ANTIMICROBIAL PEPTIDES IN SEMEN EXTENDERS: 
A VALUABLE REPLACEMENT OPTION FOR ANTIBIOTICS 
IN CRYOPRESERVATION- A Prospective Review

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ABSTRACT

Semen extenders or diluents are added to semen before cryopreservation for maintaining its fertilizing ability even after post thaw. Various types of semen extenders are commercially available for porcine, boar, bull, equine and humans. Bacteriospermia in human and animal samples appears to be concentration-time dependent which might affect quality, quantity and longevity of post ejaculated semen in either neat or with extended states. Antibiotics play a significant role in semen extender which ensures long shelf-life to spermatozoa. Also, it protects the female reproductive tract by hindering the transmission of invading pathogens. Although the use of antibiotics in semen extender is monitored by many authorities of respective concerned government, a lot of guidelines and experiments ensuring a proper usage in cryopreservation is in need. The use of antibiotics in semen extenders also pose a threat of antibiotic-resistant bacterial strains in artificial insemination centers (AI) as well as in assisted reproductive technology (ART) laboratories. Development of multi-drug resistant bacteria has been found to be the primary concern when mixture of a wide range of conventional antibiotics is used in semen extenders. Several potent strategies and critical point analysis are of urgent need to hinder the

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microbial contamination and also to prevent antibiotic-resistant issues. In order to overcome these problems, low evoking resistant antimicrobial peptides (AMPs) have been used by researchers to replace conventional antibiotics. Antimicrobial peptides are positively charged polypeptides with a minimum of 100 amino acids. They possess high structural diversity and proved to be effective against extensive array of pathogens. These AMPs not only act as pathogen defenders but also exhibit immunomodulatory actions. Naturally male and female genital tract contains many antimicrobial peptides that could play not a suitable role against invading pathogens. This review discusses the different types of antimicrobial peptides that could be used in semen extenders during cryopreservation.

1 Introduction

Cryopreservation of semen is used routinely in many areas and fields including assisted reproduction, artificial insemination, military persons, in case of pre-radiation and chemotherapy treatments and so on (Schulze et al., 2017). It is treated as fertility insurance for the males undergoing vasectomy and storage of donor semen. Testicular semen samples collected from non-obstructive azoospermia patients were cryopreserved for later usage in in vitro fertilization (IVF) treatment cycles. Cryopreservation allows a single biopsy collected semen samples from testicular sperm aspiration (TESA) and percutaneous epididymal sperm aspiration (PESA) target patients to be used for many intra-cytoplasmic sperm injection (ICSI) cycles. In order to preserve the semen samples in a better way for a long time and to ensure the feasibility of vital biological functions at subzero temperatures, semen extenders are used. Many types of semen extenders like bovine serum albumin (BSA) based, lecithin based, plant components based, animal protein free and tris based are available commercially in the market for different organisms (Paulenz et al., 2000). Various antibiotics are added to semen extenders to remove the contaminants developed by bacteria and other microbes (Martin et al., 2010). Government agencies and directives are monitoring the antibiotic usage throughout the world.

Given the fact that animal food production related industries rely on cryopreserved semen for artificial insemination (AI), a huge amount of antibiotics are currently used in semen extenders by animal breeders during AI practices. Disadvantage of using a broad range of antibiotics in semen extender is the development of antibiotic resistance over the period of time in the microbe and host organism (Tiwari et al., 2013). In fact the emerging and rising problem of antimicrobial resistant is creating public health concern worldwide (Tiwari et al., 2013; World health organization, 2014; Roca et al., 2015). To overcome this particular problem during cryopreservation, recently researchers have been trying to find an appropriate replacement for antibiotics in semen extender for cryopreservation purposes. Many researchers believed that the valuable replacement option against a broad range of antibiotics in semen extender is the usage of antimicrobial peptides (AMPs) (Da Costa et al., 2013; Schulze et al., 2015).

