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Immunocontraceptives: A noval approach to fertility control

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Abstract

The population of the globe is growing by one billion every twelve years, surpassing the 6.891 billion milestone. Approximately 80 million women worldwide become pregnant unintentionally or unwantedly each year and 45 million of these women have abortions. A pressing issue for the nation, constrained by limited resources, is the challenge of overpopulation. There is undoubtedly a global demand for an improved technique of birth control. In India, 48.2% of couples use family planning techniques. In order to further develop more advanced technology in this area, the Indian government implemented the National Family Planning Programme. Since the middle of 1970s, the Indian Council of Medical Research and the Department of Biotechnology have been working to create birth control vaccinations that work for both the sexes. Immunocontraception offers an attractive approach to the growing global problem of overpopulation.

Keywords: Antigens, immunocontraception, lactate dehydrogenase-c, sperm antigens, reproductive hormones

Introduction

Immunocontraception

Immunocontraception is defined as a process that produces humoral immune response using a reproductive protein that leads to infertility in animals for a defined period of time (Fayrer-Hosken *et al* 2000) ^[30]. The idea behind an immunocontraceptive could be put this way: A man or woman injects or swallows a formulation of specific molecules, which causes the body to produce circulating antibodies or immune effector cells that stop reproductive processes and prolong infertility period without causing any negative side effects. In this process immune response is stimulated against gametes or reproductive hormones (Miller 1995, Stevens 1999, Delves *et al* 2002) ^[30, 31, 21]. Immunocontraception may provide an alternative method of contraception.

Differences between traditional vaccines and immunocontraceptives

The paradigm of vaccination was established almost two centuries ago when an 8- year-old boy with cowpox received an effective vaccination against smallpox. Since then, numerous vaccines have been developed with the aim of protecting against life-threatening microorganisms (Smith 2011) ^[32]. An antifertility immunogen or an immunocontraceptive is designed to prevent fertility or early pregnancy. Traditional immunization against microorganisms is the only means available for controlling a given disease whereas alternative methods are already available in the case of contraception (Riedel 2005) ^[33].

Another significant difference is that standard vaccinations are targeted against foreign antigens present on the pathogen, whereas antifertility immunization targets isologous antigens from the egg, sperm or specific reproductive hormones (Delves *et al* 2002) ^[21]. Lastly, the traditional vaccines provide lifetime protective immunity, which is typically enhanced by exposure to the natural antigen; whereas immunocontraceptives provides relatively short- lived reversible immunity varying from few months to years (Christian 1996) ^[17].

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Process for the development of an immunocontraceptive

The process of creating an immunocontraceptive involves a number of procedures.

These consist of the following

1. The fundamental identification and characterization of suitable immunogens derived from reproductive hormones or from sperm, egg, egg investments or accessory reproductive organs. This step is highly complicated and is further subdivided into following steps-
 - Defining reproductive events or processes that is susceptible to immune intervention.
 - Identifying molecules (immunogens) whose removal or neutralization will affect fertility.
 - Characterizing the immunogen's structure biochemically.
 - Determining whether the immunogen is specific to the targeted reproductive event or tissue and not present in other tissues.
2. The development of techniques for producing the immunogens to high standards of purity by genetic engineering of genes encoding particular immunogens, peptide synthesis or isolation of the antigen from natural sources.
3. Production and purification of immunogens.
4. Dosage formulation of immunogen.
5. Immunogen formulations are tested for immunogenicity, safety and efficacy in small animals and primates.
6. Immunogenicity, safety and efficacy are assessed in human trials.
7. Diagnostics are developed to track the infertility status of recipients of the effective immunogens (Christian 1996) ^[17].

Targets of immunocontraception

The stages of reproduction that are susceptible to immune intervention should be taken into account while determining which molecules would be most effective for a contraceptive vaccination. Mammalian reproduction can be simplified by separating it into few crucial stages, each of which could present a chance for intervention: gamete production (oogenesis and spermatogenesis); gamete shedding and transport (ovulation and oviductal transport); gamete interaction (capacitation, the acrosome reaction, penetration of the egg investments and fusing with the oolemma, transportation of blastocyst and implantation). Theoretically, all of these events can be stopped by inducing an immune response against molecules that have critical structural and functional roles and are accessible to antibodies (McLaughlin and Aitken 2011) ^[34]. A potential candidate for an effective contraception should have relevance with the process of reproduction, exhibit tissue specificity, present in transient amount to avoid chronic risk of immune complex formation and should be accessible to antibodies (McLaughlin *et al* 2003) ^[35].

