Research Article

Expression and Cellular Distribution of INHA and INHB before and after *In Vitro* Cultivation of Porcine Oocytes Isolated from Follicles of Different Size

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Cumulus-oocyte-complexes (COCs) were collected from small (<3 mm), medium (3–5 mm), and large (>5 mm) porcine follicles, and the INHA and INHB expression and cellular localization were studied. Developmentally competent (BCB+) COCs were cultured for 44 h. Samples of mRNA were isolated before and after *in vitro* maturation (IVM) from oocytes collected from follicles of different size for RQ-PCR assay. The INHA and INHB protein distribution within the oocytes was observed by confocal microscopy. INHA mRNA expression was increased in oocytes from large compared to medium and small follicles before IVM ($P < 0.001$), and to oocytes of small follicles after IVM ($P < 0.001$). The INHB expression was not different before IVM, but the IHNB mRNA level was gradually higher in oocytes from large follicles after IVM ($P < 0.01$). INHA was not differently expressed before IVM; however, in large follicle oocytes the protein was distributed in the peripheral area of the cytoplasm; in oocytes from small follicles it was in the entire cytoplasm. After IVM, INHA was strongly expressed in oocytes from small follicles and distributed particularly in the *zona pellucida* (*ZP*). Similarly and both before and after IVM, INHB protein was highly expressed in small follicle oocytes and within the cytoplasm. In summary, INHs can be recognized as a marker of porcine oocyte quality.

1. Introduction

As yet, the oocyte developmental capacity *in vitro*, that is, to undergo maturation, fertilization, and to reach the blastocyst stage, has differed significantly from *in vivo* situation [1]. Though the *in vitro* cultivation (IVC) conditions try to mimic the *in vivo* environment, the maturation potential of the gametes is quite different. Although the efficiency of *in vitro* maturation (IVM) of porcine oocytes has been improved, there still exist problems with abnormal male pronucleus formation and an increased polyspermy rate [2]. It is accepted, that mammalian oocytes require both nuclear and cytoplasmic maturation to achieve developmental capacity [3–5]. Therefore, to optimize IVC systems in pigs, the determination of possible differences in a gene expression profile and/or cellular distribution of encoded proteins after IVC are of high interest.

Several intrinsic and extrinsic factors are associated with the maturation ability of porcine oocytes to reach MII stage [5, 6]. In order to determine developmental competence of oocytes many authors use brilliant cresyl blue test (BCB) because of its simplicity and reliability relative to many species. The aim of the test is to evaluate activity of glucose-6-phosphate dehydrogenase (G6PDH) key enzyme

of pentose phosphate pathway that produces ribose-5-phosphate, erythrose-4-phosphate, and NADPH which are used in nucleotide synthesis, aromatic amino acid synthesis, and in reductive biosynthesis, respectively. Immature oocytes that require higher supplementation of energy to become developmentally competent will have higher concentration of enzyme in relation to matured oocytes. This will lead to reduction of BCB stain by G6PDH in this cell resulting in colorless cytoplasm (cells described as BCB$^-$). On the other hand, fully maturated oocytes have lower concentration of G6PDH that is insufficient to reduce the stain resulting in blue colorization of cytoplasm (cells described as BCB$^+$).

Moreover, it was found that the follicular size is one of the factors which are linked with the expression of genes encoding proteins responsible for maturation and fertilization of oocytes. It was shown by Sun et al. (2001) [7] that the follicular size exerts no effect on the resumption of meiosis of oocytes recovered from small follicles, but it significantly affects the developmental potential of porcine oocytes. In addition, Antosik et al. [8] analyzed mRNAs expression of *zona pellucida* glycoproteins; pZP1, pZP2, pZP3, and pZP3 alpha; integrins ITGB1 and ITGB2 and pZP3 and ITGB2 proteins in porcine oocytes, isolated from follicles of various size. They found that the differential expression pattern of mRNAs and of encoded proteins, responsible for fertilization in pigs, was associated with the follicular size. Consequently, the ability of COCs which were recovered from distinct follicles may differ. Inhibins, described also as gonadal glycoprotein hormones, belong to the transforming growth factor beta (TGFB) superfamily and are involved in pituitary FSH secretion [9]. Inhibin exists in two forms, each comprising alpha subunit and covalently associated with one of two distinct subunits, respectively, termed inhibin beta-a (INHBA) and inhibin beta-b (INHBB). The genes encoding INHs are expressed in ovarian granulosa-cells. Their affected expression profile is an example of a primary marker of recurrent and residual ovarian granulosa-cell cancers [10]. Furthermore, mutation of INHA is the main cause of premature ovarian failure (POF) and other ovarian functional abnormalities [11–13]. In addition, Parrish et al. [14] compared expression of 20 different genes responsible for follicular growth in mouse ovarian follicles *in vivo* and cultured *in vitro*. They showed that expression of INHBA as well as expression of other members of TGF superfamily, including TGFB and BMP15, was downregulated in *in vitro* cultured follicles compared to *in vivo*.

