viability (Aitken and Brindle, 1993; de Lamirande et al., 1997; Lapointe et al., 1998), *in vitro* oocyte maturation (Guerin et al., 2001), *in vitro* fertilization, and *in vitro* embryonic development (Tervit et al, 1972; Quinn and Harlow, 1978) have been studied previously. All these studies demonstrated that a certain concentration of ROS is critical for reproductive events that occur *in vitro*, but they do not determine the source of production and regulation mechanism of ROS *in vivo*. Given that the effect of ROS on these processes can be blocked by antioxidants (Lapointe and Bilodeau, 2003) and ROS must be presented extra-cellularly to exert their effects. Because the oviduct is the venue of all these events *in vivo*, it is likely that a system for control of ROS levels is present in the oviduct.

Relatively small numbers of sperm reach the isthmal region of the oviduct where capacitation begins *in vivo* knowing that this environment is rich in antioxidant enzymes (Lapointe and Bilodeau, 2003; El Mouatassim et al., 2000). Furthermore, sperm produce only small amounts of ROS, which is insufficient to achieve the 10-100µmol/l concentrations (Aitken et al., 1998b; Twigg et al., 1998) apparently required to promoting capacitation, acrosomal reaction (AR) and egg-sperm binding, alternative sources should be considered. One possibility might be that ROS are generated by an oviductal NOX enzyme system serving as a novel mechanism of sperm activation and function in the bovine oviduct. This suggestion was confirmed by O'Flaherty et al. (2005) who obtained results indicating that extra-cellular ROS is required when spermatozoa undergo NOX-dependent AR and activation *in vitro*.

The activity of NOX enzymes is acutely regulated by cytoplasm subunits or intra-cellular Ca^{2+} concentrations. The ability of cells to generate ROS is not only depending on the NOX activity but also it appears to be under influence of many biological factors such as cytokines and hormones. In the present investigation, it is assumed that the expression of NOX mRNA *in vivo* may be under control of the endocrine/paracrine system. Therefore, treatment of the cells with physiological concentration of E2, P4, AA or PGE_2 may give an idea for the regulation of NOX enzymes in the bovine oviduct. The primary oviductal cell culture treated

with the above mentioned hormones revealed a distinct expression pattern of different NOX enzymes, although no significant differences were observed for NOX2 and DUOX1 expression between the different estrous cycle stages. But their responses in treated cell culture may indicate for a presence of other factors involved in the regulation of expression of these NOX members *in vivo* to maintain the levels of ROS in the oviduct. In human monocytes, activation of NOX enzyme by pro-inflammatory cytokines through up-regulation of $p47^{phox}$ can be diminished by E2 which was found to down-regulate the $p47^{phox}$ mRNA and protein expression (Sumi et al., 2003). Additionally, *in vitro* studies by Wagner et al. (2001) demonstrated that using a physiological concentration of E2 induced time- and concentration-dependent decrease of the expression of NOX2 at both the mRNA and protein levels. It was shown that a significant E2-dependant inhibition at the mRNA level could also be demonstrated after 8h exposure for the expressions of $p22^{phox}$ and $p47^{phox}$, but not for the $p67^{phox}$, which seemed to be attenuated by E2 application. Subsequently, in estrogen-deficient ovariectomized mice, Wassmann et al. (2005) showed that superoxide release and NOX activity increased in vascular endothelial cells and that E2 replacement prevented this increase, whereas P4 substitution enhanced ROS production and NOX activity. This can be attributed to P4 down-regulating the ROS scavenger expression, and E2 deficiency was associated with up-regulation of the activators of NOX, $p22^{phox}$ and $p67^{phox}$. mRNA of most NOX enzymes was detected in the oviductal cells *in vitro*, but mRNA of NOX5 was undetectable. This could be due to the low levels of NOX5 expression in oviductal cells or may indicate to other tight mechanism of regulation involved in NOX5 expression. Moreover, among the examined NOX components, NOX2, DUOX and $p47^{phox}$ maintained a higher absolute expression level and the range of NOX mRNA expression in the cell culture was between 0.02 to 10 fg/µg total RNA. As mRNA expression of theses enzyme components revealed a significant difference between the untreated and treated cells, theses findings may indicate for the presences of endocrine/paracrine components or involvement of other local factors regulating the enzyme expression in the bovine oviduct. For example, the central component of the NOX assembling and activation is $p47^{phox}$. AA released by $cPLA_2$, is essential for activation of the assembled phagocyte NOX enzyme complexes (Dana et al., 1998) and $p47^{phox}$ activation was induced by AA (Shiose and Sumimoto, 2000). $cPLA_2$ is required for the activation of proton transferring through NOX2 in a phagocytic cell line and that AA itself activates the generation of superoxide (Mankelow et al., 2003). Furthermore, in the respiratory epithelial cells, PGE_2 was found to reduce ROS production through inhibiting the activation and assembly of 47^{phox} in the phagosomal membrane (Serezani et al., 2007). In

conclusion, the general expression pattern of NOX obtained in this study suggested a different role in oviductal function. Adjusted level of ROS in the mammalian oviduct may support the fertilization ability of the sperm. Around estrus time, the reproductive tract is exposed to many pathogens and local immunity is at low level, while fertilization and suspected embryo formation occurs during luteal phase. Therefore, NOX may be up-regulated around ovulation time to support the reproductive immune defense, and compensate its activity during luteal phase by positive correlation between the enzymes and activation subunits ($p22^{phox}$, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$) as shown *in vitro* through up-regulation of $p22^{phox}$, $p67^{phox}$ and $p47^{phox}$ by E2 and P4.

