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Male Reproductive Tract Origin Sperm-quiescent Proteins: Prospects in Semen Cryopreservation of Livestock Species

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ABSTRACT

Cauda epididymis is known to preserve mature sperm in a quiescent state for several weeks without causing potential decline to fertilizing capacity of the sperm. This unique property of cauda epididymis has been attributed to the unique biochemical composition of cauda epididymal plasma and the presence of certain proteins having decapacitating and motility–inhibitory activities. Cryo-capacitation and low progressive motility are two common changes in cryopreserved semen across species and have been considered the underlying causes for low fertility rate of cryopreserved semen. Considering their potential roles in reversible inhibition of sperm capacitation and motility these naturally-occurring proteins may be suitable tools for minimizing cryo-capacitation and improving post-thaw progressive motility of cryopreserved semen by sparing available energy in sperm during cryopreservation. The present review attempts to bring out the status of purification, functional and mechanistic characterization of these sperm-quiescent proteins across species. In addition the status of recombinant production of these proteins by using different host systems and the scope for application of these proteins in semen cryopreservation of livestock species has also been described.

Introduction

Cryopreservation of semen is a useful tool for long-term preservation of animal germ-plasm of higher genetic merits, for conservation of animal species or breeds that are in the threat of extinction and also for genetic up-gradation of indigenous livestock species for enhancing their productive and reproductive efficiencies. However, poor sperm recovery, shorter post-thaw sperm survival and reduced fertilizing capability are the major limitations of cryopreserved semen across livestock species (Salamon and Maxwell, 1995a). The damage incurred to sperm during cryopreservation is multi-factorial involving cold-shock, osmotic stress, ice crystal formation and oxidative stress (Aitken and Drevet, 2020).The sub-lethal cryodamagein sperm during freezing-thawing of semen mainly involves the membranous structures including acrosome, plasma membrane and mitochondria causing capacitation-like changes such as efflux of membrane cholesterol and tyrosine phosphorylation of membrane proteins (Salamon and Maxwell, 1995b, Paul et al., 2020). Reactive oxygen species (ROS) generated during freezing-thawing attack on various macromolecules in sperm such as lipid, protein and DNA causing oxidative change to them (Len

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et al., 2019). Extensive research has been carried out across species to minimize cryodamage in frozen-thawed spermatozoa. These included use of different freezing-thawing protocols, various cryoprotectants, a lot many number of semen additives including enzymatic and non-enzymatic antioxidants, amino acids, sugars, plant extracts, honey etc. (Salamon and Maxwell, 1995b; Ugur et al., 2019; Aitken and Drevet, 2020; Dalal et al., 2020; Ros-Santaella et al., 2021). Many of these have resulted in improvement in post-thaw motility and other sperm qualities; however, much more effort is required to obtain significant improvement in post-thaw sperm functions and fertility rates of cryopreserved semen.

Cauda epididymis of mammalian species can store mature sperm in a non-motile and quiescent state for several weeks without affecting the fertilization potential remarkably (Brooks, 1990). The unique physio-biochemical properties of cauda epididymal plasma, in particular, the presence of certain sperm-quiescent proteins were considered responsible for this. Several sperm-quiescent proteins have been isolated from caudaepididymal plasma of rat, pig, goat and sheep (Usselman and Cone, 1983; Iwamoto et al., 1992; Dungdung and Majumder, 2003; Das et al., 2010; Ghosh et al., 2018; PyareLal, 2019). Some of these proteins also demonstrated de-capacitating activity (Pyare Lal, 2019). Cauda epididymal origin another protein namely cysteine-rich secretory protein 1 (CRISP-1) binds to sperm membrane in a reversible manner and regulate capacitation and protein tyrosine phosphorylation (Roberts et al., 2003). Regucalcin, a calcium-binding multi-functional protein of male reproductive tract regulate intracellular $[Ca]_i$ in sperm and apoptosis in germ cells and favors cell survival (Yamaguchi and Tai, 1991; Pillai et al., 2018). Recombinant rat CRISP-1 protein produced in HEK293 cell line demonstrated full bioactivity and possessed glycosylation. However, recombinant rat and ovine CRISP-1 proteins when produced in *E. coli* were insoluble and demonstrated only partial activity (Reddy et al., 2008; Jorasia et al., 2021). Considering the potential involvement of the sperm-quiescent proteins in regulation of sperm capacitation, motility as well as apoptosis these could be a useful tool for improvement of post-thaw quality and survival of cryopreserved sperm across species. However, this has not been explored yet in any species.

