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Human Contraceptives: Current Status, Sperm Antigen Inhibitors and an Insight into PCSK4

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Abstract

Rapid growth of global human population has been implicating to food shortage, social problems and environmental degradation. Contraceptive devices have long been applied as a major method to reduce natality. Current application of this technology relies upon hormonal administration, condom, withdrawal and recently hormonal vaccino-contraceptive. Discoveries of antisperm proteins have been directing current researches toward developments of antisperm antibody (ASA) contraceptions. Actions of ASA are targeting antigens either on the head or on the tail of sperm. Antibodies targeting head antigens aimed at blocking gamete fusion, ZP penetration and/or acrosome reaction. Molecules working on sperm tails are aimed to block sperm motility or energy production. PCSK4 is one sperm antigen firstly expressed on the human sperm acrosome during its initial development on the round spermatid and retains on the acrosome until sperm is matured. It is known to contribute to the post-capacitatorial hyperactivation of sperm essential for zona penetration. Rat models injected with rabbit-anti human PCSK4 developed incompetent sperm and allowance of these male rats to fertile female rats resulted significant reduction of conception rate. Apart from antibody, synthetic inhibitors of PCSK4 have also been developed. Future developments of ASA contraception are discussed.

Keywords: contraception, ASA, PCSK4, synthetic peptide

1. Introduction

The world population had grown enormously during the period of 40 years since 1960 to 2000, doubled from 3 to 6.1 billion peoples [1–3]. In 2010, it became 6.9 billion and was estimated to reach 9.3 billion in 2050 or to increase by 35% from that in 2010 [2, 4, 5]. The growth seems slower after 2000 and the global population growth in the next decades until 2050 was estimated to be slower by three times compared to those during the period of 1960–2000 [1–5].

Despite a slower growth, negative impacts of high human population on environment as well as on human have been remaining high. In one hand, the need to provide more foods to feed more peoples has triggered environment degradation through the use of chemical fertilisers, practices of intensified farming and farming mechanisations, to boost the amount of yields and frequency of harvests [6]. Moreover, increased land uses for housings and agricultures have led further environment degradation in tropical regions through deforestation [7]. On the other hand, population growth along with poverty was reported to link to further

serious social problems and terrorism in some communities [8]. Therefore, efforts to further effectively suppress the world population growth needs updated strategies that fits with current issues.

A method that globally has been used as a measure to control birth is contraceptive practice. Contraception includes all means to prevent pregnancy resulted from an intercourse. Contraceptions currently used widely among women are hormonal (pills, blotch and injectable forms), natural (near ovulation abstinence and before ejaculation withdrawal), intravaginal or intrauterine (vaginal rings, diaphragm, spermicides or intrauterine devices) and sterilisation, while popular men contraceptives are withdrawal method (*coitus interruptus*), condom and vasectomy [9].

More than 6.5 billion of global population have used at least one contraception method, with the increase of new users are of 75 million couples annually [3]. The largest number of contraceptive users are women while male contraceptions are practiced only by 30% of couples worldwide [3, 9–11]. Existing women contraceptive methods however, pose discomforts as well as adverse effects. For example, pills have to be taken daily. Interruptions due to some practical reasons such as forgets have been commonly heard and would risk its infectivity. Moreover, the hormonal pill contraception in selective cases might risk occurrence of venous thrombosis and breast cancer [12]. Intra uterine devices are thought to be effective for long term usage but some users reported side effects such as bleeding, discomforts, infection and pain [13].

Likewise, current male contraceptives utilisation have been known to cause several side effects which might have attributed to the low utilisation among men. Condom uses cause sexual discomforts and low rate failures have been reported (5–15%) [3]. In addition, the application of steroid male contraceptive such as testosterone enanthate (TE) was reported to cause obesity, testicular atrophy in 25% of cases and reduced level of high density lipoprotein (HDL) in 10% of cases [14].

Vasectomy, the *vas deferens* ligation, is by far the most effective and economical male contraceptive method and as many as 7% (40–60 million) of worldwide couples practice this method [12]. One advantage of vasectomy is, that it can be reversed by *vasovasostomy* surgery to restore fertility with the rate of pregnancy after the reversal was between of 30–60% but, the rate decreased when the *vasovasostomy* was performed 8 years after vasectomy was conducted [3]. Fertility restoration and pregnancy rate after vasectomy reversal are influenced by, among others, the interval of vasectomy to reversal surgery, the presence of azoospermia, presence of sperm granuloma, the age of female partner and the same female partner [15].