5.3 Expression of NOX1

The obtained results from the present study provide the first evidence for the expression of NOX1 transcripts in the bovine oviduct. The pattern of expression of NOX1 has almost similar expression levels during the estrous cycle and between the different regions of oviduct. However, NOX1 mRNA has low absolute expression levels. This is consistent with the primary oviductal cell culture showing no significant differences between the treated and non-treated cells. The results suggested that NOX1 mRNA is a novel source of continuous ROS flow in bovine oviduct environment throughout the cycle, a function that is mediated in concert with expression of cytosolic subunits. Although the protein content of NOX1 is not quantified in the present study, but the strong positive correlation with the activation subunits ($p22^{phox}$, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$) throughout the cycle may indicate to higher activity of this enzyme in bovine oviduct during the estrous cycle, particularly around ovulation time and during late luteal phase. These regulatory subunits have been known to play a central role in the assembling of NOX1 and its ability to produce ROS at the mucosal surface and in different tissues (Leto et al., 1994; Takeya et al., 2003; Ueyama et al., 2006). Furthermore, the correlation between NOX1 and $p40^{phox}$ was only seen during the luteal phase and could be related to control the activity of the enzyme as $p40^{phox}$ needed $p67^{phox}$ and $p47^{phox}$ for their translocation and connection with NOX1 at the cell membrane (Wientjes et al., 1996).

Correlation of NOX1 and its subunits to hormonal treatment may point to NOX1 as one of the mediators of PGE_2 action in the oviduct function, as PGE_2 treatment up-regulates $p22^{phox}$ and $p47^{phox}$ expression. Furthermore, it was found that PGE_2 hastens the oviductal transport of equine unfertilized oocytes and early embryos (Weber et al. 1991) and its local production also was suggested to enhance a higher contractile activity of the bovine oviduct (Wijayagunawardane et al., 1998, 1999). Earlier studies indicated that PGE_2 regulated the

oviductal motility of both the cow and the sow during the estrous cycle (Gimeno et al., 1984). Whereas Cong et al. (2007) recently provided evidence indicating that NOX1-generating ROS act as second messenger mediating the action of PGE_2 in smooth muscle contraction.

5.4 Expression of NOX2

Because NOX2 expression appeared to be regulated during the estrous cycle, oviductal cell culture was treated with E2 or P4 to test this hypothesis. The findings of the performed *in vitro* study showed that E2 treatment significantly up-regulated the expression of NOX2 mRNA in bovine oviductal cells. However, the P4-dependant increase of NOX2 expression *in vitro* does not mirror the *in vivo* situation. *In vivo*, the decrease in the NOX2 expression during the luteal phase (day 6-18) may reflect the precise regulation of this enzyme and possibly involvement of other local regulatory factors. In other cell types, the effects of E2 and P4 in NOX2 expression are as well contradictory. Some investigators showed that neither E2 nor P4 had any significant effect on NOX2 expression (Wassmann et al., 2005), while other groups indicated that E2 treatment may decrease the expression of NOX2 (Wagner et al., 2001). These findings may suggest a complex endocrine/paracrine regulations of NOX2 mRNA expression in the bovine oviduct. The present data are consistent with findings that NOX2 was expressed in mucosal cells from different tissues and cell line (Sallesa et al., 2005). Although NOX2 mRNA was detected mainly in phagocytic cells, but expression in other non-phagocytic cells has been also described (Cross and Jones, 1991; Kikuchi et al., 2000). Other investigators showed that NOX2 was expressed in mucosal cells of reproductive organs (pancreas, ovary, placenta, prostate) and the gastrointestinal tract (Cheng et al. 2001; Sallesa et al., 2005) been associated with cell aging and apoptosis. The present results suggest that NOX2 expression in bovine oviduct may be involved mainly in initiate immune response at the mucosal surface. Therefore, it was concluded that NOX2 may serve as novel mechanism to adjust production of ROS at suitable level to favor the microenvironment in the bovine oviduct.