However, only few reports are available which indicate expression of INHs in oocytes. Such an expression may suggest their role not only in folliculogenesis but also in oogenesis where TGFB superfamily proteins are involved in crucial steps of oocyte growth and achievement of developmental capacity [15, 16]. Taking into account all of these data, the aim of this study was to analyze the expression of INHA and INHB mRNAs as well as the distribution of their encoded proteins in porcine oocytes recovered from follicles of different size before and after IVM.

2. Material and Methods

2.1. Animals.
A total of 46 postpuberal crossbred Landrace gilts, with mean age of 155 days (range 140–170 days) and mean weight 100 kg (95–120 kg) were used in this study. The experiments were approved by the local Ethics Committee.

2.2. Collection of Porcine Ovaries and COCs.
The ovaries and reproductive tracts were recovered from gilts immediately after slaughter and transported to the laboratory within 20 min at 38°C in 0.9% NaCl. Follicles were classified into three size categories: small (<3mm), medium (3–5 mm), and large (>5 mm) using a caliper.

The follicles were opened by individual puncturing with a 20-G needle attached to a 5-mL syringe into a sterile petri dish. The recovered cumulus-oocyte complexes (COCs) were washed three times in a modified PBS, supplemented with 36 μg/mL pyruvate, 50 μg/mL gentamycin, and 0.5 mg/mL bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA). They were selected under an inverted Zeiss microscope (Axiovert 35, Lübeck, Germany), counted, and morphologically evaluated with special care, using the scale suggested by Jackowska et al., (2009) [17]. Only COCs of grade I with a homogenous ooplasm and having uniform and compact cumulus cells were considered for use in the subsequent steps of the experiment.

2.3. Assessment of Oocyte Developmental Competence by Brilliant Cresyl Blue (BCB) Test.
Before cultivation, COCs were washed twice in a modified Dulbecco PBS (DPBSm, Sigma-Aldrich), supplemented with 50 IU/mL penicillin, 50 μg/mL streptomycin (Sigma-Aldrich), 0.4% [w/v] BSA, 0.34 mM pyruvate, and 5.5 mM glucose (DPBSm). Then, COCs were treated with 26 μM brilliant cresyl blue (BCB; Sigma-Aldrich, St. Louis, MO, USA) diluted in DPBSm at 38.5°C, under 5% CO_2 in air for 90 min. After treatment, the oocytes were transferred to DPBSm and washed twice. During the washing procedure, the oocytes were examined under an inverted microscope and classified as either having stained blue (BCB$^+$) or remaining colourless (BCB$^-$). Only BCB$^+$ oocytes, which had completed their growth phase and may have reached developmental competence, were used in the experiment.

2.4. In Vitro Maturation of Porcine COCs.
The selected BCB$^+$ COCs were cultured in Nunclon Δ 4-well dishes (Nunc, GmbH, Co. KG, Germany) in 500 μL standard porcine *in vitro* maturation (IVM) medium (TCM-199 with Earle's salts, and *L*-glutamine, Gibco BRL Life Technologies, Grand Island, NY, USA) supplemented with 2.2 mg/mL sodium bicarbonate (Nacalai Tesque, Inc., Kyoto, Japan), 0.1 mg/mL sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 10 mg/mL BSA (Sigma-Aldrich), 0.1 mg/mL cysteine (Sigma-Aldrich), 10% (v/v) filtered porcine follicular fluid, and gonadotropin supplements at final concentrations of 2.5 IU/mL human chorionic gonadotropin (hCG; Ayerst Laboratories, Inc., Philadelphia, PA, USA) and 2.5 IU/mL

TABLE 1: Oligonucleotide sequences and primer probes used for RQ-PCR analysis.