Sperm-quiescent factors present in cauda epididymal plasma

The quiescence of cauda sperm in rat was reported to be mediated by a highly viscous protein called 'immobilin' (Usselman and Cone, 1983); however, in other species the viscoelasticity of cauda fluid was not related to immotility of cauda sperm (Carr et al., 1985; Turner and Reich, 1985). Both motility promoting as well as inhibiting proteins were reported in rat seminal vesicle secretion (Peitz, 1988). Jeng et al. (1993) purified two sperm motility inhibitors (SMI-1 and SMI-2) from boar seminal plasma. Bass et al. (1983) found some non-dialyzable factors in bovine seminal plasma (SP) that affected the viability and motility of sperm. Human SP also contains a sperm motility inhibitor that originates from seminal vesicles as a 52 kDa precursor and is degraded into smaller peptides by prostatic proteases shortly after ejaculation (Iwamoto and Gagnon, 1988; Robert et al., 1997).

Dungdung and Majumder (2003) purified a 100 kDa sperm motility inhibiting factor (MIF) from goat cauda sperm membrane. Das et al. (2010) purified a 160 kDa motility inhibiting protein (MIF-II) from goat epididymal plasma (EP). The MIF-II was reported to be a homodimer consisting of two 80 kDa sub-units and acts via cAMP-dependent pathway. Very recently, another sperm quiescent protein of 59 kDa was purified from goat EP (Ghosh et al., 2018). The protein was reported to be a monomer and is thermostable. Recently, we showed that the sperm motility-inhibiting activity in ram EP was present in the protein-rich fraction but not in the protein-free plasma fraction (Paul et al., 2018a). Further, in a recent study we could partially purify two motility-inhibitory proteins of approximate sizes 80 and 60 kDa from ram EP (PyareLal, 2019). The 80 kDa protein exhibited de-capacitating activity on cauda sperm.

Involvement of sperm-quiescent proteins in sperm motility, capacitation and associated cellular-signaling

Both sperm motility and capacitation are known to be associated with cAMP-mediated increase in intracellular $[Ca^{+2}]$. Sperm motility-inhibiting factor I and II (MIFI & II) from goat EP were reported to inhibit motility of cauda sperm at a micromolar concentration (Dungdung and Majumdar, 2003; Das et al., 2010). The MIF II also reduced the concentration of cAMP in a dose-dependent manner. Another sperm MIF 'quiescent factor' (QF) purified from goat EP was reported to inhibit sperm forward motility at 2 µM within 10 min (Ghosh et al., 2018). The sperm motility inhibition by QF is caused by reducing the activity nitric oxide synthase (NOS) enzyme and subsequently decreasing the intracellular nitric oxide (NO)

and cGMP concentration. However, it does not modulate intracellular Ca^{+2} or cAMP concentration (Ghosh et al., 2018). The 80 and 60 kDa proteins purified from ram CEP also demonstrated motility-inhibition on prewashed cauda sperm at 5 µg/ml level. The 80 kDa protein also exhibited de-capacitating activity in BSA-induced sperm capacitation study (Pyare Lal, 2019).

Although the presence of calcium regulatory cation channels namely cation sperm protein 1 (CatSper1) and transient receptor potential M8 (TRPM8) was reported on bovine and rat sperm, respectively (Chung et al., 2014), but the same is yet to be recognized on other domestic animals. Similarly, the involvement of these ion channels in sperm motility and capacitation are yet to be tested in any livestock species. The regulation of CatSper1 and TRPM8 mediated calcium signaling by CRISP proteins during sperm motility was described in rat sperm (Gibbs et al., 2011; Ernesto et al., 2015). However, the possible role of sperm motility quiescent factors reported earlier on these ion channels has not been studied in any species, including sheep and goat.

