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Original Research Article

A critical assessment of the effect of serine protease inhibitors on porcine fertilization and quality parameters of porcine spermatozoa *in vitro*

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ABSTRACT

Proteases play an important role during mammalian fertilization. Their function is frequently investigated using specific inhibitors. We analyzed four serine protease inhibitors [4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride (AEBSF), soybean trypsin inhibitor from glycine max (STI), N_{α}-tosyl-L-lysine-chloromethyl ketone hydrochloride (TLCK) and N_{p}-tosyl-L-phenylalanine-chloromethyl ketone (TPCK)] for their *in vitro* effect on fertilization and sperm quality in pigs. Inhibitor concentrations were chosen based on the reduction of fertilization rate during preliminary dose–response experiments with cryopreserved epididymal spermatozoa. The inhibitor effects on *in vitro* fertilization (IVF) and sperm parameters (membrane and acrosomal integrity, motility and mitochondrial membrane potential – MMP) were evaluated using diluted fresh semen. AEBSF (100 μM), TLCK (100 μM) and TPCK (100 μM) decreased total fertilization and polyspermy rates by at least 50%. STI (5 μM) lowered total fertilization rates but not the level of polyspermy. AEBSF and TPCK reduced fertilization parameters to a similar degree using cryopreserved epididymal spermatozoa (dose–response experiment) or diluted fresh semen. Inhibition by STI was more pronounced using cryopreserved epididymal spermatozoa, whereas TLCK inhibited IVF only with diluted fresh semen. AEBSF and STI had no effect on sperm parameters, and TLCK significantly reduced motility. TPCK diminished MMP and motility and affected membrane and acrosomal integrity in a negative way. In summary, serine protease inhibitors differed in the way they reduce the fertilization rate. These results emphasize the necessity of inhibitor testing before they can be applied in fertilization studies. AEBSF and STI can be used in the future IVF studies without compromising sperm quality.

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1. Introduction

Serine proteases play a key role in many biological pathways as well as in the modulation of cellular and extracellular proteins [1]. Their involvement in fertilization has been acknowledged in mammalian as well as in non-mammalian species [2,3]. Based on early inhibitor studies reporting a decrease in fertilization rate by serine protease inhibition [4–7], sperm serine protease activity has been mainly associated with sperm binding to and penetration of the zona pellucida (ZP) [7–10]. Since then, the sperm specific serine protease, acrosin has been described in several mammalian species and serine protease activity has also been associated with other fertilization events such as sperm capacitation, dispersion of acrosomal content and activation of oocyte transmembrane receptors [11–13]. Various serine proteases have been detected in the acrosome and on the sperm and acrosomal membranes by using techniques such as immunochemistry, western blotting, *in situ* hybridization and proteomics (for review see [14]). Still, researchers have not been able to establish a direct link between a single serine protease and a specific physiological role in fertilization.

The research on the role of serine proteases during fertilization has been carried out mainly in the mouse [11,15–17]. However, the various characteristics of the sperm proteases described so far point out that protease systems substantially differ between mice and other animals [18]. It is, therefore, not appropriate to extrapolate results from mice to other mammalian species. In the pig, the sperm-specific serine protease, acrosin and an acrosin-like protease have been described [19–26]. The proteasome, a multi-subunit protease including trypsin- and chymotrypsin-like serine protease activity, has been implicated in sperm penetration of the ZP [27,28]. Nevertheless, a general view on how trypsin- and chymotrypsin-like serine proteases contribute to the different steps of porcine fertilization is still lacking.