Reproductive hormones as antigens

GnRH Vaccination

GnRH is a decapeptide synthesized by hypothalamus that regulates the anterior pituitary to secrete gonadotropins-Luteinizing hormone (LH) and Follicle stimulating hormone (FSH) both these hormones contribute majorly in oogenesis and spermatogenesis. GnRH based vaccines offer a promising approach in contraception. These vaccine triggers an immunological response against GnRH, which in turn

influences the chain of reaction of reproductive hormones (Killian *et al* 2008) ^[36]. Decrease in LH and FSH levels ultimately affects spermatogenesis and oogenesis (Bremner and Matsumoto 1986) ^[11]. The vaccine's contraceptive effect fades with time in most species (Miller *et al* 2008, Massei *et al* 2012) ^[38], ⁴¹, while infertility can persist for a few years (Killian *et al* 2009, Gionfriddo *et al* 2011, Massei *et al* 2012) ^[39, 40, 41].

GonaCon, an injectable, single-dose gonadotropin releasing hormone (GnRH) vaccine, is one of the contraceptives that has been repeatedly demonstrated to reduce fertility in a variety of ungulates such as white-tailed deer (*Odocoileus virginianus*), wild boar (*Sus scrofa*), horses (*Equus caballus*) and bison (*Bison bison*) for a period of one to six years (Miller *et al* 2000, Miller *et al* 2004, Killian *et al* 2008, Massei *et al* 2008, Gray *et al* 2010, Massei *et al* 2012) ^[42, 42, 36, 44, 45, 41]. In 2004 a research was done at the Idaho Fish and Game Wildlife Health Laboratory in Caldwell, Idaho (USA) to find out if a GnRH vaccination was successful in lowering pregnancy in bison and to investigate the safety of injecting GnRH in pregnant bison. Pregnant and non-pregnant female bison were given a single injection of the GnRH vaccine. After GnRH immunization, the pregnant bison who had received the shot gave birth to healthy calves. The immunized non-pregnant bison were found to be non-pregnant even after mating depicting the contraceptive and effective nature of the GnRH vaccine (Miller *et al* 2004) ^[42].

Similar study was performed by Massei and his co-workers in 2008. They examined the physiology and behaviour of two groups of captive female wild boar in two consecutive trials (Trial 1 and Trial 2) to determine the efficacy and possible adverse effects of the single-dose GnRH vaccine GonaCon. They observed that no treated female delivered during the first trial and progesterone levels dropped to baseline levels in trial 2. Their findings suggested that GonaCon can inhibit wild boar reproduction without having any major impact on physiology or behavior of the animal (Massei *et al* 2008) ^[44]. Later in 2011 the long-term effects of the GnRH contraceptive vaccination on the breeding behavior, hormone levels, antibody levels specific to GnRH and fertility in male and female white-tailed deer (*Odocoileus virginianus*) was investigated. Reduced fawning rates, changed estrous behaviour, lower progesterone and inability to sustain pregnancy after conception were the results reported by them. Their findings showed that the GnRH vaccination successfully caused reversible infertility with infertility lasting up to two years without booster doses (Gionfriddo *et al* 2011) ^[41].

GnRH and pZP- GnRH vaccination was later assessed with other contraceptive techniques. Killian *et al* (2008) assessed the contraceptive effectiveness of the human intrauterine device (IUD) 380 Copper 'T', porcine zona pellucida (pZP) vaccine SpayVac and gonadotrophin-releasing hormone (GnRH) vaccine GonaCon in mustang mares. The rate of contraception was reported to be 100% for SprayVac, 94% for Gonacon and 80% in the mares treated with IUD's. They reported that a single dose of either SpayVac or GonaCon vaccination can provide mustang mares with long-term contraception.