Host defense peptides (HDPs) are the first line of defense against microbial invasions. These possess direct effect against various bacteria, virus and fungi (Schulze, et al., 2015). Moreover HDPs possess many biological functions like inhibition of lipopolysaccharide (LPS) induced inflammation, immunomodulation (Brown & Hancock, 2006), and anti-tumor activity, especially these also enhances wound healing. These useful biological properties combined with the raising problem of multiple antibiotic resistance development there is an immediate urgency to shift towards the use of AMPs as better replacement for wide range of conventional antibiotics as semen extenders (Hancock & Sahl, 2006; Giuliani et al., 2007; Baltzer & Brown, 2011; Seo et al., 2012). The functions of AMPs have been studied very well by several researchers especially during cryopreservation of boar semen samples for artificial breeding (Bussalleu et al., 2017). Many scientists studied widely about the use of AMPs in semen extenders for replacing conventional antibiotics to minimize the problem of antibiotic resistance development (Martin et al., 2010). Herein, we tried to review various AMPs from different sources and their use in semen extenders as a better replacement for conventional antibiotics for semen cryopreservation purposes.

2 Semen extenders and their types

Semen extenders or semen diluents constitute an aqueous solution used to increase the volume of the ejaculated semen and to maintain the functional characteristics of sperm until artificial insemination (AI) (Foote, 2002). Semen extenders have been classified by duration into three different groups like short term, long term and medium term extenders. Short term semen extenders are used in small distance transport and where the semen doses can frequently be made (Paulenz et al., 2000). Long-term semen extenders are used when the semen dose production area and insemination sites are too long. Long time semen
extenders advantages include: enables transport of semen to longer distances, can facilitate prediction of pregnancy outcome before insemination, and help in distributing the semen doses to different centers. In 1980’s, diluents were based on the glucose solutions without freezing the samples, and later the egg yolk and citrate buffers were used in semen extenders (Paulenz et al., 2000). The important innovation of semen extender research started in early 1960 with the addition of chelating agent like Ethylenediaminetetraacetic acid (EDTA) (Plisko, 1965), which are used to block the action of calcium that helps in capacitation and acrosome reaction during cryopreservation. The major components present in commercial semen extenders are glucose, sodium citrate, sodium bicarbonate, potassium chloride, EDTA, acetylcysteine, Heps, Tris, citrate, bovine serum albumin (BSA), cysteine, trehalose, polyvinyl alcohol (PVA), 3-(N-morpholino) propanesulfonic acid (MOPS) and glycerol. Semen extenders have also been classified into bovine serum albumin (BSA) based, lecithin based, soya bean based, skim milk based and tris based extenders (Westendorf et al., 1975; Gadea et al., 2003). The liquid diluents/ extenders are used in a way that should possess the following, provide nutrients and help to maintain the sperm cell metabolism, provide valid protection against the cold shock due to cryopreservation, could control the pH and osmotic pressure and the major concern is to control the development and growth of microorganism during cryopreservation (Gadea et al., 2003). Different types of semen extenders and the kind of antibiotic used by various researchers in the recent years has been tabulated in Table 1. Commercial availability of successful extenders with its antibiotic concentration for use in boar, equine, porcine and human has been tabulated in Table 2.

3 Importance of antibiotics in semen extenders

Microbial contamination occurs mainly during the collection process of the semen (Rillo et al., 1998). Antibiotics in semen extender are added to overcome the growth of contaminated bacteria which is promoted by the presence of glucose in the extenders. The temperature range of 15 to 16°C also promotes the growth of most common Gram negative bacteria in the ejaculate. The addition of antibiotics at an appropriate concentration is needed for sperm survival during cryopreservation and this enhances pregnancy outcomes (Althouse et al., 2000; Gadea et al., 2003). Penicillin and streptomycin were the first used antibiotics in semen extender for cryopreservation. Later, antibiotic cocktail including tylosin (50 μg), gentamicin (250 μg), spectinomycin (300 μg) and lincomycin (150 μg) was used first time in long term bull semen extenders. Most recently, antibiotics like cefiofur were also used in semen extenders and are being used, but there are no conclusive and evident results on resistance development (Gadea et al., 2003; Schulze et al., 2016).

4 Why should antibiotics be replaced in semen extender preparation?

Due to lack of hygienic standards in AI centers, the antibiotic usage is not regulated in many centers. To counteract the progress of antibiotic resistance in contaminant bacteria, there is a need of highly sophisticated hygienic standard management in AI centers and proper identification of hygienic critical control points (HCCP) (Schulze et al., 2017). Many alternates are there for antibiotics such as physical removal of bacteria during semen collection and processing time. Further alternatives are using single layer centrifugation, and to carry out the process in aseptic conditions. In very recent years, the use of antimicrobial peptides (AMPs) instead of antibiotics has been found to have great importance for the researchers as well in AI centers and cryobanks for better results (Dietrich et al., 2017). The disadvantage of using antibiotics and advantage of using antimicrobial peptides are represented in figure 1. The antibiotic resistances in semen extenders were discussed by Morrell in articles (Morrell & Wallgren, 2014; Morrell et al., 2016).