Transcript	Sequence (5′–3′ direction)	Probes number	Gene accession no.	Product size (bp)
GAPDH	F: TCCCTCAAGATTGTCAGCAA	28	NM001003142	68 bp
	R: TGGATGACTTTGGCTAGAGGA			
β-actin	F: GATCACCATTGGCAACGAG	12	NM001003349	111 bp
	R: CCCAGGAAAGAAGGTTGGA			
INH B	F: ATCATCCAGGGGTCCTGTG	26	NM001164842	101 bp
	R: GACTGGGTGGGTCAACAAAG			
INH A	F: AGCGTGTGGACCTGAAGC	28	NM214189.1	105 bp
	R: ACTGCACGTCCAGGTTGAG			

equine chorionic gonadotropin (eCG; Intervet, Whitby, ON, Canada). Wells were covered with a mineral oil overlay and cells were cultured for 44 h at 38°C under 5% CO_2 in air. The COCs were incubated with bovine testicular hyaluronidase (BTH; Sigma-Aldrich, St. Louis, MO, USA) for 2 min at 38.5°C to separate the cumulus cells. These cells were removed by vortexing the oocytes. The cumulus cell-free oocytes were used for further analysis.

2.5. Confocal Microscopic Observations. Oocytes were isolated from small, medium, and large follicles (each $n = 10$ per slide). Oocytes were fixed with 2.5% paraformaldehyde in PBS and 0.2% Triton-X 100 for 30 min at room temperature (RT) and washed three times in PBS/PVP (0.2%). In order to block nonspecific binding, the samples were incubated with 3% BSA in PBS plus 0.1% Tween 20 for 30 min at RT. Oocytes were incubated for 12 hours at 4°C with goat polyclonal anti-INHA antibody (Ab, sc-22048) or rabbit polyclonal anti-INHB (Ab, sc-50288) both from Santa Cruz Biotechnology (Santa Cruz, CA, USA), diluted 1 : 500 in PBS/1.5% BSA/0.1% Tween 20. After several washes with PBS/0.1% Tween 20, samples with goat polyclonal anti-INHA Ab were incubated for 1 hour at RT with fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG Ab, produced in rabbits, and diluted 1 : 200 in PBS/0.1% Tween 20. Samples with rabbit polyclonal anti-INHB Ab were incubated for 1 hour at RT with crystalline tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit IgG Ab, produced in goats, and diluted 1 : 200 in PBS/0.1% Tween 20. Following washing in PBS/0.1% Tween 20, the oocytes were mounted on glass slides in antifade drop and observed under confocal system LSN 510 in Carl ZEISS microscope, Axiovert 200 M. FITC was excited with 488 nm from an argon laser, and emissions were imaged through a 505–530 nm filter. TRITC was excited with 543 nm from an argon laser and the emissions were imaged through a 565–615 nm filter.

2.6. Gene Expression Analysis. Total RNA was isolated from BCB^+ oocytes collected from large ($n = 50$), medium ($n = 50$), small follicles ($n = 50$) each before and after IVM using an RNeasy mini column Qiagen GmbH (Hilden, Germany). RNA samples were treated with DNAse I in order

to purify the samples from DNA contamination, and the quality was examined by the microchannel electrophoresis using an Agilent Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA). The RNA samples were resuspended in 20 μL of RNase-free water and reverse-transcribed into cDNA. Real-time quantitative PCR amplification reactions were carried out in an AB 7500 HT Real-Time PCR System (Applied Biosystems) using Universal PCR Master Mix (Roche, Mannheim, Germany). All reactions were performed in a 20 μL volume with 6 pmol forward and reverse primers and 4 pmol of probe (Table 1). The INHA and INHB expression was studied using primer probes (cat. no. 04687604001, cat. no. 04687574001, resp., Roche, Mannheim, Germany) according to the manufacturer's instructions. All reactions were executed in triplicates. In case of the negative control cDNA was not added. All primers used in the experiment were designed in the way that primers cover ends of two neighbour exons. In that case there was no need of performing reverse transcription control.

The relative expression levels of INHA and INHB transcripts in each sample were standardized by the internal standard of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In order to ensure that granulosa cells did not contaminate the oocytes, we demonstrated the absence of cytochrome P450 aromatase (cat. no. 69-04688686001), Roche, Mannheim, Germany) transcript by RT and RQ-PCR.