Mechanism of action of CRISP-1 protein in sperm de-capacitation

Although the exact mechanism involved with the CRISP-1-mediated inhibition of sperm capacitation is largely unknown, the previous studies have reported it as a potent de-capacitation factor. Mouse CRISP-1 was identified in the fraction of mouse epididymal plasma having de-capacitation activity (Nixon et al., 2006). Furthermore, addition of purified native CRISP-1 to rat sperm *in vitro* was able to inhibit manifestation of tail protein tyrosine phosphorylation, a robust marker of capacitation, and reduce the number of sperm undergoing acrosome reaction in response to cholesterol removal from the sperm plasma membrane (Roberts et al., 2003). Recombinant CRISP-2 CRISP domain was capable of inhibiting Ca^{2+} flow through RyR2 in a non-voltage-dependent manner and activated RyR1 opening in a weakly voltage-dependent manner (Gibbs et al., 2006). Since, CRISP-1 also inhibits sperm capacitation and protein tyrosine phosphorylaton and both of these processes require increase in intracellular $[Ca^{+2}]$, it is likely that specific cationic channel similar to CatSper, RyR2 or TRPM8 might be involved in this signaling pathway (Fig. 1); however, this needs to be validated.

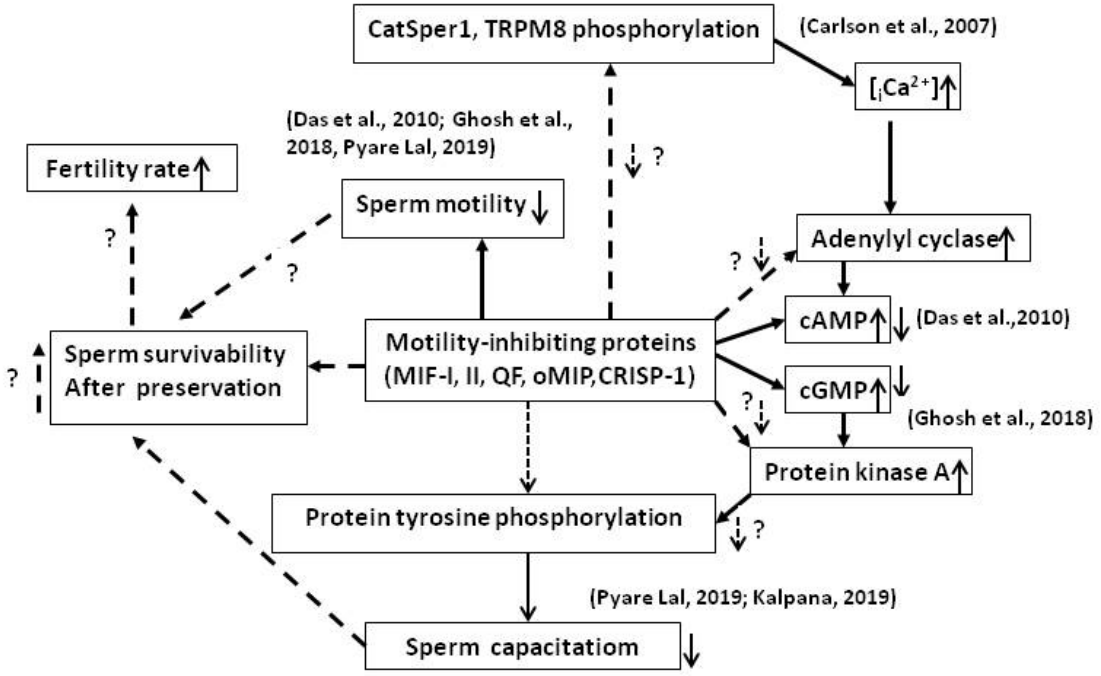

Fig. 1. Schematic outline of the known and unknown mechanism of actions of sperm-quiescent proteins

The mechanism of action of regucalcin (RGN)

The regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by RGN is achieved by controlling the activity of Ca^{2+} channels, Ca^{2+}-ATPase in the membrane of mitochondria and endoplasmic reticulum (Yamaguchi and Mori, 1989) and (Ca^{2+}–Mg^{2+})-ATPase in the plasma membrane (Yamaguchi et al., 1988; Takahashi and Yamaguchi, 1993). RGN also plays an important role in the regulation of Ca^{2+}-a dependent enzyme such as cAMP phosphodiesterase, which degrades cAMP regulating latter's level in cells (Yamaguchi and Tai, 1991; Yamaguchi and Kurota, 1997). RGN also regulates protein kinases and phosphatases, which in turn regulate the activity of other proteins (Kurota and Yamaguchi, 1997). RGN also inhibits NOS (Yamaguchi et al., 2003) and Ca^{2+}-dependent endonuclease activities; as anti-apoptotic actions (Yamaguchi and Tai, 1991). RGN probably inhibit apoptosis by up-regulating the expression of Akt-1 and Bcl-2 while down-regulating the expression of Caspase-3 (Nakagawa and Yamaguchi, 2005).