Among the different approaches that can be used in fertilization studies, protease inhibitors have the advantage that they can be used to distinguish active proteases from inactive zymogens, in contrast to methods such as immunochemistry or proteomics which allow only to demonstrate the localization of a protease but not its functionality. In general, inhibitors are designed to bind to the active site of the protease and to interfere with the catalytic mechanism of the protease. In this study, we investigated the effects of inhibitors of trypsin-like and chymotrypsin-like serine proteases on total fertilization and polyspermy during porcine *in vitro* fertilization (IVF) in relation to their effects on four important sperm quality parameters: sperm membrane integrity, motility, mitochondrial membrane potential and acrosomal integrity. Four inhibitors were used in the study: 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride (AEBSF), soybean trypsin inhibitor from glycine max (STI), N_{α}-tosyl-L-lysine-chloromethyl ketone hydrochloride (TLCK) and N_{p}-tosyl-L-phenylalanine-chloromethyl ketone (TPCK). AEBSF is a broad spectrum serine protease inhibitor and irreversibly inhibits proteases belonging to the trypsin-, chymotrypsin- and elastin-like serine protease family. STI and TLCK inhibit trypsin-like serine proteases, respectively in a reversible and irreversible manner [29]. TPCK is used to inhibit proteases of the chymotrypsin-like family of serine proteases and inhibits these proteases in an irreversible manner [1]. AEBSF, TLCK and TPCK are of low molecular weight, whereas STI is a high molecular weight inhibitor (20.1 kDa). Results show that serine protease inhibitors differ in the way they reduce fertilization rates. AEBSF and STI inhibited fertilization rate without compromising sperm quality and were found suitable for further research on the role of serine proteases during porcine fertilization.

2. Materials and methods

2.1. Media

All chemicals used in this study were purchased from Sigma–Aldrich (St. Louis, MO, USA), unless otherwise stated. The basic medium used for the collection and washing of cumulus oocyte complexes (COCs) was a modified HEPES-buffered Tyrode balanced salt solution (HEPES-TM) with 10 μg/mL gentamycin sulfate, 10 mM HEPES and 3 mg/mL bovine serum albumin (BSA). Oocyte maturation medium consisted of BSA-free 'North Carolina State University' 23 (NCSU23) [30] supplemented with 0.57 mM cysteine, 10 ng/mL epidermal growth factor, 10 IU/mL equine chorionic gonadotropin (eCG) (Folligon®, Intervet, Boxmeer, The Netherlands), 10 IU/mL human CG (hCG) (Chorulon®, Intervet) and 10% porcine follicular fluid. The basic medium for IVF was Tyrode's albumin lactate pyruvate medium (TALP medium) [31] supplemented with 0.3% BSA (FERT-TALP). Presumed zygotes were washed in HEPES-buffered TALP medium (HEPES-TALP), *i.e.*, TALP medium with 25 mM HEPES. The embryo culture medium was NCSU23 with 0.4% BSA. The pH of oocyte maturation medium, FERT-TALP, HEPES-TALP and embryo culture medium was respectively 7.4, 7.5, 7.4 and 7.4. Prior to IVF and fluorescent staining, boar spermatozoa were washed (390 × *g*, 3 min) in Androhep extender (pH: 6.7) (Minitüb®, Tiefenbach, Germany).

2.2. Protease inhibitors

All inhibitors were used at different concentrations within the range of effective (or lower) concentrations reported by the manufacturer (Sigma–Aldrich). STI (T6522) and TLCK (90182) were stored desiccated. AEBSF (A8456) was dissolved in deionized water (stock solution of 10 mM) and TPCK (T4376) in dimethyl sulfoxide (DMSO) (stock solution of 20 mM). Protease inhibitor dilutions were prepared in HEPES-TM with 10 μg/mL gentamycin sulfate, 10 mM HEPES and 3 mg/mL BSA. For the sperm experiments, all dilution steps were performed with HEPES-TM medium.

For IVF experiments, the preparation of inhibitor working solution included a final 1:9 dilution step in fertilization medium. From this inhibitor dilution in fertilization medium, 10 μL was added to the fertilization droplets (90 μL) approximately 15 min before matured COCs were assigned to the different treatments. AEBSF was stored in deionized water. To adjust the concentration of medium compounds, a 1:1 dilution was made of stock solution in deionized water and medium