The two housekeeping genes GAPDH and β-actin (cat. no. 119-04693531001 and cat. no. 44-04688040001, resp., Roche, Mannheim, Germany) were amplified as the reference for mRNA quantification.

In order to quantify specific genes expressed in the oocytes, the levels of the expression of specific oocyte mRNAs in each sample were calculated relative to those of GAPDH and β-actin using the method described. Expression of GAPDH and β-actin mRNAs was measured in cDNA samples from isolated oocytes. All reactions were applied in at least five replicas. Determination of gene expression was based on $2^{\Delta\Delta Ct}$ method according to Roche technical guide.

2.7. Statistical Analysis. Two-way ANOVA followed by the Tukey posttest was used to compare the results of the real-time PCR reaction. The differences were considered to be significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$. The software program GraphPad Prism version 4.0 (GraphPadSoftware, San Diego, CA, USA) was used for statistical calculations.
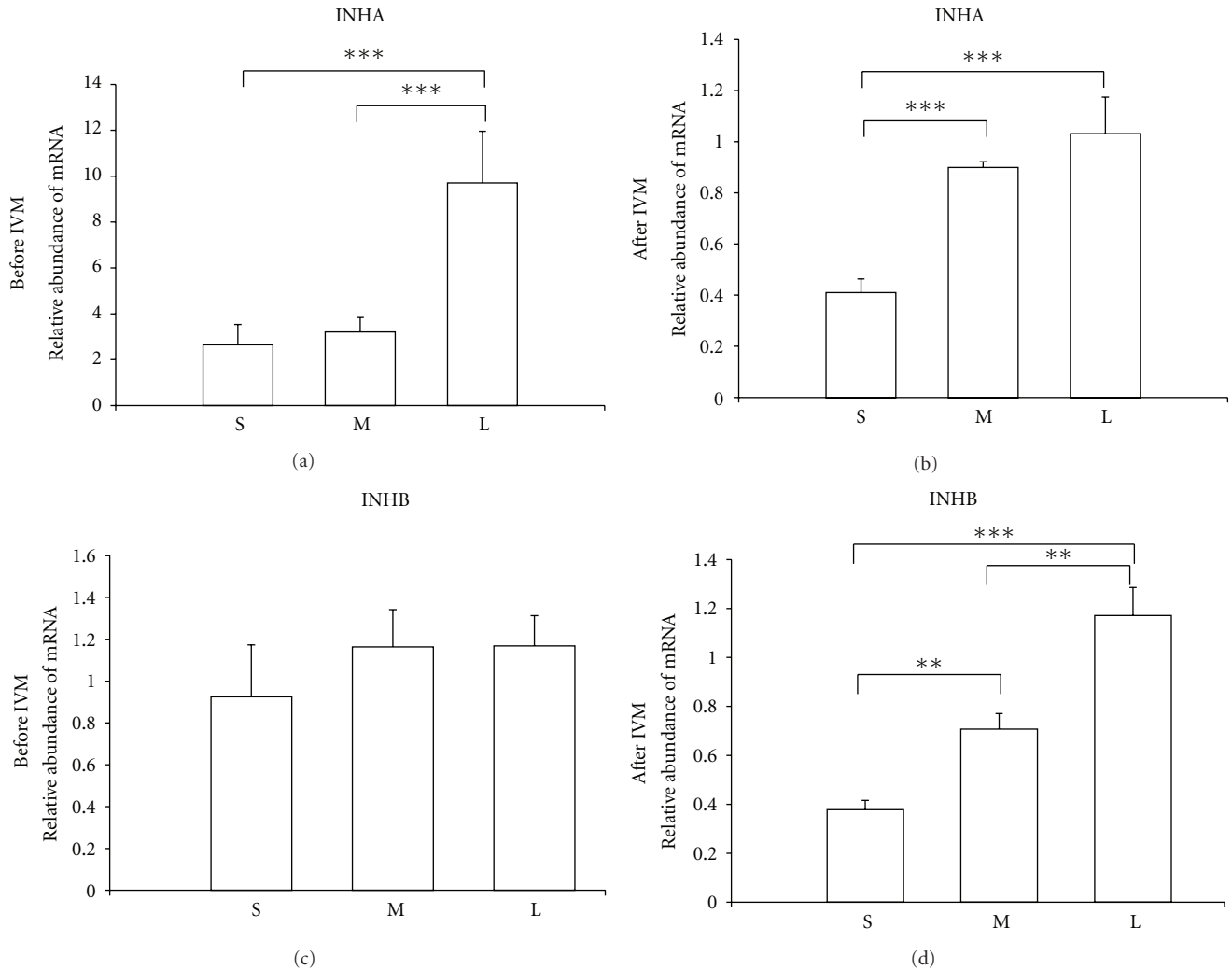
FIGURE 1: Relative abundance of INHA and INHB transcripts in porcine oocytes isolated from different size of follicles before and after IVM. The porcine oocytes were isolated from follicles of pubertal gilts and immediately used to isolate RNA, which was reverse-transcribed into cDNA. The relative abundance of INHA (a), (b) and INHB (c), (d) mRNAs was evaluated by RQ-PCR analysis before ((a), INHA, (c), INHB) and after ((b), INHA, (d), INHB) IVM. The oocytes analyzed before or after IVM were isolated from small (<3 mm), medium (3–5 mm) and large (>5 mm) follicles. Results are presented as mean ± SEM with the level of significance, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