Purification of sperm-quiescent factors from cauda epididymal plasma

Dungdung and Majumder (2003) purified a 100 kDa sperm MIF from goat cauda sperm membrane to apparent homogeneity by Sepharose-6B affinity chromatography and DEAE-cellulose ion-exchange chromatography. Das et al. (2010) purified a 160 kDa MIF-II from goat EP by hydroxylapatite gel adsorption chromatography, DEAE-Cellulose ion-exchange chromatography and chromatofocusing. The MIF-II was purified to apparent homogeneity and the molecular weight estimated by Sephacryl S-300 gel filtration was 160 kDa. The MIF-II was reported to be a homodimer consisting of two 80 kDa sub-units and acts via cAMP-dependent pathway. Very recently, another sperm quiescent protein of 59 kDa was purified from goat EP (Ghosh et al., 2018). The purification was achieved by a combination of gel elution of active band, DEAE-cellulose ion exchange chromatography followed by molecular sieve HPLC. The protein was a monomer and thermostable. Recently, we showed that the sperm motility-inhibiting activity in ram CEP was present in the protein-rich fraction but not in the protein-free plasma fraction (Paul et al. 2018b). Further, in a recent study we could partially purify two motility-inhibitory proteins of approximate sizes 80 and 60 kDa from ram CEP (PyareLal, 2019). The 80 kDa protein exhibited de-capacitating activity on cauda sperm.

Production of recombinant sperm-quiescent proteins

The recombinant rat sperm epididymal DE protein (CRISP-1) produced in bacteria was demonstrated to have partial activity in inhibiting sperm-egg fusion. Although **c**arbohydrates have no role in DE-mediated gamete fusion, disulfide bridges are required for full biological activity of the protein (Ellerman et al., 2002). The putative mature forms of recombinant murine CRISP-1, 2, 3 and 4 were expressed in *E. coli* as thioredoxin-tagged fusion proteins. The peptides were expressed exclusively as insoluble forms in inclusion bodies even after adopting some measures to enhance proper folding and solubilization such as culture of cells to mid-log phase at 37°C in Luria broth (LB) containing 0.5% glucose to repress basal expression and the subsequent induction of expression with 0.1 mM IPTG in LB containing 0.5% glycerol at 20°C (Reddy et al., 2008). On the other hand, murine CRISP-3 protein when expressed in eukaryotic system involving HEK 293 cell line it was expressed as soluble, correctly folded and post-translationally-modified form which was effectively purified from the conditioned media. The purified recombinant protein demonstrated full biological activity as revealed by its interaction with known serum binding partner- alpha 1-B glycoprotein (Volpert et al., 2014). In contrary, Vadnais et al. (2008) reported very low yield of recombinant porcine CRISP-1 protein compared to CRISP-2 following protein expression by using pcDNA3.1/V5-HisA and PEGE cell line. Recent study in our lab showed that prokaryotic expression of ovine CRISP-1 Crisp peptide using pET32b vector in *E. coli* BL21 (DE3) resulted in insoluble expression of the peptide, which after solubilization and refolding demonstrated both motility inhibiting and de-capacitating activities on ram sperm (Jorasia et al., 2021).

Scope for application in semen preservation

The reversible inhibition of sperm motility and capacitation caused by CRISP-1 and other motility-inhibitory proteins of male genital tract renders these proteins as potential semen additives for temporal inhibition of sperm motility and capacitation during semen processing and preservation. The inhibition of sperm motility soon after dilution is beneficial in preserving the energy available in sperm both during liquid and cryopreservation. In addition, sperm quiescence also leads to lesser production of ROS and hence reduction in lipid peroxidation. Together, these may result in higher post-preservation sperm motility and membrane functionality. Although several attempts

were made for production of functionally active recombinant CRISP proteins and exploration of their possible mechanism of action; however, the possible usage of these proteins in semen preservation has not been explored yet in any species. Therefore, future research on semen cryopreservation should be focused on this aspect to develop an alternative tool for enhancing post-thaw quality and fertility rates of cryopreserved semen across species.

Conflict of interest

The authors declare absence of any conflict of interest between them

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