5 Antimicrobial peptides (AMPs) and their functions

Evolution has gifted every single organism with a wide variety of tools for survival. Evolution and natural selection are concurrent processes which helps human to develop innate mechanisms against microbial evolution. This scenario happens in microbes as well, making them more virulent. The shorter lifespan of microbes makes them accumulate more evolutionary change than humans, thus giving the microbes a winning edge (Jeralia & Porro, 2004).

AMPs are produced as first line of defence by human innate immunity system. For the first time, AMPs were initially isolated in 1980’s from varieties of insects and frogs (Zasloff, 1987). After that, a large number of AMPs were identified and are in use for various clinical and medical applications. In general, AMPs possess different functions that cover a broad array of activities. While identifying AMPs in the initial days, researchers pointed that AMPs will have only bactericidal activity, but later found that AMPs also posses antifungal (De Lucca & Walsh, 1999), antiviral (De Lucca & Walsh, 1999), antitumor (Papo & Shai, 2005), immunomodulatory activities (Mcphie & Hancock, 2005). Many researchers claimed surprisingly a single AMP can exhibit all of these activities (Mcphie & Hancock, 2005). β-defensins an antimicrobial peptide is the best example for immunomodulatory peptides which possess both innate and adaptive immune
response, and they reveal direct antimicrobial activity (Dietrich et al., 2017).

6 Uses of AMPs:

The use of AMPs in semen extender was first used with the boar semen ejaculate, and antibiotic resistance was not reported in that case (Paulenz et al., 2000). The use of cyclic hexapeptides and synthetic magainin derivatives was also investigated by many researchers to be used in semen extenders for preserving boar and other species ejaculates (Coelho et al., 2017). The AMPs were found with little/no on eukaryotic cells, and this is the major prerequisite for the application of AMPs in semen extenders. Proteolytic stability, thermodynamic stability, bacterial selectivity and sensitivity, made hexapeptides as the best replacement for antibiotics in semen extenders for long time storage of semen samples especially for humans (Hall-Stoodley et al., 2012).

Many higher species including humans possess innate immune defense mechanisms, of which AMPs were found to be necessary components. The biological activity of each AMPs varies as the primary and secondary structure differences exhibited by the peptides affect their functionalities (Hall-Stoodley et al., 2012).

This is the crucial step that has to be focused while selecting AMP for the replacement of antibiotics in semen extenders. Conversely, the cationic charge and amphipathicity of AMPs were found to be conserved characteristics which explain its selective action on bacterial membranes that are highly negatively charged lipid molecules. Lysozyme isolated from human saliva (130 amino acids), by Alexander Fleming in 1922 still stands as the widely used antimicrobial protein of human origin, (Fleming, 1922).

Many peptides have been found to be rich in particular amino acids especially phenylalanine, tryptophan or arginine. For this reason the antimicrobial motifs in natural proteins, acts as potent candidates for designing selective and effective AMPs in clinical and medical applications (Chan et al., 2006). Many alternate methods were there to replace antibiotics in semen extender like doing Single Layer Centrifugation (SLC), carried out with more strict aseptic conditions may be very helpful in reducing the bacterial contamination (Morrell & Wallgren, 2014), but this technique (SLC) has limitations too. The best alternative is to replace the antibiotics with AMPs in semen extenders. Many researchers have worked with different AMPs for substituting conventional antibiotics in semen extenders. Critical studies related to AMPs in semen extenders and their implications were tabulated in table 3.