Some studies reported the association of vasectomy and prostatic cancers but, studies claimed as such were mainly conducted before year 2000, while more recent studies indicated the opposites [16]. A cohort study involved 2 million samples of Danish men, reported only weak statistical association between prostatic cancers and vasectomy [17]. Likewise, a systematic review of 53 epidemiological studies with total samples of more than 2 millions men indicated that the role of vasectomy in the development of prostatic cancers was weak if any, and further analysis after controlling potential bias indicated that the association did not exist, statistically [16]. Other population based study reported no association between vasectomy and prostatic cancer [18]. Besides these unclear statistical associations, there have not been biological evidence supporting the causal role of vasectomy to prostatic cancers [16]. These indicate that being vasectomised are safe from risk of contracting prostatic cancers. However, vasectomised men experienced post operational discomforts such as pain, infection, scrotal area swelling and granulomatous inflammation of *vas deferens* [3].

Those limitations of existing contraceptive methods indicate the need to develop new procedures. The latest should be effective in preventing pregnancy but ideally

acting specifically to reproductive organs, easy in application, eliminating side effects, and reversible. The aim of this chapter is to describe the current knowledge in regard to antisperm antibody (ASA) contraception and the characteristics of proprotein convertase subtilisin/kexin type 4 (PCSK4) as an ASA contraceptive candidate as well as to shed light into the direction of future ASA developmental researches.

2. Novel immune-contraceptive methods and the discovery of ASA

With the continued human population growth, its consequential problems and limitation of present contraceptive approaches, alternatives methods of contraception have been reported and immune-contraceptive modes has become one popular theme. Immuno-contraception is a mean of contraception conducted by administering immunogenic substance into human body to induce antibody formation against particular antigens involved in the conception process [19]. Immuno-contraceptive agents target at least one of three reproductive physiology pathways i.e. preventing gamete production (target antigens: FSH, LH, GnRH), stopping embryonic development (target antigen: hCG), or inhibiting gamete function (target antigens: Zona Pellucida, spermatozoa) [9].

Vaccine candidates to prevent gamete production have been studied. Heterologous ovine LH based vaccine was reported to cause muscle wastage, reduced testicular weight and alopecia in monkey [20]. Vaccines against LH were known to elicit steroidal side effect and hormonal depression in humans therefore, cannot be used for the species [21]. Vaccine against FSH in men inhibited spermatogenesis to oligospermia but not azospermia, thus only partially affected fertility [21]. A vaccine against GnRH has been proven to reduce serum testosterone to castration level and has undergone phase II clinical trial to treat prostate carcinoma [22]. However, its use for contraception in humans is currently not practical due to feared side effects, such as loss of body hair, atrophy of prostate and impotency after chronic exposure [21, 23]. In contrast, clinical trials on hCG based vaccine have been conducted in women from several countries including India, Brazil, Finland, Sweden and Chile under the International Committee on Contraception Research of Population Council, New York. These trials indicated that the vaccine has no apparent side effects [24]. The vaccine has prepared to enter a further phase of clinical trial in India [24].

Inhibition of gamete function, such as debilitating the function of sperm using ASA, has also been seen as one promising alternative of immuno-contraceptive approach [21, 25–27]. ASA were discovered in 2–30% of either infertile men or in their women partners and in 70% of men following vasectomy [21, 25, 28, 29]. ASA might become a potential method of immuno-contraceptive in mammals, provided that they fulfil a few criteria: (1) Antigens are expressed only in sperm, therefore the risk of side effect of cross reaction with other somatic cells will be omitted. (2) Antigens are expressed on the outer surface of sperm cell membrane, making them highly exposed to antibody recognition. (3) Sperm antigens play roles in fertilisation process so that the inactivation will impair sperm fertilizability [30].

It has been reported that ASA or other sperm antigen inhibitors could influence fertilisation process through at least one of four ways: (1) blocking gamete fusion, (2) preventing sperm-zona pellucida binding and penetration, (3) inhibiting acrosome reaction or (4) immobilising sperm [31–48].