Similar study was performed in a population of wild female horses (*Equus caballus*) by Gray and his co-workers to examine the effectiveness and possible adverse effects of

two contraceptive formulations: GonaCon-B and SpayVac formulation. The reproductive status of the females was examined for three years. They observed that both the contraceptive methods dramatically decreased fertility. Their findings showed that even without booster shots, both the vaccinations were capable of considerably lowering fertility for several years (Gray *et al* 2010) [45].

The contraceptive impact and immune response in rats were studied using two new injectable immunocontraceptive formulations targeting the GnRH: Mycobacterium avium cell wall fragments- Imaxio (MAF-IMX294) and (MAF-IMX294P) conjugates (both identified for having potential as oral contraceptives). Both the formulations caused 100% of rats to develop anti-GnRH antibody titres and significantly reduced fertility as compared to control animals (Pinkham *et al* 2022) [47].

To investigate the effectiveness of GonaCon in black-tailed prairie dogs (*C. ludovicianus*) a study was conducted in USA. Female Prairie dogs weighing ≥ 660 g were live trapped and injected with 0.4 ml of GonaCon (0.032% w/w GnRH) and the control females were injected with sham vaccine (without GonaCon). Adults and juveniles were counted at six different locations to determine the initial Gonacon effectiveness and the persistence of contraception. It was observed that the juvenile:adult ratio was three times higher in the areas where females were injected with sham vaccine. GonCon was reported to be very effective in the first year of the study but not in the second year (Shiels *et al* 2024). Other than Gonacon Improvac, Equity, Bopriva, Improvest and Repro-Bloc are the other GnRH based vaccines that are currently under investigation (Gupta and Minhas 2017). Along with its contraceptive behaviour the GnRH based vaccines have shown various secondary drawbacks including decrease in testicular size, possible decline in libido and alterations in secondary sexual characteristics that impedes the commercial acceptance of this formulation.

Immunization with LH and FSH

LH and FSH hormones are known to work in conjunction to regulate testicular and ovarian function. A variety of steroid hormones and proteins are secreted by the gonads in response to their secretion. Gonadal steroids perform a variety of tasks, such as maintaining secondary sexual traits and accessory sex tissues, stimulating hypothalamic regions linked to aggressive and sexual behaviour and controlling the secretion of Luteinizing hormone releasing hormone (LHRH), LH and FSH through feedback processes (D'Occhio 1993) [25]. Hormones are easily accessed by antibodies since they are carried by the general circulation. Therefore, it has been suggested that antibodies that are specific for a given hormone will attach to that hormone in the bloodstream, thereby inhibiting its biological effects. Notably, these hormones are not immunogenic; consequently, immunization with these hormones requires conjugation to antigenic carrier proteins (Ladd *et al* 1990) [48].

Ovine and human FSH have similar structural characteristics, using ovine FSH instead of human FSH reduces the likelihood of cross-reactive antibodies with human LH and FSH (Moudgal *et al* 1992) [50]. Male bonnet monkeys that received ovine FSH vaccination developed testicular dysfunction, oligozoospermia and ultimately sterility. Notably, there was no correlation between

infertility and changes in testosterone levels, which adds another benefit to the use of FSH-based vaccines (Murty *et al* 1979) [51]. After ovine FSH tests were successful, two distinct forms of ovine FSH- ovine FSH $\alpha\beta$ heterodimer or isolated ovine β - chain were used in a phase 1 clinical trial. Alum was used as an adjuvant in both the cases. Following immunization, all immunized individuals developed antibodies specific to FSH. However, since the concentration of the antibodies produced was low, it did not affect the sperm concentration (Moudgal *et al* 1997) [51]. When adult female sheep (ewes) were immunized with LH they did not become pregnant for two breeding seasons. The preovulatory surge of LH was thought to be the mechanism of action, which prevent ovulation (Roberts and Reeves 1989) [52].