Figure 1: Antimicrobial peptides in semen extender wards off the disadvantages associated with conventional antibiotics usage. 1- Conventional antibiotics in semen extender, 2- usage of antibiotics in semen extender leads to decreased semen quality, 3- usage of antibiotics results in evolution of antibiotic resistant bacteria, 4- replacement of conventional antibiotics in semen extenders with antimicrobial peptides makes development of antibiotic resistance a less possible event.
Table 1 List of different semen extenders with its antibiotics used by researchers

<table>
<thead>
<tr>
<th>Semen extender used</th>
<th>Remarks</th>
<th>Antibiotics used in semen extender</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS Citrate</td>
<td>Short term storage of Ram semen</td>
<td>20 µM Penicillamine/ 50 µg mL⁻¹ of gentamicin</td>
<td>Acharya et al., 2017</td>
</tr>
<tr>
<td>TRIS Milk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Many different extenders were used with</td>
<td>Boar semen/ lipoprotein fraction from ostrich egg yolk</td>
<td>50 µg mL⁻¹ of gentamicin is the major antibiotic used in all the different extenders</td>
<td>Dziekonska et al., 2017</td>
</tr>
<tr>
<td>supplementation of lipoprotein fraction from</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ostrich egg yolk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRIS citrate, Milk, Soybean with Ringer’s solution and</td>
<td>Cocktail semen preservation with mentioned semen extenders</td>
<td>70 µg mL⁻¹ of gentamicin and 20 µM Penicillamine</td>
<td>Schneider et al., 2017</td>
</tr>
<tr>
<td>Glucose solution 5%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boars semen extender with soyabean and lecithin</td>
<td>Boars semen to check the quality after addition of betaine in semen extender</td>
<td>Sperm parameters were maintained with betaine with addition of gentamicin antibiotics 70 µg mL⁻¹</td>
<td>Lugar et al., 2017</td>
</tr>
<tr>
<td>Human semen extenders (E4), TRIS extender with antioxidants</td>
<td>Human semen extenders supplemented with Tea Polyphenol-T. Arjuna Bark, antioxidant extender</td>
<td>Penicillin-streptomycin 50 µg mL⁻¹</td>
<td>Parameswari et al., 2017</td>
</tr>
<tr>
<td>Boar semen preservation, TRIS semen extender</td>
<td>Boar semen</td>
<td>Gentamicin varying concentration with median 220.37 mg/L</td>
<td>Schulze et al., 2017</td>
</tr>
<tr>
<td>Boar semen extenders, TRIS Semen extenders</td>
<td>Boar semen samples preservation</td>
<td>Replacement of antibiotics with cationic antimicrobial peptides</td>
<td>Schulze et al., 2016</td>
</tr>
<tr>
<td>Turkey, EK, Lake and Chicken semen extenders, TRIS semen extenders</td>
<td>Indian Red Jungle Fowl (<em>Gallus gallus marghi</em>)</td>
<td>Gentamicin/penicillin 50 µg mL⁻¹</td>
<td>Rakha et al., 2016</td>
</tr>
<tr>
<td>Two different egg yolk based semen extenders and soyabean lecithin based semen extender</td>
<td>Bull semen samples</td>
<td>1000000 IU penicillin, 100 mg streptomycin</td>
<td>Abdussamad et al., 2016</td>
</tr>
<tr>
<td>Boar semen extenders</td>
<td>Liquid semen storage of boar semen and effect of bacteriospermia</td>
<td>Streptomycin 50 µg mL⁻¹</td>
<td>Kuster &amp; Althouse, 2016</td>
</tr>
<tr>
<td>Egg yolk and soybean extenders</td>
<td>Evolution from egg yolk to soybean-based extenders</td>
<td>Gentamicin/penicillin 50 µg mL⁻¹</td>
<td>Layek et al., 2016</td>
</tr>
<tr>
<td>egg yolk and soya milk-based extenders</td>
<td>buffalo semen sample cryopreservation</td>
<td>Streptomycin 50 µg mL⁻¹</td>
<td>Chaudhari et al., 2015</td>
</tr>
<tr>
<td>Magnetized semen extenders containing BSA</td>
<td>Boar semen sample long time preservation</td>
<td>Gentamicin/penicillin 50 µg mL⁻¹</td>
<td>Lee &amp; Park, 2015</td>
</tr>
<tr>
<td>Modified Beltsville extender</td>
<td>Rooster post-thaw semen quality</td>
<td>Streptomycin 50 µg mL⁻¹</td>
<td>Amini et al., 2015</td>
</tr>
<tr>
<td>Four different semen extenders, TRIS, soyabean, Lecithin, Milk based</td>
<td>Human semen samples preservation</td>
<td>Gentamicin/penicillin/ streptomycin 50 µg mL⁻¹</td>
<td>Vickram et al., 2015</td>
</tr>
<tr>
<td>Twelve different semen extenders</td>
<td>Boar semen samples</td>
<td>Gentamicin/penicillin/ streptomycin 50 µg mL⁻¹</td>
<td>Akandi et al., 2015</td>
</tr>
<tr>
<td>Soybean lecithin-based semen extender</td>
<td>Ram semen samples</td>
<td>Streptomycin and different antioxidants 50 µg mL⁻¹</td>
<td>Sharafi et al., 2015</td>
</tr>
<tr>
<td>Soybean-Based Extenders</td>
<td>Friesian Bull semen samples</td>
<td>Gentamicin/penicillin/ streptomycin 50 µg mL⁻¹</td>
<td>Rehman et al., 2014</td>
</tr>
<tr>
<td>Different types of bovine and canine commercial available semen extenders, soyabean and lecithin semen extenders</td>
<td>Brown bear semen samples</td>
<td>Gentamicin 50 µg mL⁻¹</td>
<td>Gomes-Alves et al., 2014</td>
</tr>
<tr>
<td>plant-based soybean lecithin semen extenders</td>
<td>Goat semen sample preservation</td>
<td>Streptomycin and different antioxidants 62 µg mL⁻¹</td>
<td>Salmani et al., 2014</td>
</tr>
</tbody>
</table>
Antimicrobial peptides in semen extenders for cryopreservation