Several differences in the expression profile of INHA and INHB mRNAs as well as in localization and/or cellular distribution of encoded proteins were observed in oocytes collected from follicles of different size. Using RQ-PCR analysis we found an increased expression of INHA mRNA in oocytes isolated from large follicles as compared to oocytes from medium and small follicles before IVM ($P < 0.001$), (Figure 1(a)). When oocytes were analyzed after IVM, higher INHA mRNA level was revealed in oocytes isolated from large and medium follicles ($P < 0.001$), (Figure 1(b)). To the contrary, no modifications were observed before IVM in the INHB mRNA expression profile between oocytes recovered from follicles of different size (Figures 1(c) and 1(d)). After IVM, a gradual increased expression of the mRNA was noted in oocytes

with relation to the follicle size: large > medium > small follicles ($P < 0.001$, $P < 0.01$, $P < 0.01$, resp.) (Figure 1(d)).

The confocal microscopy observations revealed no differences in INHA protein expression within the oocytes isolated from different sized follicles. However, in oocytes from large follicles before IVM, INHA protein was distributed in the peripheral area of the oocyte membrane, whereas in oocytes from medium and small follicles INHA protein was significantly stained in the cytoplasm (Figures 2(a), 2(b), and 2(c)). After IVM, INHA protein manifested a strong *zona pellucida* expression in oocytes from all follicles (Figures 2(d), 2(e), and 2(f)). Additionally, an increased expression was observed in *zona pellucida* of oocytes from small follicles (Figure 2(f)). Before IVM, INHB protein expression was higher in oocytes collected from small follicles (Figure 3(c)). INHB was distributed in the cytoplasm rather than in the *zona pellucida* of oocytes of all follicle
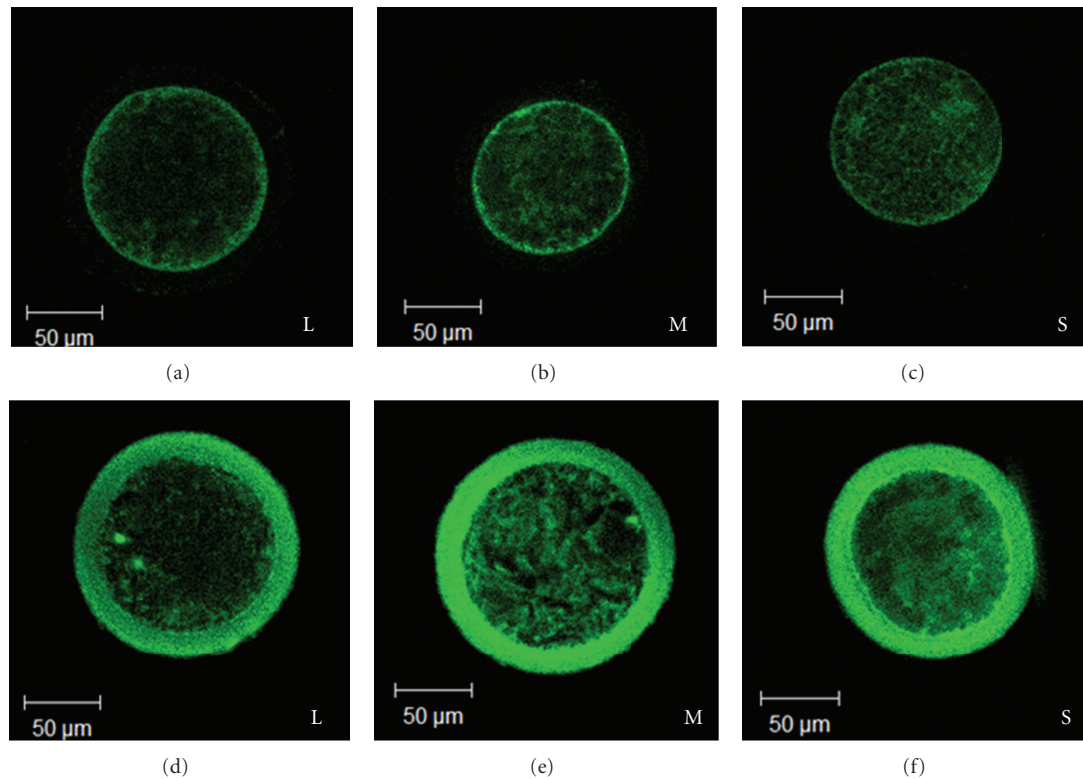
FIGURE 2: Confocal microscopic observation of INHA cellular distribution in porcine oocytes isolated from small, medium, and large follicles before and after IVM. Porcine oocytes isolated from small, medium, and large follicles were stained before and after IVM with porcine INHA (before IVM; (a), (b), and (c) and after IVM; (d), (e), and (f)), (goat polyclonal anti-INHA, sc-22048). The treated oocytes were labeled for 40 min with FITC-conjugated anti-goat IgG Ab, which emits a green fluorescent signal after excitation at 488 nm at a 1 : 200 dilution in PBS. Bars are 50 μm. L: large, M: medium, S: small follicles.