2.1 ASA that block gamete fusion

Zona-free hamster-egg system was used for sperm-egg fusion which is an important step of fertilisation process [39]. Using this system, several mammalian

sperm antigens have been studied for their role in sperm-oocyte membrane fusion including acrosomal protein equatorin (EQT), A disintegrin and metalloproteases (ADAMs) family proteins, DE, sperm acrosomal membrane-associated protein 32 (SAMP32), SAMP14, cluster of differentiation 46 (CD46), human equatorial segment protein (hESP) and Izumo [31–39].

The EQT is found in various mammalian sperm, including human [40]. Equatorin protein is first detected on the budding acrosomal membrane of round spermatids, retaining on acrosome during its remodelling in elongating spermatids and translocating to the equatorial region of acrosome during the acrosome reaction [40, 41]. Anti-equatorin antibody was reported to block sperm–oocyte fusion *in-vitro* [37]. In other *in-vivo* study, antibody-containing and control solutions were injected directly into the right and left oviductal ampullae, respectively. The results revealed that the rates of pregnancy in mice injected with antibody-antieuatorin-containing solution were significantly lower than that in the control group [37].

The ADAMs (also known as fertilin β) were believed to play some role in fertilisation of mammalian gametes [31, 42]. Sperm from mice lacking ADAM2 were incapable of binding to egg membrane, migrating inside female reproductive tract and binding to the zona pellucida [43]. In human, many ADAMs proteins are expressed in many different organs, while many other ADAM genes presents only as pseudogenes [42]. However, only three human ADAMs (ADAMs 2, 20 and 30) are specifically expressed in testicular tissues. Courtesy of Human Protein Atlas, www.proteinatlas.org [49]. ADAM2 are expressed in abundant in early and round spermatid, ADAM20 presents in abundant in late and elongated spermatids and ADAM 30 are expressed in cells along seminiferous duct. Courtesy of Human Protein Atlas, www.proteinatlas.org [49]. However, ADAM2 and ADAM20 are not detected either in the human sperm [44] or in ovarian tissue. Courtesy of Human Protein Atlas, www.proteinatlas.org [49]. These imply that these two human ADAMs could be important in sperm maturation but might not be important in human fertilisation process. Further, although aforementioned mice study indicated the role of ADAMs in gamete fusion, the role of human ADAM30 in fertilisation process remains unclear.

Epididymal glycoprotein DE (37 kDa) of rat is secreted by the epithelium of the proximal epididymis and attached to the head of spermatozoa during its transit in the epididymis. It participates in the gamete fusion process by binding its ligand on the ovum surface [33]. Immunisation of male rats with DE induced specific antibodies and produced a significant reduction in the animal fertility until as low as 0–33% as shown by an *in-vitro* study. The antibody did not interfere with the synthesis or secretion of DE, with its attachment to the sperm membrane, or with changes in sperm motility, viability, or ability to undergo capacitation and acrosome reaction but, antibody against DE debilitated sperm ability to fuse with zona free-egg [33].

The SAMP32, also called sperm acrosome-associated 1 (SPACA1), was expressed in the inner membrane of equatorial segment of human sperm acrosome and other acrosomal segments [34, 45]. An anti-rSAMP32 was demonstrated to block fusion of capacitated human sperm with zona-free hamster eggs *in-vitro* [34]. A study to monitor the outcomes of *in-vitro* fertilisation (IVF) confirmed that the rate of zygotes developed into blastocysts were much lower when the sperms weakly expressed SAMP32 on their acrosome, than sperms with high expression of SAMP32 [45]. These studies indicate that SAMP32 could prevent conception through at least one of two mechanisms i.e. inhibiting gamete fusion or interfering zygote development.

Another member of SAMPs family, SAMP14 was shown to be specifically expressed in the testis. The protein is localised on outer and inner acrosomal membranes and in the acrosomal matrix of human sperm. However, it retains on the inner acrosomal membrane after the acrosome reaction. SAMP14 might have a

role in gamete interaction, as antibodies anti-recombinant SAMP14 inhibited the binding and the fusion of human sperm to zona free hamster eggs *in-vitro* [35].

Human CD46 is a protein involved in immune response against external antigenic exposure [40]. Human CD46 or Membrane Cofactor Protein (MCP) are also known to anchor membrane of sperm and involved in fertilisation process. Antibodies against MCP significantly inhibited human sperm binding to hamster oocytes, *in-vitro* [36].