### Table 2 Commercial semen extenders and the antibiotics used

<table>
<thead>
<tr>
<th>Commercial successful semen extenders</th>
<th>Model for semen cryopreservation</th>
<th>Antibiotics used</th>
</tr>
</thead>
<tbody>
<tr>
<td>AndroPRO® Plus</td>
<td>synthetic long-term porcine semen extender</td>
<td>Ampicillin, Apramycin, Enrofloxacin 60 µg mL⁻¹</td>
</tr>
<tr>
<td>AndroMed®</td>
<td>Long-term Bull semen extender</td>
<td>Antibiotic cocktail includes Tylosin 50µg, Gentamicin 250µg, Spectinomycin 300 µg and Lincomycin 150 µg</td>
</tr>
<tr>
<td>Triladyl® &amp; Biladyl®</td>
<td>Long-term Bovine semen extender</td>
<td>Tylosin, Gentamicin, Spectinomycin, Lincomycin</td>
</tr>
<tr>
<td>EquiPRO CoolGuard</td>
<td>Long term Equine semen extender</td>
<td>Amikacin &amp; Penicillin</td>
</tr>
<tr>
<td>Kobidil+ semen extender</td>
<td>Short term semen extender for Boar semen samples</td>
<td>Gentamycin sulphate 200 mg/L</td>
</tr>
<tr>
<td>Beltsville semen extender</td>
<td>Short term semen extender</td>
<td>Gentamycin sulphate 250 mg/L</td>
</tr>
<tr>
<td>Acromax semen extender</td>
<td>Long term Boar semen extender</td>
<td>Lincomycin 13.3 mg/L and spectinomycin 26.6 mg/L</td>
</tr>
</tbody>
</table>