classes (Figures 3(a), 3(b), and 3(c)). There was an increase of INHB protein expression in oocytes from large follicles after IVM as compared to before IVM (Figure 3(d)). Overall, the highest expression of INHB was found in oocytes recovered from small follicles (Figure 3(f)). Furthermore, a distinct cytoplasmic distribution of INHB was noted in oocytes of all follicles groups (Figures 3(d), 3(e), and 3(f)). The negative control was performed without primary antibody against investigated proteins (Figures 4(a) and 4(b)).

4. Discussion

Porcine models are successfully applied in many fields of medicine including tissue engineering, cardiovascular disease, skin physiology, and reproductive biology (reviewed by [18]). Due to the good availability of porcine oocytes, they are of growing interest of studies at the molecular level of reproductive processes.

In several studies it was clearly shown that the maturation ability of mammalian oocytes is regulated on the molecular level by expression of genes and encoded by them proteins, and which are responsible for mitotic/meiotic progress and for morphological changes [19–22]. Genes belonging to TGFB superfamily are important regulators; they are

called also "checkpoints of oocyte maturation potential" [23]. INHA and INHB are members of TGFB superfamily and it is suggested that they regulate important stages of oocyte maturation *in vivo* and *in vitro* [24–26]. However, the influence of several factors, for example, the follicular size and/or *in vitro* cultivation, on INHA and INHB mRNA expression profile or encoded proteins distribution within the porcine oocytes, is still not entirely known. Our study aimed to examine the role of the follicle size and IVC on the expression of INHA and INHB genes and the expression of respective proteins.

It has been shown in many species that the follicular size is correlated with developmental competence of oocytes. Despite differences in oocytes diameter across species used in the definition of small, medium, and large oocyte applied by authors, the general hypothesis is still consistent. Bovine oocytes are a good example of such a situation where differentiation in groups is not the same, but authors agree that follicles of a higher diameter contain more developmentally competent oocytes [27, 28]. This trend is preserved in many other species including camel, dogs, and pigs [29, 30].