5.5 Expression of NOX4

The results of the present study provide new data concerning the expression of NOX4 in the oviduct in terms of mRNA levels. These findings are consisting with other findings which showed that NOX4 mRNA was expressed in the mucosal surface in the kidney (Geiszt et al., 2000). Kim et al (2008) have published data concerning the expression of the NOX4 transcript in airway epithelial cells. The findings of the present study showed that NOX4 may constitute as another source of ROS that may have roles in the bovine oviduct function in addition to other NOX enzymes and may be essential for regulation of ROS production because this enzyme revealed an estrous cycle-dependant pattern of expression. A significantly increasing level of NOX4 mRNA was observed during post- and pre-ovulatory phases of the cycle. Although mRNA of NOX4 was detected in all regions of bovine oviducts without any significant differences, but there was a general increase in NOX4 expression in ampulla and isthmus around ovulation time suggesting that expression of NOX4 enzyme by oviductal cells may be implicated in the production of ROS catalyzed by $p22^{phox}$-dependent NOX. $p22^{phox}$ was up-regulated when oviductal cells exposed to physiological levels of E2, P4 for 2h or PGE_2 after 6h of treatment. This indicates that NOX4, similar to its phagocytic counterpart, has a role in host defense, since NOX4 is highly expressed during the pre- and post-ovulatory phases. Furthermore, $p22^{phox}$ appears to be the only subunit required for NOX4-dependent radical generation. A knockdown and gene transfection study by Martyn et al. (2006) confirmed that ROS generation by NOX4 requires $p22^{phox}$ and ROS was abolished in cells lacking $p22^{phox}$. Furthermore, epithelial cell lines were found to stably express the NOX4/$p22^{phox}$ complex at the plasma membrane providing a NOX4 based superoxide-generating system (Serrander et al., 2007). NOX4 produces small amounts of superoxide in a constitutive manner (Geiszt et al., 2000; Shiose et al., 2001). An increase in $p22^{phox}$ expression seems to facilitate the NOX4-dependent ROS production (Kawahara et al, 2005). Particularly, Guzik et al. (2004) demonstrated a strong correlation between $p22^{phox}$ and NOX4 expression and between superoxide production and the enzyme activity. Neither co-expression of $p47^{phox}$ nor that of $p67^{phox}$ has any effect on the NOX4-dependent ROS production (Kawahara et al, 2005). Taken together, these findings indicate that NOX4 may be involved in regulation and enhancement of ROS level in the bovine oviduct to support the oviductal defense mechanism at the time when needed, and the activity of this enzyme may be regulated by $p22^{phox}$ subunit.

5.6 Expression of NOX5

Identification of NOX5 transcripts has been demonstrated in this work in the bovine oviduct during the estrous cycle in different regions of ipsi- and contra-lateral oviducts. The data presented here demonstrated that NOX5 has a distinct expression pattern in the bovine oviduct during the estrous cycle being up-regulated during post-ovulatory and early-to-mid luteal phases. The results presented here showed that NOX5 mRNA could be expressed in mucosal cells, which is consistent with other investigations. It is therefore likely that NOX5 is expressed in the oviduct at the place where gamete maturation and early reproductive events occurs (Hawk, 1987; Ellington, 1991; Way et al., 1997). Up-regulation of NOX5 after ovulation may reflect the involvement of this oxidase in the preparation of sperm for fertilization event. Further support for this idea came from the results demonstrated that the gene for the NADPH oxidase family member, NOX5, is expressed in human primary spermatocytes (Banfi et al., 2001), and that the presence of the enzyme has recently been confirmed in mature sperm (Sabeur and Ball, 2007). Therefore, the presence of NOX5 mRNA with a significantly increasing level in ipsi-lateral oviduct after ovulation may be involved in sperm AR and capacitation. Indeed, ROS was first implicated in oocyte maturation because the formation of ROS was reported to increase the developmental potential of oocyte during *in vitro* maturation to produce embryos (Blondin et al., 1997). In a preliminary study, Attaran et al. (2000) reported that optimal levels of ROS in the follicular fluid might be a potential marker for predicting success in *in vitro* fertilization. Taken together, these findings suggest NOX5 to enhance early embryo formation and development. Generally, NOX5 has been described as one of the important factors involved in the processes of sperm maturation and fertilization. Cheng et al. (2001) demonstrated that NOX5 mRNA and protein were expressed in varieties of human adult and fetal tissues including uterus, ovaries, prostate, and placenta as well as in epithelial cell lines. Moreover, increasing NOX5 expression during the luteal phase may enhance NOX2 activities when the expression of NOX2 is down-regulated. But real-time PCR failed to detect any NOX5 mRNA signal neither in the control cell culture nor in E2, P4, AA, or PGE_2 treated cells. So it appears that NOX5 expression is tightly regulated during the estrous cycle and different factors may be involved in its expression regulation.

An other possible role for activation of spermatozoa, that NOX5 may act to couple an increase in intracellular Ca^{2+} with other cell-signaling events via an increase in superoxide generation. This observation is supported by numerous studies, which have demonstrated that ROS generation by spermatozoa can be stimulated by treatment with calcium ionophores (Aitken et al., 1992). Although the ROS generation by NOX5 requires a relatively high Ca^{2+}

level, the concentrations required are at least consistent with those generated during activation of spermatozoa (Banfi et al., 2004). Because NOX5 does not require assembly of regulatory subunits as does other NOX, calcium activation leads to a rapid production of ROS similar to that observed when spermatozoa are treated with calcium ionophores. Although studies to date have not conclusively indicated the role of NOX5 in ROS-mediated signal transduction in spermatozoa, but it appears that NOX5 may be another candidate for generation of superoxide anions in the bovine oviduct.