Pan *et al* (2024) [53] in prepubertal and pubertal mammals, investigated the effects of recombinant LH receptor (LHR) and recombinant FSH receptor (FSHR) subunit vaccines on testicular development, spermatogenesis and testosterone secretion using male Sprague Dawley (SD) rats as a model. The LH receptor- diphtheria toxin (LHR-DTT) and FSH receptor- diphtheria toxin (FSHR-DTT) vaccines both markedly reduced the prepubertal rat's serum testosterone level ($p < 0.05$), but it had no effect on the pubertal rat's testosterone secretion. Additionally, in both prepubertal and pubertal rats, the vaccines reduced spermatogenesis and the number of cell layers in the seminiferous tubules. Subunit vaccination FSHR-DTT reduced testicular index and sperm motility in pubertal rats and decreased sperm density in the epididymis in both prepubertal and pubertal rats. In contrast, LHR-DTT only decreased sperm density in the epididymis in pubertal rats. Their findings suggest that the FSHR subunit vaccination is potentially a useful method for immunocastration. Despite the possibility of using LH vaccination as a contraceptive method, its widespread acceptance is hindered by the possibility that it may impact sex steroids

Targeting egg-surface antigens

In mammals, the ZP is an extracellular transparent glycoproteinaceous matrix that envelops the egg (Talbot *et al* 2003, Nixon *et al* 2007) [54, 55]. It functions as a comparatively species-specific "docking site" for spermatozoa-oocyte interaction, triggers acrosomal exocytosis in spermatozoa, inhibits polyspermy and is crucial for the protection of a pre- implanted blastocyst (Zhao and Dean 2002, Nixon *et al* 2007) [56, 55]. ZP glycoproteins have been considered as potential antigens for immunological contraception because of their vital involvement in reproduction. Zona matrix is made up of three or four different glycoproteins. The ZP matrix in mice is made up of three glycoproteins: ZP glycoproteins-1 (ZP1), ZP glycoproteins-2 (ZP2) and ZP glycoproteins-3 (ZP3) (Spargo and Hope 2003). ZP matrix in humans and non-human primates is made up of four glycoproteins: ZP1, ZP2, ZP3 and ZP glycoproteins-4 (ZP4).

Whole native porcine zona pellucida (pZP) collected post-mortem from pig ovaries is a heterologous antigen that has been used for fertility control in feral and exotic animals. Many mammalian species including lagomorphs, equids and primates have been proven to experience infertility as a result of active vaccination trials using this vaccination (Bhatnagar *et al* 1992) [6]. Previous researches has shown that pZP is a potent heteroimmunogen in most species and

has been effectively used to control fertility in a variety of animals including wallabies, horses, white-tailed deer (Turner *et al* 1996, Kitchener *et al* 2002, Locke *et al* 2007, Miller *et al* 2008) [57, 58, 59, 38], bonnet monkeys (Miller and Killian 2002, Hardy *et al* 2002) [36, 61], bears (Lane *et al* 2007) [62] and elephants (Delsink *et al* 2007) [20].

In North-America pZP immunocontraception via remote dart delivery was delivered to control the population of wild horse. An analysis was conducted to assess the efficacy of the treatment as well as the impact of the treatment on annual birth rates and population demographics for a period of four years. With no noticeable change in the foaling season or duration, a downward trend in the foaling rate and number of mature mares was reported at monthly intervals, demonstrating decline in yearly seasonal breeding peaks. After four years 58% decrease in foaling and a mere 10% conception rate was recorded (Schulman *et al* 2024) [63]. Long-term researches have revealed that vaccination with zona antigens may trigger an immune response against a large number of eggs within the ovary, potentially resulting in early ovarian failure (Paterson *et al* 1992) [64]. Hence, sufficient data must be obtained before placing it in the market (Naz 2005) [65].

Targeting sperm antigens

Many studies have been carried out to induce immunological contraception by injecting women with bovine sperm phospholipid or fresh semen. For the development of immunocontraceptive vaccines out of the available choices, sperm constitute the most promising and exciting target. Even though sperm contains many antigens but only the antigens that are sperm-specific can be ideal candidates for immunocontraception. Since crude sperm extracts have shown some efficacy as contraceptives, the main focus of future researches has shifted to discovering and characterizing specific sperm molecules with comparable antifertility properties. Numerous antigens that are initially formed during spermatogenesis and subsequently remain on mature sperm have been reported in many studies. Compared to the extrinsic or coating antigens of sperm, these antigens collectively are frequently referred to as "intrinsic differentiation antigens". These differentiation antigens frequently meet the crucial requirement of tissue specificity (Feuchter *et al* 1981) [66].