The other family of MCP, the CD52 sperm antigen has been detected on the mature sperm and seminal plasma; Antibody anti-CD52 showed sperm immobilisation properties *in-vitro*. As anti-CD52 is reported to cross react with CD46, CD55 and CD59 cofactor proteins [46] and because substances are expressed in many somatic tissues as humoral immunity agents, their use as contraceptive agent could compromise immune system.

hESP is known to localise to the equatorial segment of human sperm. ESP first appears in the early phase of acrosomal biogenesis in round spermatids, persists during acrosomal maturation and isolated to the edge of the mature acrosome [47]. Antisera to recombinant human ESP inhibited both binding of oolemma and fusion of human sperm in the hamster egg penetration assay. ESP immunoreacted with 27% of 15 antisperm antibody (ASA)-positive serum samples from infertile male patients and 40% of 5 ASA-positive female sera indicating the possible role of ESP in some cases of infertility [38].

Both mouse and human Izumo proteins are detectable on sperm surface only after the acrosome reaction. Thus, it was suggested that Izumo is hidden under plasma membrane and exposed only after the acrosome reaction occurs. When an antibody anti-human Izumo was added to the mixture of mouse sperm and hamster egg, no fusion was observed, whereas the fusion was observed in the control assay [39]. Izumo1 was hypothesized to act at a molecule designated as Juno, as its receptor on mouse eggs and in other mammals [48].

2.2 ASA that prevent sperm-zona pellucida binding and penetration

A notable sperm antigen that acts in sperm-zona pellucida interaction in mammals is fertilisation antigen-1 (FA-1) [50]. Immunofluorescent reactivity of FA-1 was detected in acrosome region of human sperm [51]. This protein is known to react strongly with 55 kDa Zona Pellucida protein-3 (ZP3) [52]. Zona pellucida pre-incubated with human sperm FA-1 failed to bind to sperm, indicated that FA-1 blocked sperm binding to zona pellucida. Similar blockage was also observed when the antibody against FA-1 was pre-incubated with sperm before insemination, indicating that the FA-1 is localised on the sperm [50].

In an *in-vivo* study, female mice injected intradermally with a sperm-specific FA-1 DNA vaccine caused a long-term systemic and local immunity resulting in anti-fertility effects. The effects were further enhanced when the vaccine was mixed with YLP12 DNA vaccine and oligodeoxynucleotide (ODN) [53]. Further study suggested that almost half of infertile women studied had circulating antibodies against human FA-1 antigen and YLP12 peptide sequence [54]. The development of FA-1 based for male contraceptive vaccine warrants further studies.

2.3. ASA that inhibit acrosome reaction (YLP-12 peptide)

Most of mammalian sperm are incapable of fertilising eggs when ejaculated and fertilisation occurs only after an exocytotic process called the acrosome reaction [39]. The halts of acrosome reaction thus, might potentially debilitate the competence of sperm to fertilise egg.

Two sperm antigens have been studied for this property and for their potential to base a development of contraceptive agents; YLP12 and testis specific antigen-1 (TSA-1). A dodecamer sequence designated YLP12 is a peptide sequence that have been identified to specifically localise on the acrosome and tail of spermatozoa [55]. It is known to recognise ZP3 component of human ZP proteins, to involve in sperm-ZP binding and the antibodies against synthetic 12-mer peptide based on YLP12 sequence was reported to specifically inhibit human sperm-ZP binding [56]. The antibody anti-YLP12, in other study, was reported to show a concentration-dependent inhibition of acrosome reaction but did not affect the sperm motility [57]. Immunisation of murine model with synthetic YLP12 produced antibodies affected fertility by reducing sperm capacitation, acrosome reaction and sperm-oocyte binding in an *in-vitro* assay but, immunised murine remained fertile and were capable of delivering the equal number of pups compared to control [55]. In contrast, other study reported that a sperm-specific YLP12 DNA vaccine injected intradermally in female mice caused anti fertility effects [53].

A study reported that TSA-1 is localised to the regions of acrosome, equatorial, mid-piece and tail of human sperm. An *in-vitro* test discovered that TSA-1 concentration-dependently inhibits human sperm acrosome reaction [58].

2.4 Various ASA that immobilise sperm

Sperm tail is widely known as an organelle of motility. Proteomic study showed that proteins extracted from tail fraction of sperm can be classified according to their functions into two main groups: proteins related to metabolism and energy production from endogenous sources, and those related to tail structure and motility [59]. These groups however, are at the end function together to support sperm motility.