5.7 Expression of DUOX

Identification and quantification of DUOX1 transcripts in the bovine oviductal mucosa and absence of DUOX2 transcripts in the present study is consistent with findings by other authors. Studies by Geiszt et al. (2003b) revealed that there was a variation in the expression of DUOX isoforms between epithelial cells of exocrine glands and mucosal surfaces including the gastrointestinal and respiratory tracts. Epithelial cells in salivary excretory ducts and rectal glands express DUOX2, whereas tracheal and bronchial epithelial cells express DUOX1. Furthermore, they detected DUOX1-dependent H_2O_2 release in cultured human bronchial mucosal cells, but not of DUOX2. There was no relation between the expression of two DUOX enzymes. The results presented here showed that DUOX1 mRNA has a distinct expression pattern during the estrous cycle. It appeared that DUOX1 is up-regulated during the phase after ovulation compared to phases before ovulation. These observations suggested that DUOX1 as a source of ROS may support antimicrobial defense mechanisms at mucosal surfaces of the bovine oviductal cells when required during different phase of the estrous cycle. To date, most work in these areas has focused on the roles of activated inflammatory leukocytes or environmental agents as the sources of toxic ROS at mucosal surfaces. The discovery of DUOX1 as a novel source of ROS in bovine oviductal mucosal cells provides a new candidate mediator of the antimicrobial process. These suggestions come from the transcription and localization studies of DUOX1 protein expression in different tissue: DUOX1 can function as a novel source of ROS in mucosal surface in different tissues (Dupuy et al., 1999; De Deken et al., 2002; El Hassani et al., 2005).

In the present study, *in vitro* investigation of the expression regulation by E2 or P4 hormones showed that DUOX1 expression reached in response to P4 higher expression level after 4h treatment. However, E2 depressed DUOX1 mRNA expression throughout the experimental period and the suppressed effects were more pronounced after 2h compared to untreated cells. Obviously, P4 exerted an opposing E2 effect on DUOX1 expression in the oviductal cell

culture. The present results are in good agreement with previous work in endothelial cells for the NOX enzymes family (Wagner et al., 2001; Gragasin et al., 2003; Wassmann et al., 2005). In contrast to that effect DUOX1 was down-regulated by P4 *in vivo* during the luteal phase. This may suggest a different and more complex regulation mechanism of DUOX1 during this estrous cycle phase. Further investigations in this area are necessary to elucidate the precise regulation of DUOX1 in the oviduct during the estrous cycle. Furthermore, the increase in the expression level of DUOX1 after ovulation may indicate the release from the suppressing effect of E2. Moreover, decrease of DUOX1 expression before ovulation and during luteal phases may indicate protective mechanism for gamete and oviductal cells from the harmful effects of ROS, as there is no longer needed for increased ROS.

5.8 Expression of activation subunits

The mRNA of $p22^{phox}$, $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$ components of the superoxide-generating oxidase systems are present in bovine oviductal cells, confirming the existence of complete potential active NOX enzymes in the bovine oviduct. The present study clearly demonstrated that NOX enzymes were expressed together with their activators along the bovine oviduct during the estrous cycle.

The obtained expression pattern of these subunits in the present investigation suggested that they are important co-factors for full NOX enzyme activity, as they were found to be expressed throughout the cycle with almost similar levels in different regions of the oviduct. Increased ROS formation was associated with enhanced mRNA levels of the small membrane subunit p22phox (Görlach et al., 2000). In contrast to the NOX homologues, which exhibit tissue-specific expression (Cheng et al., 2001), $p22^{phox}$ mRNA is widely expressed in high abundance (Parkos et al., 1988; Vignais, 2002), but $p22^{phox}$ protein levels in cells not expressing NOX homologues are low reflecting the close relationship between the expression of $p22^{phox}$ and active enzyme. Moreover, these authors reported that in NOX2-deficient leukocytes, $p22^{phox}$ protein is undetectable. Most likely, NOX2 prevents rapid degradation of $p22^{phox}$ when they are forming a heterodimer active complex at the cell membrane (Babior, 1999).

The expression of $p67^{phox}$ was found in oviductal cells from different regions of ipsi- and contra-lateral oviducts throughout the estrous cycle. It is probable that expression of $p67^{phox}$ along with other subunits function as part of an NADPH oxidase activity in bovine oviduct. Moreover, $p67^{phox}$ was expressed without any significant variation during the estrous. The absolute expression of $p67^{phox}$ mRNA (0.1-2.0 fg/µg total RNA) indicates the presence of

relative low levels of this subunit in the oviduct, which may reflect a low level of protein expression. In particular, the mRNA for $p67^{phox}$ was found always less abundant than for $p47^{phox}$. It has been reported that there is two to three times more $p47^{phox}$ in neutrophils compared with $p67^{phox}$ (Heyworth et al., 1994). Importantly, an increase in NADPH oxidase activity does not require a measurable increase in $p67^{phox}$ protein (Stringer and Edwards, 1995), but presence of $p67^{phox}$ enhances ROS production.

Furthermore, no differences were observed in the expression of $p47^{phox}$ between either all the cycle phases or the different regions of the oviducts. The *in vitro* results demonstrated that $p47^{phox}$ could be inducible, as E2 or P4 treatments significantly increased the expression of $p47^{phox}$ after 6h treatment. It is likely that both E2 and P4 seemed to have similar effects in the expression of $p47^{phox}$ on oviductal cell cultures for more than 2h. However, direct effects of ovarian steroids in the expression of $p47^{phox}$ by mucosal cell have not yet been studied.