Immunocontraceptives associated with sperm or egg antigens are commonly referred to as pre- or post-fertilization immunocontraceptives based on their hypothesized mechanism of action. The first theory states-immunocontraception that occurs before fertilization in which an antibody to a component of the sperm membrane will act in the oviduct to stop fertilization or agglutinate/lyse sperm during passage through the female tract is referred to as a pre- immunocontraceptive. In contrast, an immunocontraceptive based on an antibody against hCG which target an early embryo within the uterus to induce early abortion is considered as a post-fertilization immunocontraceptive. hCG is secreted by the trophoblast layer which stimulates corpus luteum to secrete progesterone that maintains the uterine lining. Drop in progesterone levels will ultimately lead to shedding off of uterine lining further impeding implantation (Kayisli *et al* 2003) [67].

One in five couples of reproductive age suffers from infertility. Antisperm antibodies are possessed by some of

these individuals in their blood and reproductive tract secretions. 5- 30% of infertile patients and 50-70% of vasectomized men produce antisperm antibodies (Liskin *et al* 1983, Ohl and Naz 1995) [68, 69]. Sperm consists of both auto- and iso- antigens thus can produce antibodies in both men and women. Fertilization and fertility are affected by antisperm antibodies both in vitro and in vivo conditions (Naz *et al* 2005) [65]. Immunizing males or females of different species including human beings with sperm or sperm extracts results in the development of antisperm antibodies leading to infertility (Baskin 1932) [4]. Interaction between some of the sperm membrane antigen moieties and antibodies can be the main reason of immune infertility, as antisperm antibodies are unable to penetrate through the plasmalemma of live sperm cells. Knowledge of the nature of sperm antigens implicated in immune responses might aid in the development of contraceptive vaccines for humans, domestic and wild animal populations (Frayne and Hall 1999) [70].

Sperm- specific antigens can induce immune responses as they are synthesized and incorporated into spermatozoa at puberty after neonatal period (Tung 1998) [71]. By this time, the contact between immune system and sperm antigens is confined, as during the last stages of development the male germ cells are sequestered behind the blood-testis barrier (Pelletier 1998) [72]. For the purpose of immunization, the whole spermatozoa cannot be used as it shares several internal and external antigens with the somatic cells (Naz 1999) [73]. The most attractive target for immunocontraception is the sperm- zona pellucida binding site. Only sperm specific antigens can be employed for contraceptive vaccine. These antigens develop in the testis or during the passage through epididymis which is the maturation site for sperms.

Some of the well characterised sperm antigens are sperm adhesion molecule 1 (SPAM 1), sperm protein (SP-10, SP-17), metalloprotease disintegrin cysteine (MDC), NZ-1, NZ-2, fertilization antigen (FA-1), lactate dehydrogenase-C4 (LDH-C4), YLP-12 peptide, sperm agglutination antigen (SAGA-1), rabbit sperm membrane protein-B (rSMP-B), human equatorial segment protein (hESP), BS-17, 80 kDa human sperm antigen (HSA) and sperm acrosomal membrane-associated protein (SAMP-32). Moreover, during transit through epididymis spermatozoa acquires few glycoproteins from epididymal secretions on its surface, including, epididymal protease inhibitor (Eppin), dorsal head and equatorial proteins (DE), sperm flagella protein (SFP-2), Izumo and A- kinase anchoring protein (AKAP) (Kaur and Prabha 2014) [74].

Sperm competence in animal fertilization necessitates the coordinated actions of several sperm-specific proteins, which are typically allo-immunogenic in females. The allo-antigens of sperm membrane are potential contraceptive targets as they block the protein's function during gamete interactions. A targeted proteomic technique was used to identify significant allo-antigens in pig sperm membranes and lipid rafts. In gilts with high allo- antibody reactivity to proteins in sperm membranes or lipid rafts, fewer offspring (73%) were generated than in adjuvant-only or non immune control animals. Allo-antisera has been shown to have around 20 different sperm membrane proteins and 5 different lipid raft proteins. ADAMs fertilin, fertilin β and cyritestin are alloantigens recognized by mass spectrometry. Myo-inositol monophosphatase-1, ATP synthase F1 β