Epididymal protease inhibitor (Eppin) is expressed in the testis and epididymis tissue and on the acrosome and tail of human sperm [60–62]. An *in-vitro* assay showed that the monkey anti-eppin antibodies decreased the progressive motility of human spermatozoa in terms of distance travelled and speed [62]. Other study suggested that blockade of Eppin epitope by anti-eppin antibody would halt the acrosome reaction through reduction of ionophore-induced calcium influx [60]. Further pre-clinical trial was performed *in-vivo* in non-human primates and showed that Eppin immunised *Macaca radiata* developed high titers to Eppin (78%) and all of these immune-converted monkeys were sterile [63].

Heparin-binding serpin, protein C inhibitor (PCI), is a nonspecific serpin that inactivates many plasmatic and extravascular serine proteases. Mutant male mice lacking PCI gene are infertile but apparently healthy. Histologic examination showed that Sertoli cells and their barrier were destroyed. The resulting sperm are malformed, lack of tail and deformed head and immobilised, similar to those seen in some cases of men infertility [64], thus the effect of PCI on testicle tissues is apparently cytotoxic. In *in-vivo* fertilisation experiments, only 0.5% (n = 416) eggs are fertilised by sperm of mutant male compared to the rate of 92% (n = 415) of eggs develop into blastocysts when intact mice is used [64].

The 80 kDa human sperm antigen (HAS) is known to present on the head of the human and rat spermatozoa, in the testes and epididymis but not in other somatic tissues. Active immunisation of male and female rats with 80 kDa HSA caused infertility in all the immunised animals [65]. Further, active immunisation of male rabbits, rat and marmosets with synthetic peptide-1 of HSA induced reversible infertility in 100, 100% and 6 of 7 of the respective animals [66]. It strongly indicates that antibodies anti-HSA have potential as immuno-contraceptive agents. Additionally, anti-HSA antibody induced *in-vitro* agglutination of human, rat and monkey sperm [67].

Two proteins, A-kinase anchoring protein 3 (AKAP 3) and especially AKAP 4, are proteins abundantly present in fibrous sheath of mid-piece tail of human and mice sperms [68, 69]. AKAP 3 is synthesised earlier during round spermatid formation and involves in the arrangement of the basic structure of the sperm tail, while AKAP 4 is expressed later during late phase of spermatid formation and plays role in the maturation of sperm tail structure [69]. A study showed that male mice lacking AKAP4 was not implicated in sperm numbers but, progressive motility of its sperm was failed and the male mice were infertile [70].

rSMP-B, following its discovery from mid-piece and tail of rabbit sperm [71], was identified to present on the same regions of human sperm [72]. Antibody-anti rSMP-B possessed immobilising activities against human and mouse spermatozoa, but no agglutinating activity, *in-vitro* [72].

Lactate dehydrogenase-C4 subunit (LDH-C4) was expressed specifically in the testis and most abundant in human sperm. Courtesy of Human Protein Atlas, www.proteinatlas.org [49]. Its high activity in the testis is associated with human sperm biogenesis and motility [73]. An *in-vitro* assessment of sperm from LDH-C4 immunised mature male baboon (*Papio sp.*) indicated that sperm binding capacity to Zona pellucida is reduced in seroconverted animal model [74].

Etomoxir is not an ASA. It is a mitochondrial carnitine palmitoyltransferase inhibitor which block serial reaction that transport fatty acid from cytosol into intermembrane space of mitochondria, thus halt the oxidation and energy production from the substrate [75, 76]. The incubation of sperm with etomoxir results in a decreased sperm motility in concentration-dependent manner but does not affect the sperm viability [59]. A systemic usage of Etomoxir for contraceptive is not applicable, as the substance is hepatotoxic [77]. However, more studies with Etomoxir could shed more light of contraceptive targeting sperm antigens responsible for energy production.