### Table 3 Important studies related to antimicrobial peptides (AMP’s) in semen extender and cryopreservation:

<table>
<thead>
<tr>
<th>Theme of the study</th>
<th>Implications</th>
<th>Conclusion</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Can antimicrobial peptides be an alternative for antibiotics in semen extenders?</td>
<td>There is a possibility of multi-bacterial resistant in offspring via artificial insemination (AI). Enormous steps have been taken to employ AMPs to substitute conventional antibiotics in semen extenders</td>
<td>Magainin and cyclic hexapeptides, antimicrobial peptide derivate can be used in boar semen extenders to replace the existing conventional antibiotics</td>
<td>Schulze et al., 2016</td>
</tr>
<tr>
<td>Can cationic synthetic peptides be used in semen extender for cryopreservation?</td>
<td>The antibacterial activity of two types of cyclic synthetic peptides (c-WWW, c-WFW) and one helical peptide called magainin II against Gram-positive and Gram-negative bacteria</td>
<td>These antimicrobial peptides, not a complete alternative to conventional antibiotics and still to avoid multi-strain resistant. Cyclic and helical peptides may be the possible alternative for antibiotics in boar semen preservation</td>
<td>Speck et al., 2014</td>
</tr>
<tr>
<td>hCAP-18 (human cationic antimicrobial peptides produced from epithelium of epididymis and contributes counter immunity against female reproductive tract bacteria)</td>
<td>By immunohistochemistry, hCAP-18 was able to detect in the epididymis and not in the testis. hCAP-18 was found to have antibacterial activity against broad spectrum of bacteria</td>
<td>This hCAP-18 antimicrobial peptide can probably use in many medical applications including cryopreservation</td>
<td>Malm et al., 2000</td>
</tr>
<tr>
<td>Processing of hCAP-18 to ALL-38 antimicrobial peptides in female reproductive tract</td>
<td>Seminal plasma antimicrobial peptide hCAP-18 was further processed to generate a 38 amino acid antimicrobial peptide called ALL-38 by gastricsin</td>
<td>ALL-38 was found to possess antibacterial activity against a wide range of bacteria. Further, it protects from infection after sexual intercourse</td>
<td>Sorensen et al., 2003</td>
</tr>
<tr>
<td>Is GL13K - an antimicrobial peptide are effective against Pseudomonas aeruginosa</td>
<td>GL13NH2 was not showing any bactericidal activity, later it was introduced with three lysine residues and converted into an active antimicrobial peptide called GL13K</td>
<td>GL13K - an antimicrobial peptide found to be very active against Pseudomonas aeruginosa</td>
<td>Hirt &amp; Gorr, 2013</td>
</tr>
<tr>
<td>Is Semenogelin derived antimicrobial peptides protects spermatozoa?</td>
<td>Cationic antimicrobial peptide was derived from Semenogelin protein</td>
<td>It poses number of applications including antibacterial and antiviral activity could be used in semen extenders</td>
<td>Bourgeon et al., 2004</td>
</tr>
</tbody>
</table>
7 Cationic antimicrobial peptides (CAMPs):

Cationic antimicrobial peptides exert direct antibacterial and antifungal activity. It also possesses various biological functions like inhibition of lipopolysaccharide related inflammatory, immunomodulation and immunosuppressant activity (Mulder et al., 2013). Along with the above mentioned functions, antibiotic resistance evolution in using conventional antibiotics makes cationic antimicrobial peptides a better replacement for conventional antibiotics in semen extenders for cryopreservation (Zasloff, 2016). It was found that 80-85% of the infections were with biofilm etiology in the United States (Fux et al., 2003; Davies et al., 2006). Extracellular matrix along with low growth rates associated with biofilms necessitates a 10 to 1000 fold higher concentrations of antibiotics to curtail bacterial growth (Hawkey, 2008). Antibiotic resistance among medically important microbes is on rising, and this also adds to the existing challenge (Boucher et al., 2009).

CAMPs were seen as a potential alternates to deal with the problem of antibiotic resistance development and biofilms (Schulze et al., 2014; Speck et al., 2014). In comparison to traditional antibiotics, CAMPs lower the probability of microbial resistance. Antigenic sites on bacterial membranes change as a result of antibiotic resistance evolution and less specific activity expressed by CAMPs on bacterial membranes makes development of resistance against CAMPs in bacteria impossible (Yengu et al., 2004). This circumvents the restriction of many traditional antibiotics that require bacterial growth (Ernst & Peschel, 2007). Bradshaw discussed issues for potential clinical use of CAMPs (Bradshaw, 2003). The conception that CAMP may not possess broad-spectrum activity was found wrong. Ambicin, gramicidin S are found to be the best examples for broad spectrum activity of antimicrobial peptides. Researchers showed that 13-amino-acid peptide GL13K exhibited low toxicity and broad antibacterial activity against mammalian cells and planktonic bacteria, respectively (Abdolhosseini et al., 2012). GLK13K is effective against *Pseudomonas aeruginosa* biofilms formed on pegs of a Calgary device, associated with a static biofilm system (Ceri et al., 1999). Overall, Ceri results showed that a 99.9% reduction in bacterial cell numbers in a 4-h treatment with 100 µg/mL concentration of this GLK13K. These results support the usage of GLK13K in human and animal semen preservation in extenders instead of standard antibiotics. Biofilm growth can be inhibited/controlled by employing mechanical forces, which is not possible in cryopreservation process. Hence the use of CAMPs is greatly realized. More specifically, CAMPs are highly sensitive to physiological salt concentrations. In turn, this high sensitivity limits the utilization of CAMPs in biological fluids (Goldman & Widom, 1997).