subunit and zymogen granule membrane glycoprotein-2 are among the less abundant alloantigens. Lymphocytic antigen 6K, SAMP14 and the epididymal sperm protein E12 are all immunodominant sperm lipid raft alloantigens. Eleven of the fifteen membrane alloantigens were known sperm-specific proteins with unknown fertilization roles. The existence of the remaining four sperm-specific isoforms was unknown. After *de novo* sequences of tryptic peptides from sperm membrane alloantigen that showed no obvious resemblance to known proteins, a novel sperm specific gene product "M6" was found in swine (Cormier *et al* 2018) [18].

Sperm-specific antigens with immunogenic potential

Numerous conserved, sperm-specific immunogenic proteins have been found and assessed for the development of a secure and an effective contraceptive vaccine (Jones 1990 [75], Primakoff 1994 [76], Diekman and Herr 1997, Frayne and Hall 1999) [24]. Many of these have shown promising results. Female baboons were found to be less fertile after receiving the LDH-C4 vaccination, but female cynomolgus macaques were not affected (Tollner *et al* 2002) [76]. In male guinea pigs, active immunization with protein hyaluronidase (PH-20) developed autoimmune orchitis and also completely inhibited fertility (Tung *et al* 1997) [78]. Eppin vaccination resulted in infertility and high antibody titres in 78% of immunized monkeys (O'Rand *et al* 2004) [79].

The Sperm Adhesion Molecule 1 (SPAM1): a 64 kDa glycosyl-phosphatidylinositol (GPI) is expressed in the testis, epididymis, sperm and epididymis luminal fluid (Deng *et al* 2000, Zhang and Martin-DeLeon 2003) [22, 80]. During fertilization, the hyaluronidase enzyme SPAM1 has three primary roles. These consist of ZP binding, cumulus penetration (Cherr *et al* 2001) [15] and Ca^{2+} signalling during acrosomal exocytosis (Cherr *et al* 1999) [14]. The protein is highly conserved among different animals and has been cloned from numerous species including fox (Ten Have *et al* 1998), guinea pig (Lathrop *et al* 1990) [81], pig (Day *et al* 2002) [19], rat (Hou *et al* 1996) [82], rabbit (Holland *et al* 1997) [87], monkey and man.

100% infertility was reported in male and female guinea pigs immunized with purified SPAM1. The sperm-ZP binding capacity was decreased when sperm were incubated with antibodies against SPAM1 (Primakoff *et al* 1997) [78]. In case of immunized males, autoimmune diseases were linked to infertility (Tung *et al* 1997) [78]. Whereas, sperm egg binding was prevented by antibodies that resulted in infertility in case of females. To produce proteins in sufficient quantity recombinant technology was used and recombinant SPAM 1 (rSPAM-1) was later employed to immunize both male and female rabbits, the results were not very promising as it did not cause any of the immunized animals to become infertile. This was attributed to the fact that sufficient levels of anti-rSPAM1 antibodies were not induced, necessary for preventing conception (Holland *et al* 1997) [87].

PH-20: The sperm antigen PH-20 was isolated from guinea pig (Primakoff *et al* 1997) [78]. The protein resulted in 100% infertility in both male and female guinea pigs after active immunization. They reported that fertility was regained after a drop in antibody titre but it caused autoimmune orchitis in case of males. The protein's hyaluronidase activity was discovered later.

Fertilin: earlier known as PH30 is a sperm-associated protein. It was first isolated from caudal epididymal sperm extract from guinea pigs. Fertilin α (60 kDa) and β (44 kDa) are two related subunits. The function of α fertilin is linked to sperm-egg adhesion (Blobel *et al* 1992) [9]. The existence of a cysteine-rich domain, which seems to be involved in cell adhesion, justifies its role in adhesion. However, the sperm lacking this subunit is still capable of sperm-egg fusion hindering its use in immunocontraception (Cho *et al* 2000) [16]. Fertilin β is well known for mediating membrane fusion and sperm-egg interaction (Yuan *et al* 1997) [90]. Fertilin β antibodies prevented fertilization *in vitro* (Stein *et al* 2004) [91], but were found to have no effect on fertility *in vivo*, therefore not considered a suitable choice as a contraceptive (Ramaraio *et al* 1996, Hardy and Mobbs 1997) [92, 93].