3. PCSK4 as a potential sperm antigen for ASA contraceptive developments

3.1 Biochemistry of PCSK4

One sperm antigen potential for development of contraceptive ASA is the pro-protein convertase subtilisin/kexin type 4 (PCSK4) [78–81]. PCSK4 is an enzyme protein expressed in abundant on the outer surface of acrosomal plasma membrane of mammalian spermatozoa (**Figure 1**). It has a molecular weight of 45 kDa [79, 83, 84]. Biochemically it plays role in the proteolytic activation of precursor proteins in the cellular secretory pathway and physiologically it has a specific important role in mammalian reproductive process [79, 83–85]. PCSK4 gene is a 9 kilobase (kb) DNA, consists of 15 exon and 14 intron which in humans it is located in the chromosome 19 [78, 80, 85]. It is a protein associated with bacterial subtilisin and yeast kexin where its biosynthesis takes place in endoplasmic reticulum in the form of a multi-domain preprotein [78, 80]. PCSK4 is one among nine family members of calcium-dependent serin endoprotease *Proprotein Convertase Subtilisin/Kexin* (PCSK) [79, 83–85]. These nine family members of PCSK are: PCSK1 and PCSK2 which are expressed in endocrine and neuroendocrine cells; PCSK3, PCSK5, PCSK6 and PCSK7 are widely expressed by different types of cells; PCSK4 is specifically presented by gonadal and placental cells and; PCSK8 and PCSK9 which play roles in the synthesis pathways of cholesterol and lipid acid [79, 80, 86–88]. PCSK 1 to 7 belong to sub-family of kexin while PCSK8 and PCSK9 belong to sub-family of pirolysin and proteinase-K, respectively [79, 80, 86]. PCSK 1 to 3 are also known

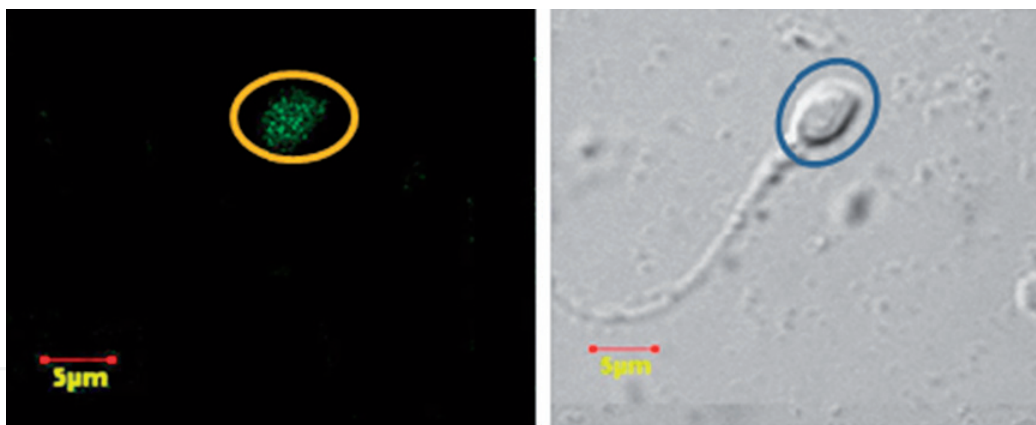


Figure 1. Using monoclonal antibody anti-human PCSK4 and rodamin as staining, PCSK4 molecules are detected in abundant on the membrane surface of the head of human spermatozoa under Laser Scanning Microscope (LSM) examination [82].

as, consecutively, PC1/3, PC2 and furin, while PCSK 5 to 9 are also recognised as PC5/6, PACE4, PC7, SKI-1/SIP and NARC-1, respectively [89].

PCSK4 is synthesised as a zymogen, proPCSK4, in the endoplasmic reticulum [81]. Its maturation process remains unclear but suggested to be by an autocatalytic process that cleaves the molecule at two sites situated between prodomain and the catalytic domain [80]. PCSK4 proprotein contains five domains located consecutively, i.e. the signal peptide (SP) domain, prodomain, catalytic domain, a domain of 150 amino acids called P (Protease) domain or homo B domain and the C-terminal domain which carries transmembrane domain [80]. The SP domain of PCSK4 contains signal peptide which directs precursor proteins toward the secretory pathway. The Prodomain acts as an intra-molecule chaperone and regulator of the catalytic activity of the enzyme, removal of this domain is essential for the activity of the enzyme [90]. The primary and secondary cleavage sites of proPCSK4 are situated between prodomain and catalytic domain. Catalytic domain of PCSK4 is the active site of the enzyme and carries Asp-His-Ser catalytic triad characteristics of serin proteinase [80].