Two different synthetic cationic antimicrobial peptides like c-WWW and c-WFW which belongs to cyclic hexapeptide group were investigated with boar semen extender for cryopreservation (Speck et al., 2014). Speck used a synthetic helical amide analog, magainin II (MK5E), for boar semen sample preservation invitro. This was found to be effective against gram positive and gram negative micro flora found in boar semen. In this study boar semen samples were initially maintained only with Beltsville Thawing Solution (BTS) with 250 µg/mL gentamicin which is treated as a control group. Boar semen samples were then grouped into three like BTS+c-WWW, BTS+c-WFW, and BTS+MK5E. Minimum inhibitory concentration (MIC) for all the AMPs was done using broth microdilution technique (Speck et al., 2014). The author concludes that both c-WWW and c-WFW was showing activity against almost all bacteria in liquid preserved boar semen samples. Speck et al finds that antimicrobial peptides in semen extenders would be a better replacement for conventional antibiotics and it will lead to limit the selection of multi-resistant strains (Speck et al., 2014).

8 Human parotid secretory protein associated AMPs in semen extender:

Human parotid secretory protein (PSP) which is found to have structural similarity with bactericidal, and two potential proteins, permeability-increasing protein, and lipopolysaccharide binding protein, could be possibly used in semen extender (Abdolhosseini et al., 2012). These two peptides were found as potential antimicrobial peptides and the sequence information was identified from PSP. This could be more relevant replacement option, as it is found to have structural similarity with lipopolysaccharide binding protein, as prostasomes were rich in lipid content and played a significant role in motility (Schiesssel, 2003). Helmut Hirt derived a 13-amino acid peptide called GL13K, from human PSP. Initially, the antimicrobial and various functions of this particular protein was attributed to amino acid residues 141 to 153 of PSP (GL13NHz) (Hirt & Gorr, 2013; Chen et al., 2014). This peptide was found to have the property of aggregating both Gram-negative and Gram-positive bacteria and capable of binding with LPS, but many times the peptide lacks bactericidal activity.

GL13K derived its name from the replacement of charged aminoacids in 5th and 11th position by lysine (K) residues. This replacement event confers bactericidal activity to the AMP but prevents it from acting as a bacterium agglutinating agent. Researchers found that this GL13K was also active against bacteria in biofilm communities as well as monospecies (Bingle & Gorr, 2004). GL13K was reported to significantly reduce the cell count in biofilms grown either under aerobic or anaerobic conditions. So, as per these results, GL13K could be the source
9 Male and female genital tract derived AMPs

Mammalian organs synthesized several types of antimicrobial peptides with multi-functionalities. Amongst them, the most focused areas are the epithelial layers of skin, and entire digestive, respiratory and reproductive tracts (Zhang et al., 2000; Gallo et al., 2002; Boman, 2003). Compared to any other tract, genital tract of human males and females produces enormous endogenous proteins and peptides which have anti-infectious characteristics. In humans, the most studied cationic AMP’s are β-defensins and cathelicidins (Frew & Stock, 2011). Defensins contain a series of cysteine molecules that are considered as part of immune defense system, in the genital tract. Many β-defensins are synthesized mainly in the epithelial cells of epididymis during sperm maturation, later released into the lumen, finally detected on the surface of sperm membrane (Com et al., 2003). This attachment refers to not only the function of antimicrobial activity but also in the proper function of fertilization. In a study, defensin depleted mutant spermatozoa was found with impaired motility, morphology, a destabilized microtubule structure and finally results in infertility (Zanich et al., 2003; Zhou et al., 2004).