The follicular size has an influence on the expression profile of several genes involved in oocyte maturation and fertilization, too [8, 31, 32]. Glister et al. [33] investigated the role of genes of the TGFB superfamily, including INHA,
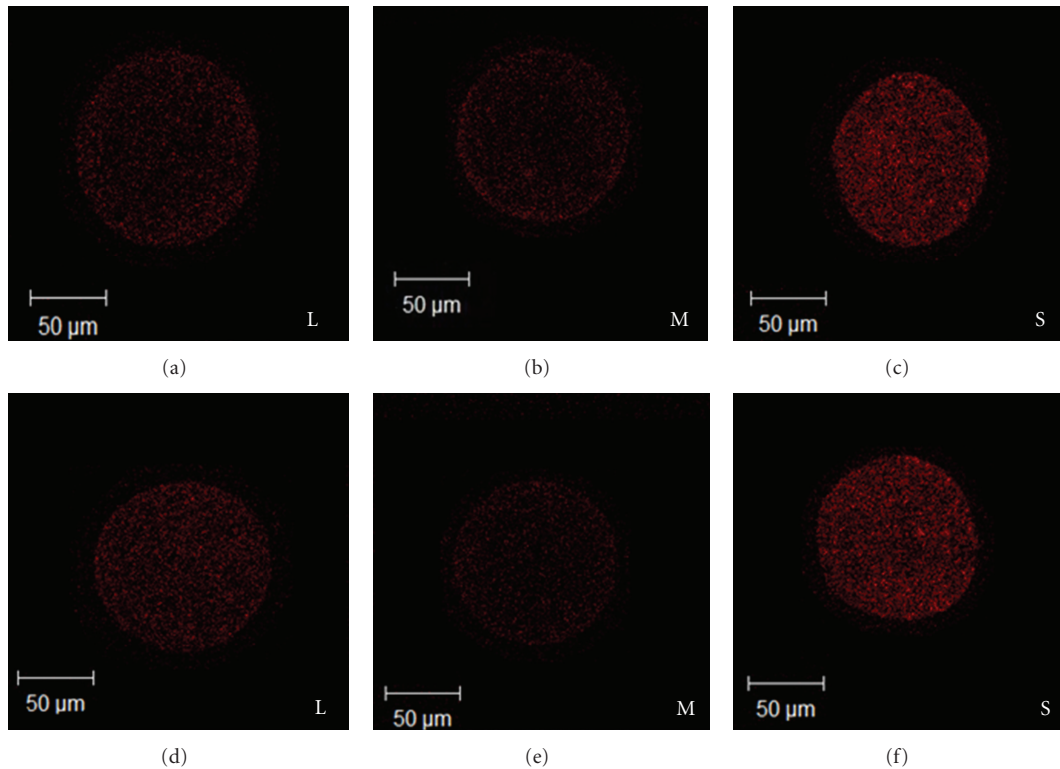
FIGURE 3: Confocal microscopic observation of INHB cellular distribution in porcine oocytes isolated from small, medium, and large follicles before and after IVM. Porcine oocytes isolated from small, medium, and large follicles were stained before and after IVM with porcine INHB (before IVM; (a), (b), and (c) and after IVM; (d), (e), and (f)), (rabbit polyclonal anti-INHB, sc-50288). The treated oocytes were labeled for 40 min with TRITC (crystalline tetramethylrhodamine isothiocyanate), which emits a red fluorescent signal after excitation at 543 nm at a 1 : 200 dilution in PBS for INHB. Bars are 50 μm. L: large, M: medium, S: small follicles.
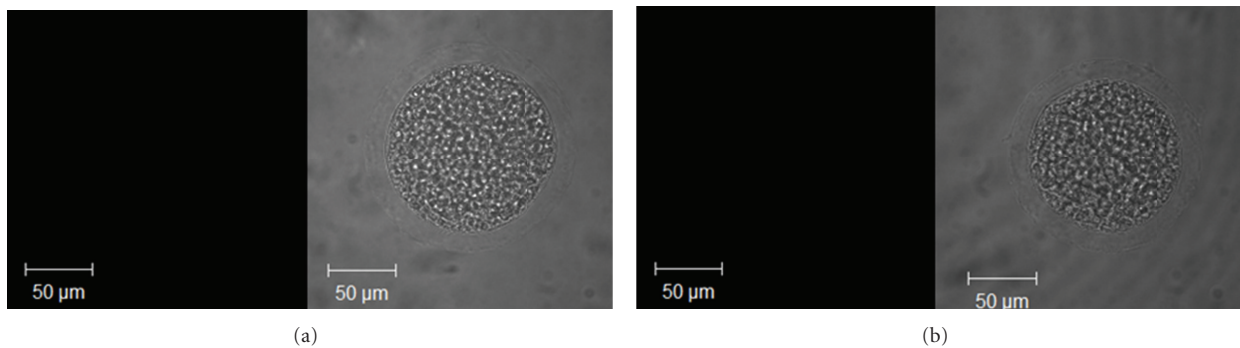