The P domain of PCSK4 plays an important role for proper folding, contains beta fold sandwich of galactose bond domain capable of mediating carbohydrates, phospholipids or membrane receptors. This P domain acts as a regulator site of ultimate enzyme activities under influences of optimum pH and calcium, but also in stabilising the structure of catalytic domain [80, 90]. It helps to balance asymmetric surface at the region of catalytic domain bond, owing to the specific characteristic of multi-basic residues of this enzyme (the consensus of substrate order is Arg-X-Lys-Arg-Arg, where X can be an amino acid; the site of peptide dihydrolysis) [80, 83, 91]. The C-terminal variable domain influences intracellular localisation, in-out recycle of proteins and protein–protein interactions. N-glycosylation sites are located at P domain and at C-terminal domain [80].

Expressions of PCSK4 are discovered in abundant in reproductive organs, especially in testis. In testis, they can be found in epididymic and germinal cells i.e. in acrosomal granules of round spermatids, in acrosomal ridges of elongated spermatids and on the acrosomal plasma membrane of spermatozoa [80, 81, 84, 92]. The PCSK4 or named as proprotein convertase PC4 in a study, is also expressed in the human placenta and macrophage-like cells in the ovary [87, 88].

3.2 Physiological roles of PCSK4

Intracellularly, PCSKs takes action in the limited endoproteolytic regulation mechanism of the secretory pathway. Limited endoproteolysis constitutes the

post-translational modifications of proteins by which cells diversify and regulate gene products [78]. Endoproteolytic process occurs during modifications to activate many precursor proteins in biological cell functions including zymogen activations, formations of peptide hormones, complement activations, blood clotting and blood clot lysing, angiogenesis and tissue re-modelling. Secretory pathway processes of eukaryotic cells are mainly assisted by carboxyl residues of Lys or Arg (P1) in the order R/K – (X)_n – X/K/R – R (where: X = amino acids other than Cys; n = 1, 3, or 5; K or R = the place of P2 amino acid) [78, 86, 91].

PCSK4 present in acrosome region of sperm thus, is hypothesised to play role in capacitation and acrosome reaction [80, 84]. SPCSK4-null sperm has normal appearance, normal motility competence and undergo normal capacitation process but, following capacitation they suffer reduced hyperactivated motility [93]. Post-capacitation hyperactivity is a qualitative characteristics of sperm which thought to be important to assist sperm penetrating zona pellucida of an egg. PCSK4-null sperms show a reduced fertilisation competence *in-vitro* and the embryo resulted from PCSK4-null sperm fails to develop further [93].

3.3 Substrates of PCSK4

Two molecules have been known as the natural substrates of PCSK4; pro-pituitary adenylate cyclase-activating protein (proPACAP) and Insulin-like growth factor II (IGF-II) [87, 94]. The proPACAP has two active isoforms; PACAP38 and PACAP27 residues. PACAPs are expressed in hypothalamus, in extra-hypothalamic regions of the brain, in the granulosa cells of the developing ovarian follicles of the rat and transiently in rat spermatid cap but, are absent at the other stages of spermatogenesis. They are also expressed in Sertoli cells and Leydig cells. Gonadal PCSK4 is the only enzyme that activates proPACAP both in the testis and the ovary of the mice [94]. A study reported that PACAP null female mice failed to implant its embryo to the uterus [95], suggesting proPACAP activation conducted by PCSK4 is pivotal in embryonic implantation. PCSK4 null male mice lack of PACAP activation and produce normal but incompetent sperm. It leads to the hypothesised that PACAP may not important in maturation process of the sperm but involves in the production of molecules required for the functional mature sperm [94].

Other substrate of PCSK4, IGF-II, is discovered in placenta and its inactivity has been shown to be involved in the pathophysiology of intrauterine growth restriction (IUGR) of human foetus; a major cause of perinatal death. In this pathway, Placental PCSK4 activates pro-IGF-II to form a half-matured IGF-II and successively mature IGF-II as a result of the cleavage of its terminal basic residues by carboxypeptidases [87]. Inhibition of Placental PCSK4 by a PCSK4-specific inhibitor blocks pro-IGF-II processing resulting a reduced trophoblast cell migration [87], likely due to reduced effectivity of trans-placental diffusional exchange leading to reduced nutritional supply [96]. The locality and functionality of the two substrates of PCSK4 indicate that PCSK4 are physiologically important during zona penetration and embryonic development.