The precursor protein hCAP18, which is considered to be the only representative for antimicrobial activity from the human male genital tract, was predominantly synthesized in the epididymis (Hammami-Hamza et al., 2001). CAP18 molecules have also been identified in head, tail and neck region of the sperm cell as well in seminal plasma. The majority of hCAP18 proteins were associated with proteasomes. The primary functions of the prostasomes were sperm cell-proteasome interaction, enhancing sperm cell rapid motility, induction of sperm cell capacitation, induction of acrosome reaction and finally the fertilization (Gombart et al., 2005). So, once the sperm cells are deposited in the female reproductive tract, though the cell may be matured to fuse with the ova, it requires many series of reactions in order to fertilize. Prostasomes fulfilled these series of steps in female reproductive tract. Prostasomes are rich in cholesterol-phospholipid ratio. Therefore it is opined that during fusion event of prostasomes with spermatozoa, the protein like hCAP18 which is present on the surface will also be transferred along with cholesterol to the sperm cell membrane and here the transport is mediated by the sperm into the uterus. Once the proteolytic processing of hCAP18 was over, the AMP LL-37 get released from the precursor protein. When it was releasing, the disruptive nature of these peptides were protected by the cholesterol enrichment in the sperm membrane (Yarbrough et al., 2014). This particular antimicrobial peptide can also be isolated from the male genital tract and can be processed to use in the semen extender for cryopreservation of sperm cells against contamination produced by many bacteria as well to maintain the integrity of sperms (Ciornelci et al., 2003). Many antimicrobial peptides were synthesized by sperm cells and seminal plasma and transferred to the female reproductive tract, but female reproductive tract itself is also capable of synthesizing some antimicrobial peptides to evade the pathogens and immunomodulatory functions. Lipophilin, an antimicrobial peptide derived from female reproductive tract (FRT) showed broad spectrum activity. This type of peptide can also be used in semen extender during cryopreservation (Dcruz et al., 1995).

10 Development of AMPs resistant pathogens:

There are some bacteria which are resistant to even AMPs through many mechanisms. The major mechanism of getting resistance is through the selective production of bacterial proteases (Hashemi et al., 2017). After the penetration, peptides are very prone for transportation outside the cell through the energy-dependent mechanism and finally resided at the cytoplasmic membrane of bacteria. In addition to this, the formation of extracellular polymers as a biofilm further makes the microbes insensitive to AMP effects. A study was conducted with pexiganan and reported that resistance of AMPs was predicted when some bacterial population is regularly exposed with AMPs (Perron et al., 2006). These types of reports put many questions on researchers and on ART professionals to include AMPs in semen extender and its practices, but there is no timescale limit to predict the development of AMP resistant pathogens (Van Nierop et al., 2008).

11 Plant-derived AMPs:

AMPs can also be isolated from different plant species, within those different parts including flowers, leaves, fruits, roots, tubers, and especially seeds (Pelegrini et al., 2008; Benko-Iseppon et al., 2010; Silva et al., 2011; Astafieva et al., 2013; Singha et al., 2016). Plant-derived AMPs have many characteristic features in common that includes, low molecular mass, amphipathic properties, net positive charge at physiological pH, having more repeats of cysteine residues connected in pairs forming disulfide bridges, resulting in stable peptides (Astafieva et al., 2013). These types of AMPs have an explicit interaction with particular cellular membranes and active against certain pathogens, with these properties, AMPs from capsicum have some role in preventing the infections of various pathogens evading. Different fractions of enriched peptides have been isolated from capsicum including PEF1, PEF2, and PEF3 that exhibited a potent antifungal activity against different yeasts (Dias et al., 2013). The N-terminal sequence of these peptides was shown with highest similarity level with serine protease inhibitors (SPI) when analyzed through proper database systems. Taveira et al. identified a protein that is equal to the compound thionin from capsicum that has severe...
antifungal and antibacterial activity, and this shows effective therapeutic reactions against Candida species (Taveira et al., 2014; Taveira et al., 2016). In this research, the author concluded that antimicrobial peptides isolated and purified from capsicum demonstrated in vitro inhibitory activity against a range of fungi and sometimes with bacteria of agronomic importance.

Conclusions

Antibiotics are said to be the mandatory component while preparing semen extender for cryopreservation. High level increase in the resistance rate against conventional antibiotics in semen extenders demands an alternative for antibiotics in semen extender. In this review, we suggest that the only best alternative to traditional antibiotics is antimicrobial peptides in semen extender. Especially, the antimicrobial peptides derived from male and female genital tract could be the best peptides that render the antibiotic functions in semen extender.

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Conflicts of interest:

All authors declare that there exist no commercial or financial relationships that could in any way lead to a potential conflict of interest.

References


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Antimicrobial peptides in semen extenders for cryopreservation

John Doe

Abstract

Introduction

Methods

Results

Discussion

Conclusion

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