The present study demonstrated that $p47^{phox}$ expression can be induced in bovine oviductal cells when treated with physiological concentration of AA and PGE_2. However, the expression of $p47^{phox}$ showed a marked decrease after 6 hours in AA treated cells. PGE_2 treatment provoked a significant increase in the expression of $p47^{phox}$ mRNA compared to untreated cells. Despite this in PGE_2 treated cells the $p47^{phox}$ mRNA expression appears to be down-regulated during the experimental period compared to the untreated cells. These changes in $p47^{phox}$ expression levels suggested that $p47^{phox}$ may be regulated by AA and PGE_2 and involved in enhancement of defense mechanism by NOX enzymes. There is no available data on the effect of AA or PGE_2 in the expression of $p47^{phox}$ by different cells types. Evidences obtained by Wijayagunawardane et al. (1998) indicated that PGE_2 levels are increased after ovulation and decreased during late luteal phase. In the present investigation, it was noted that $p47^{phox}$ expression level was relatively increased during post- and early-to-mid luteal phase. However, some investigators showed the effects of AA and PGE_2 on the NOX activation in leukocytes as these two factors interact with $p47^{phox}$ to change the enzyme activity. It is well known that AA act as a second-messenger molecule and it is released from membrane phospholipids (Balsinde et al., 2002). In activated cells, AA is a stimulator of neutrophils leading to the oxygen-dependent respiratory burst (ROS production). There are several mechanisms by which AA stimulates the respiratory burst. These include the direct binding of AA to subunit proteins ($p47^{phox}$) which regulate the assembly of the NADPH oxidase (Shiose and Sumimoto, 2000; Peng et al., 2003) as well as the activation of key signaling molecules which control the respiratory burst (O'Flaherty et al., 2001). Exogenous AA has long been known to activate neutrophils superoxide production (Badwey et al., 1981).

All these findings may stand for an interaction between the AA, PGE_2 synthesis and level of ROS produced by NOX. Furthermore, an autocrine/paracrine regulation of NOX-dependant ROS production in the oviduct may be suggested. It was concluded that inducible expression of functionally active $p47^{phox}$ by PGE_2, in turn, may contribute both to the inflammatory response and to the attenuation of the antioxidative defense of the oviductal cells, leading to the enhancement of NOX enzyme activity and possibly, through an increased PGE_2 production, to oviductal environment stability.

The third cytosolic subunit $p40^{phox}$ has been as well demonstrated to be expressed in bovine oviductal cells at almost similar levels between the different phases during the estrous cycle. Furthermore, the relative up-regulation of $p40^{phox}$ in the ampulla, the place where the gamete maturation and fertilization take place (Hawk, 1987), may support the suggested role for NOX in the fertilization process. The obtained expression pattern of this subunit suggested $p40^{phox}$ as important subunit for the increase of the enzyme activity as it is necessary for the translocation of $p47^{phox}$ and $p67^{phox}$ from the cytoplasm and increase their affinity to bind NOX at the cell membrane. Indeed, $p40^{phox}$ could promote the conformational change in $p47^{phox}$ that is believed to be necessary to bind with NOX enzyme complex at the cell membrane (Fuchs et al., 1995; Ito et al., 1996). Additionally, Wientjes et al. (1996) demonstrated that $p40^{phox}$ could bind to $p67^{phox}$ to increase the affinity of these components for $p47^{phox}$, thereby facilitate their translocation which is required for recruiting them to the cell membrane.

In general, there was a significant difference in the responses of NOX subunits expressed by the oviductal cells when exposed to physiological levels of E2, P4, AA or PGE_2 *in vitro* (**Table 7**). For example, as E2 or P4 treatment seemed to up-regulated $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ subunits. All these findings taken together may indicate to the involvement of these subunits in the regulation of NOX enzymes in the bovine oviduct during the estrous cycle and to enhance their activities.

	Expression changes induced *in vitro*			
NOX subunits	**E2**	**P4**	**AA**	**PGE_2**
$p22^{phox}$	↑	↑	NS	↑
$p67^{phox}$	↑	NS	↓	NS
$p47^{phox}$	↑	↑	↓	↑
$p40^{phox}$	NS	NS	NS	NS

Table 7: Expression of NOX enzyme subunits in the oviductal cells culture treated with E2, P4, AA or PGE_2 for 6h compared to untreated cells. NS: no significant. ↑: up-regulation; ↓: down-regulation

6 Conclusion and suggestions

Up to date, most of the research in the field of fertility and infertility in mammals have not suitably considered the relation between the redox status and reproductive events in the oviduct. The present study is a first attempt to explore the existence of such systems in the bovine oviduct throughout the reproductive cycle. It is evident from the findings reported here that an oxidant system (NOX enzymes) was present in the bovine oviduct during the normal estrous cycle. Furthermore, considerable focusing has been done to reveal the expression pattern of the known NOX enzymes during all phases of the cycle and in different regions of ipsi- and contra-lateral oviducts. The importance of expression of NOX is indicated by the different expression pattern along the oviduct during the estrous cycle. In the present study, NOX enzyme mRNA is expressed differentially in the bovine oviduct with higher significant level around the time of ovulation. The observed cyclic expression pattern among NOX members is expected due to the influences of local factors or hormonal fluctuations during the estrous cycle. Therefore, up-regulation of NOX mRNA expression during the estrous cycle, particularly around the time of ovulation has been suggested to optimize the oviductal microenvironment for successful pregnancy in different ways.