FIGURE 4: Confocal microscopic observation of control oocytes. Porcine oocytes with intact *zona pellucida* (negative control) were incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG Ab produced in rabbits for INHA, diluted 1 : 200 in PBS/0.1% Tween 20 (a), and for INHB with TRITC (crystalline tetramethylrhodamine isothiocyanate)-conjugated anti-rabbit IgG Ab produced in goats, at a 1 : 200 in PBS/0.1% Tween 20 (b). The oocytes were mounted on glass slides in an antifade drop and observed under confocal system. Bars are 50 μm.

in the bovine follicular fluid in relation to the follicle growth. They found that each stage of the follicle growth was associated with the expression of INHA protein. Moreover, a stimulatory effect of FSH was noted on investigated genes. It may be suggested that the expression profile of INHA may represent a marker and prognostic factor of the follicle growth and development. These observations were partially proven by Jyotsna and Medhamurthy [34]. They analyzed changes in the estradiol and progesterone concentration in the Buffalo cow follicles during periovulatory development, and an increased expression of INHA in granulosa cells of the dominant follicle. Although there are only few published data regarding INHs expression and the growth of the follicle, the two experimental papers suggest that

the growth of the follicle may be determined by INHA and upregulation of follicle development is associated with a higher expression profile of inhibins. Similarly, our study has revealed significant changes in both INHA and INHB mRNA expression profile in oocytes collected from porcine follicles of different sizes. Taking into consideration all these results, it may be suggested that INHs could be regarded as important factors involved in the porcine follicle growth too. Since it was documented in several studies that oocytes of an increased fertilization and maturation ability are recovered predominantly from large follicles [8, 31], our results have also shown that IHNs expression profile may be assumed to represent not only a marker of porcine follicle growth, but also a significant genetic index of the oocyte quality. Contrary to INHs mRNA expression profiles, we have observed an opposite effect of INHA and INHB protein expression and distribution in relation to the follicular size. An increased expression of these two proteins was detected in oocytes recovered from small follicles. This effect may be explained by the existence of different transcriptional and translational regulatory mechanisms and/or posttranscriptional modification of INHs mRNAs.

Although only one study describes the role of INHs in regulation of the cell cycle, the impact of other genes and encoded proteins in attainment of a full maturation by oocytes was recognized [35, 36]. Cyert and Kirschner [37] used Xenopus oocytes as an experimental model to study the activation of MPF and phosphorylation of pre-MPF and their involvement in the regulation of the cell cycle progress. They demonstrated that INH, as the pre-MPF inhibitor, has a function similar to protein phosphatase. Therefore, the activation of pre-MPF occurs via the mechanism of protein phosphorylation. The activation of INH is also regulated by another protein, called protein phosphatase-1. It is suggested that an upregulation of INH is necessary for MPF activation as a presumably first step of a mechanism that leads to the cell cycle regulation and progression. Since the cell cycle controlling system is a main mechanism associated with the maturation potential of mammalian oocytes, we investigated the INHA and INHB expression profiles in porcine oocytes before and after maturation *in vitro*. A substantially increased expression of INHA mRNA has been observed in oocytes before the maturation process; however, the INHB mRNA expression was unaffected. On the other hand, the expression of INHA and INHB proteins was significantly increased in oocytes after IVM. This observation points to the existence of a posttranscriptional regulation of INHs expression.

INHB protein was always distributed in the cytoplasm of porcine oocytes before and after IVM. Against, INHA protein was located in the peripheral area of the cytoplasm in oocytes before IVM, while after maturation the protein displayed a pronounced staining in the *zona pellucida*. An increased expression of INHA in the *zona pellucida* after IVM may indicate its role as an important gonadal glycoprotein. However, the crucial function of INHA at oocyte maturation and oocyte-sperm interaction needs to be confirmed in further experiments.

5. Conclusions

Our study demonstrated differential INHs mRNA expression profiles and distribution of the encoded proteins within porcine oocytes during the process of *in vitro* maturation. Furthermore, the relationship between the follicular size, oocyte maturation, and INHs expression profiling was shown. It is supposed that INHs are an important factor involved in the process of the follicle growth and oocyte maturation in pigs.

Acknowledgment

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