3.4 Inhibitors of PCSK4

Studies in model animals showed that individuals with PCSK4 expression disorder have significantly lower fertilisation capability [79]. In our study, intact male *Rattus norvegicus* previously injected with antibody anti-PCSK4 was allowed to fertile female rats and it showed that the number of off-springs delivered by these female rats significantly declined in accordance with the increased doses of antibody anti-PCSK4 injected [82]. These indicate that inactivation of PCSK4 by injectable anti-PCSK4 could prevent conception.

The use of synthetic PCSK4 inhibitors could also serve as an option for contraception. A synthetic inhibitor of PCSK has been developed based on the knowledge, that prodomain removal is essential for activation of the enzyme. Binding of this domain to PCSK4 active enzyme could hypothetically inactivate the enzyme. A peptide, mimicking prodomain sequence near its primary activation site, was engineered and an *in-vitro* assay using a recombinant PCSK4 showed that PCSK4-mediated proteolysis was efficiently blocked by synthetic prodomain rPC4_{101–116} peptide [90].

Other potent synthetic PCSK4-inhibitors: tetrapeptide chloromethyl ketone and the Dec-RVKR/K-cmk (Decanoyl-RVKR/K-chloromethyl ketone) were reported to inhibit PCSK4 more potently than synthetic prodomain rPC4_{101–116} peptide [90]. Another substance, synthetic enediynes amino acid containing peptides, was developed and reported to inhibit PCSK4 activity *in-vitro* [97]. Further, dimeric form of CRES was reported to moderately block the PCSK4 activity to human proIGF-2 in human placental trophoblast cell line [98].

4. Future development of ASA contraceptives

Only a few of aforementioned ASA underwent animal models *in-vivo* study. Among are EQT, FA-1, YLP12, *Eppin*, PCI, HSA, AKAP 4 and PCSK4. None of these molecules however, shows full inhibition of fertilisation in animal models, whereas YLP12 contraceptive studies reported various results from partial to lack of inhibition of fertility to animal models [37, 53, 63–65, 82] [53, 55, 70]. Therefore, further efforts are needed to make ASA contraceptive become reality.

Future development of ASA contraceptives might include the studies of under-explored proteins such as those involve in the energy production in the mitochondria of sperm. Proteomic approach assistance, in this regard, have enabled the discovery of large number of novel proteins [59] and allows further investigations of single protein of interest.

On the other hand, as the usage of single molecule have been impractical, alternative of future ASA development might include the study of efficacy of multivalent vaccines, in order to boost the final effective contraception effect as well as reducing potential toxic effect of high dose administration of a single substance. Study of multivalent vaccine targeting proteins of acrosomal sperm has been actually initiated. In the study, immunised monkeys recognised the five antigens used: ESP, SLLP-1, SAMP 32, SP-10 and SAMP 14, with the highest IgG average absorbance values were to ESP, SAMP 32 and SP-10 but at IgG lower values for SLLP-1 and SAMP 14 [99]. Further, capacitated sperm treated with sera from immunised monkeys showed fusion inhibition but only in two of five individuals [99]. This was a sound study that used proteins which were pre-tested to not cross react to each other. But the study used molecules that never had undergone *in-vivo* study so individual effect of the vaccine component was unknown. In addition, a study of FA-1 and YLP12 mixed vaccine in mice further support the more potential of multivalent vaccine in inducing contraceptive effects compared to monovalent vaccine [54].

The *in-vivo* study of anti-PCSK4 administration demonstrated in our laboratory indicates that it is possible to efficaciously administer contraceptive agents in the form of antiserum rather than injecting them to an individual for a few times as immunisation [82]. In the future, this approach might be more practical if the molecule could reach therapeutic concentration in the seminal plasma once the semen ejaculated thus well mixed with sperm along their journey to egg, mimicking *in-vitro* incubation of sperm with its inhibitors prior to a fertilisation challenge with eggs.

Topical applications such as intravaginal administration might be strategy of choice in the antisperm contraceptive application especially for molecules which have been known to be toxic when administered systemically such as etomoxir. Topical intra-ampullar oviduct administration of antisperm agent in animal model has proven that topical ASA contraceptive application can be efficacious [37].

5. Conclusions

Existing contraceptive methods have been widely practiced by couples globally. However, some side effects limit the broader utilisation. Although ASA is a promising method of contraception it is not yet practical. More studies should be done in order to enable ASA to replace common contraceptive methods.

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Conflict of interest

We authors declare that there is no conflict of interest related to the preparation of this manuscript.

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