Expression of NOX2 mRNA, the major NOX enzyme, with higher absolute levels during the estrous suggests this enzyme as main source to maintain basal levels of ROS in the oviductal microenvironment. The maintaining of a constant basal ROS level may be supported by expression of NOX1 expressed consistently throughout the estrous cycle. Moreover, the distinct pattern of correlation between different NOX enzymes and their activation subunits (**Table 5**), beside the responses pattern of the subunits to hormonal treatments may indicate to the fine-tuning mechanism for regulation of the enzyme activities during the estrous cycle. As in the present study oviductal mucosal cells have been shown to express the NOX1, NOX2 and NOX4 which requires an adapter protein such as $p22^{phox}$, $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$ for the regulation of enzymatic activity (Banfi et al., 2003). In addition, NOX1 has a strong positive correlation with the NOX subunits ($p22^{phox}$, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$), which were reported to be essential for the assembling of membrane-associated NOX to produce ROS (Babior, 1999; Geiszt et al., 2003a). The presences of these subunits along with other NOX enzymes may further indicate for their different roles in controlling the enzymes. The membrane subunit $p22^{phox}$ contributes to the presence of the core unit of NOX at the cell membrane and it was seemed to be necessary for regulation of enzyme activities as it was up-regulated with E2, P4 or PGE_2. The pattern of cytosolic $p67^{phox}$ expression and obtained low

absolute expression levels suggested this subunit as rate limiting factor in the process of ROS formation in the oviduct. Previously, it has been reported that the amount of $p67^{phox}$ may be the limiting factor for the assembly and activation of the NADPH oxidase (Uhlinger et al., 1992) as the addition of $p67^{phox}$ (1.6µM) to the cell-free system increased ROS production by 3-fold when compared to $p47^{phox}$ (2.2µM). Moreover, the pattern of expression of $p47^{phox}$ *in vivo* and *in vitro* along with the higher absolute expression level compared to other subunits, and the strong positive correlation with NOX1 during the cycle and with NOX2 during the luteal phase of the cycle may indicate for a pronounced the roles of $p47^{phox}$ in the regulation of NOX enzyme activity in the bovine oviduct. Cytosolic subunit $p40^{phox}$ may be involved to support NOX1 and NOX2 activities during luteal phase at the same time when NOX2 expression was down-regulated. Moreover, this correlation could be related to balanced levels of ROS in oviducts during the estrous cycle. Previously it has been shown that maintaining of the balance between the oxidants and antioxidants is necessary for normal oocyte and sperm functions (Agarwal and Allamaneni, 2004b) and increased ROS to a certain level has benefits on fertilization process and embryo formation *in vitro* (Blondin et al., 1997; Aitken et al., 1998b; Bedaiwy et al., 2002). Furthermore, it is postulated that during the pre-ovulatory phase increasing the NOX2 expression is required for the generation of ROS, influencing the immune protective at the mucosal surface. The secretory immune system of the reproductive tract is up-regulated during the pre-ovulatory phases due to higher E2 levels. It has also been described that E2 influences distinct functions of leucocytes, such as oxidative burst activity in cow (Roth et al., 1983).

Additionally, NOX4, NOX5 and DUOX may be involved in fine regulation of NOX enzyme activities in the oviduct providing optimal levels of ROS supporting the oviduct to receive the gametes and to be prepared for fertilization. Blondin et al. (1997) demonstrated that ROS at certain levels significantly increase gamete fusion, penetration rates and developmental competence of oocyte and these effects were reduced when the ROS level was decreased. Generally, it was concluded that presence of members of NADPH oxidase enzymes in the bovine oviduct may be considered as a novel source of ROS in the microenvironment of the bovine oviduct and may influence oviductal function in different aspects through providing balanced ROS levels (**Fig. 36**).

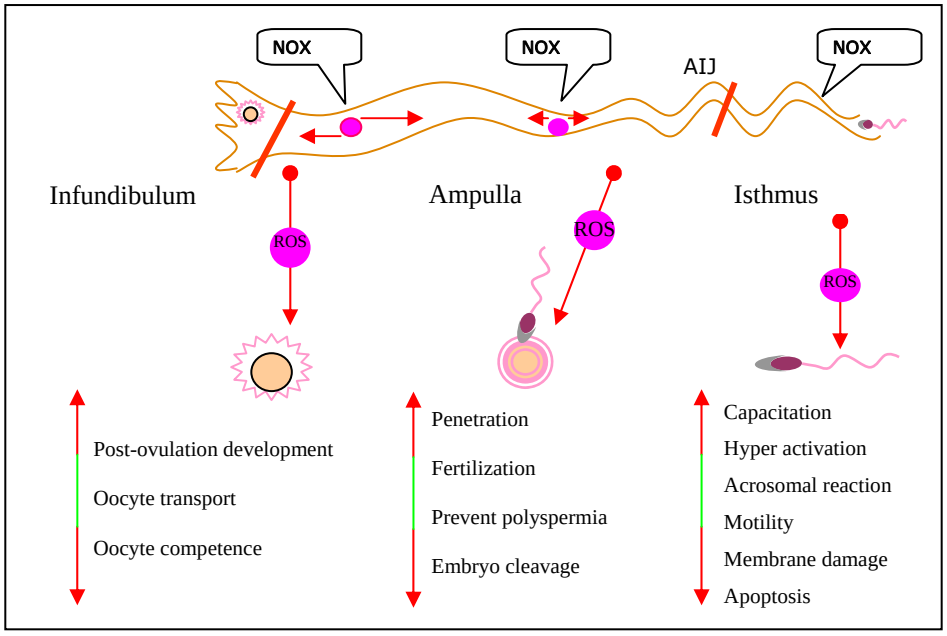

Fig. 36: Proposed roles of NOX-generating ROS in the different regions of the bovine oviduct. The figure shows that NOX enzymes were expressed along the oviduct. Green line indicated positive influences at balanced ROS levels, while red arrows indicated negative influences when ROS levels increases or decreases on the specific mentioned functions. AIJ: ampullari-isthmic junction

The presence of distinct NOX enzymes and their expression pattern were attributed mainly to providing suitable environment in the bovine oviduct via production and optimizing of ROS levels. This hypothesis needs further confirmation by future studies including measurements of the enzyme activities, localization of NOX proteins in the bovine oviductal cells, which would help in the interpretation of the importance of ROS during the estrous cycle to achieve successful pregnancy.

7 Summary

Oviductal samples from 28 cows were used to examine the expression of NADPH oxidase (NOX) enzymes and their activation subunits in the bovine oviduct during the estrous cycle. The regional expression pattern of NOX enzymes in oviducts from ipsi- and contra-lateral site was also investigated.

Bovine oviducts collected from the slaughterhouse were classified into 4 groups: post-ovulatory (day 1-5), early-to-mid luteal (day 6-12), late luteal (day 13-18), and pre-ovulatory (day 19-21) phases. For each phase ipsi- and contra-lateral oviducts were identified according to ovulation side and further sub-divided into two separate regions, the ampulla and isthmus. Thereafter, the oviductal mucosa was recovered separately from ampulla and isthmus. For *in vitro* investigation of NOX enzymes mRNA expression, a primary oviductal cells culture was used. The cells were stimulated for 6h with physiological concentrations of E2 (10pg/ml), P4 (10ng/ml), AA (10µM) or PGE_2 (0.25µM). Samples were collected for RNA isolation with intervals of 2 hours at time points 0h, 2h, 4h, or 6h of treatment. Cell cultures without hormonal treatment were used as control for each of the time points.

For the first time NOX and their activation subunits were found expressed in oviductal mucosal cells in ampulla and isthmus of ipsi- and contra-lateral oviduct throughout the estrus cycle. Only DUOX2 was absent in bovine oviductal mucosa confirmed by detection of its mRNA in bovine endometrial cells. NOX1, NOX2 and DUOX1 showed non-estrous cycle-dependant expression pattern, while mRNA expression of NOX4 and NOX5 were estrous cycle-dependant. NOX4 and NOX5 showed distinct expression pattern: NOX4 mRNA was up-regulated during the pre- and post-ovulatory phase, while the level of NOX5 mRNA expression was increased after ovulation and during early-to-mid luteal phase. In addition, NOX5 mRNA has a significant regional expression during post-ovulatory phase: its mRNA is up-regulated in the isthmus of ipsi-lateral oviduct compared to ampulla. Furthermore, all mRNA of the NOX subunits ($p22^{phox}$, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$) were continuously detected without any significant difference between the different regions of oviduct during the estrous cycle. These subunits seemed to be expressed equally between the different phases of the estrous cycle and showed a positive correlation with expression levels of NOX1, NOX2. Furthermore, NOX4 has a strong positive correlation with its activating subunits $p22^{phox}$ during the post-ovulatory phase.

For the *in vitro* expression, all NOX enzyme components were detected in the untreated and treated primary oviductal cell cultures. E2 or P4 treatments resulted in an up-regulation of

some NOX components, namely NOX2, $p22^{phox}$ and $p47^{phox}$, while DUOX1 mRNA expression was up-regulated under P4 treatment and down-regulated by E2. One observation is that NOX5 mRNA was undetectable in untreated cells or treated cells during the whole experimental period. Generally, AA as well as PGE_2 treatments showed no significant effects on the expression of NOX enzymes, although AA treatment significantly down-regulates mRNA expression of $p67^{phox}$ and $p47^{phox}$ while PGE_2 induces up-regulation of $p22^{phox}$ and $p47^{phox}$.

The obtained expression pattern of NOX components during the estrous cycle pointed to NOX enzymes as a novel source for balanced ROS levels in the oviductal environment. The enzyme activity may be regulated by endocrine and/or local factors. Presence of NOX subunits with distinct expression pattern and its responses in cell culture could be point towards precise mechanisms to regulate NOX activities. Moreover, ROS was implicated in early reproductive events occurring in oviduct and may play important new roles in physiological and pathological effects in the female reproductive tract. This leads to the hypothesis that providing controlled levels of these molecules have benefits on oocyte maturation, spermatozoa function, fertilization and early embryo development in the cow.

8 Zusammenfassung

Identifizierung und Quantifizierung der regionalen Expression von Vertretern der NADPH Oxidase (NOX) Enzym-Familie während des Sexualzyklus im bovinen Ovidukt.

Von 28 Kühen wurden Ovidukte verwendet um die Expression der NADPH-Oxidase (NOX)-Enzyme und deren Aktivierungsuntereinheiten während des Sexualzyklus zu untersuchen. Das Expressionsmuster der NOX-Enzyme in den verschiedenen Regionen des Oviduktes wurde ebenfalls untersucht.

Bovine Ovidukte wurden am Schlachthof gewonnen und in 4 Gruppen eingeteilt: post-ovulatorische Phase (Tag 1-5), frühe-mittlere Lutealphase (Tag 6-12), späte Lutealphase (Tag 13-18) und prä-ovulatorische Phase (Tag 19-21). Für jede Phase wurden ipsilatereale (Seite der Ovulation bzw. mit Corpus Luteum) und kontralaterale Ovidukte gewonnen und weiter eingeteilt in die Abschnitte Ampulle oder Isthmus. Danach wurde die Oviduktmukosaschicht von den einzelnen Abschnitten gewonnen. Für *in vitro*-Untersuchungen der mRNA-Expression der NOX-Enzyme wurde eine Ovidukt-Primärzellkultur verwendet. Die Zellen wurden für 6h mit physiologischen Konzentrationen von E2 (10pg/ml), P4 (10ng/ml), AA (10µM) oder PGE_2 (0.25µM) behandelt. Proben wurden für die RNA-Isolierung im Abstand von 2 Stunden gewonnen zu den folgenden Zeitpunkten: 0h, 2h, 4h, oder 6h. Zellkulturen ohne Hormonbehandlung wurden als Kontrollen zu diesen Zeitpunkten verwendet.

Zum ersten Mal wurde die Expression von NOX und deren Aktivierungsuntereinheiten in allen Regionen des bovinen Oviduktes während des Sexualzykluses dektektiert. Nur DUOX2 mRNA konnte im bovinen Ovidukt nicht nachgewiesen werden, sehr wohl aber in bovinen Endometriumszellen. NOX1, NOX2 und DUOX1 zeigten kein sexualzyklusabhängiges Expressionsmuster im Ovidukt, während die mRNA-Expression von NOX4 und NOX5 zyklusabhängig war. NOX4 und NOX5 zeigten folgende spezifische Expressionsmuster: NOX4 mRNA-Expression war während der prä- und post-ovulatorischen Phase erhöht verglichen mit der Lutealphase. Dagegen stieg die NOX5 mRNA-Expression nach der Ovulation und während der frühen-mittleren Lutealphase an. Zusätzlich zeigte die NOX5 mRNA-Expression ein lokal-spezifisches Expressionsmuster während der post-ovulatorischen Phase. NOX5 mRNA-Gehalte waren im Isthmus des ipsi-lateralen Oviduktes hochreguliert verglichen mit der Ampulle. Die mRNA der NOX-Unterheinheiten ($p22^{phox}$, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$) wurden auf konstanten Niveau in den verschiedenen Regionen des Oviduktes

exprimiert als auch während des Sexualzykluses. Die mRNA-Expression dieser NOX-Untereinheiten zeigte eine positive Korrelation mit der Expression von NOX1 und NOX2. NOX4 wies auch eine starke positive Korrelation mit seiner Aktivierungseinheit $p22^{phox}$ während der post-ovulatorischen Phase auf.

mRNA-Expression für fast alle NOX-Enzym-Komponenten wurden in *in vitro*-Experimenten gefunden, sowohl in unbehandelten als auch behandelten Primär-Oviduktzellkulturen. E2- oder P4-Behandlung resultierte in einer Hochregulierung von einigen NOX-Enzym-Komponenten: NOX2, $p22^{phox}$ und $p47^{phox}$. DUOX1 mRNA-Expression hingegen wurde unter P4-Behandlung erhöht und durch E2-Behandlung erniedrigt. Im Gegensatz dazu konnte keine NOX5 mRNA während der gesamten Zeit in unbehandelten oder behandelten Zellen nachgewiesen werden. Meistens zeigte die Behandlung der Zellen mit AA oder PGE_2 keine signifikanten Effekte auf die Expression der NOX-Enzyme. Ausnahmen war die Abschwächung der mRNA-Expression von $p67^{phox}$ und $p47^{phox}$ durch AA, während PGE_2 eine Erhöhung der mRNA-Expression von $p22^{phox}$ und $p47^{phox}$ induzierte.

Die erhaltenen Expressionsdaten der NOX-Enzym-Komponenten im Ovidukt während des Sexualzykluses deuten darauf hin, dass NOX-Enzyme eine neue Quelle für eine regulierte ROS-Konzentration im Ovidukt sind. Die Enzymaktivität kann durch endokrine oder lokale Faktoren reguliert werden. Die Anwesenheit der NOX-Untereinheiten und ihre Regulation auf Hormone in Zellkulturexperimenten deuten auf einen fein regulierten Mechanismus hin um die NOX-Aktivität zu steuern. ROS ist involviert in frühe Vorgänge der Reproduktion, die im Ovidukt vonstatten gehen und spielen möglicherweise eine wichtige Rolle in physiologischen und pathologischen Vorgängen im weiblichen Reproduktionstrakt. Dies führt zu der Hypothese, dass diese Komponenten unter genauer Kontrolle einen positiven Einfluss auf Oozytenreifung, Spermienfunktion, Befruchtung und Entwicklung des frühen Embryos in der Kuh spielen können.

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Selbständigkeitserklärung:

Hiermit bestätige ich, Mohamed Okasha, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 26.03.2009

Mohamed Elsir Elnageeb Okasha