Sperm Protein-10 (SP-10): a testis specific but not species specific protein was first identified in humans (Anderson *et al* 1987) [2]. *In vitro* and *in vivo* studies have proved that antibodies raised against this protein inhibit sperm-zona binding (Kurth *et al* 1997) [94]. However, the protein is restricted to outer acrosome layer and is therefore, only accessible to antibodies after the acrosome reactions are triggered.

Sperm epididymal protein (DE): The immunocontraceptive characteristics of recombinant sperm epididymal protein (DE), was evaluated to assess its involvement in the fertilization process in rats. Male and female rats were randomly assigned to one of three groups and given five injections of recombinant DE (recDE), native DE (nDE) and maltose binding protein (MBP). According to the findings recDE elicited a stronger immunological response and greater anti-DE levels than nDE. recDE injections resulted in a substantial decrease in fertility in both male and female rats with no indication of pathological consequences and has shown to cause an immunological response as well as impair fertility in both male and female rats (Ellerman *et al* 2008) [27].

Human seminal plasma inhibin (hSPI) - is a peptide with a molecular size of 10.5 kDa that regulates FSH, has also been identified as a potential antigen for controlling fertility. When male and female rats were immunized either actively or passively with hSPI or its synthetic peptides, fertility was inhibited by 75% (Vanage *et al* 1992, Sheth *et al* 1994, Vanage *et al* 2000) [96, 97, 98]. When male bonnet monkeys were actively immunized with the N-terminal 17 amino acid peptide (R-17), the ejaculated spermatozoa lost their motility and an increase in antibody titre was observed. The study has also showed that antifertility effect was reversible along with a drop in antibody titre (Bandivdekar 2014) [3].

Several sperm antigens are under investigation and majority of these have demonstrated efficacy in lowering sperm-egg interaction *in vitro*; nevertheless, immunization trials have not been very successful in terms of their ability to provide contraception.

Lactate dehydrogenase (LDH) - The LDH family is composed of at least three genes *Ldha*, *Ldhb* and *Ldhc* that translates into different subunits of proteins A, B and C respectively. The *Ldha* and *Ldhc* genes are situated on chromosomes 11 and 7 respectively, in humans and mouse genome (Edwards *et al* 1989), whereas the *Ldhb* gene is

located on chromosomes 12 in humans and on chromosome 6 in mouse (Takeno) ^[99]. According to the gene evolution theory, initially *Ldh* gene duplicated in vertebrates, giving rise to the *Ldha* and *Ldhb* genes (Markert *et al* 1975) ^[100]. Later the *Ldha* and *Ldhb* genes underwent second gene duplication, leading to the emergence of *Ldhc* gene in fish, columbid birds (Zinkham *et al* 1969, Mannen *et al* 1997) ^[101, 102] and mammals (Li *et al* 1983b, Millan *et al* 1987) ^[103, 104].

The protein family catalyzes the redox reaction involving inter-conversion of pyruvate into lactate (Everse and Kaplan 1973) ^[29]. The two subunits of LDH *viz.* A and B form homo or heterotetramers that are dispersed throughout the body in various configurations to reflect the metabolic needs of various tissues. LDH-A is more prevalent in skeletal muscle and LDH-B is the most common type in cardiac muscle. Thus, skeletal muscles may have more A homotetramers and AB heterotetramers than the heart. The tetramers differ in their kinetics, which represents the tissue's total LDH activity. For instance, compared to LDH-A, LDH-B is inhibited at higher substrate concentrations because lactate production rises during exercise, LDH-A is therefore more suited to function in muscle cells, whereas high lactate levels would inhibit LDH-B and be harmful to the metabolism of cardiac muscle cells (Goldberg *et al* 2010) ^[5]. In male germ cells, LDH-C was the first testis-specific isozyme found. LDH-C was first discovered in human spermatozoa and spermatogenic cells of the testes using gel electrophoresis. Later, it was found that this testis-specific variant of the LDH family, earlier known as LDH-X, was a distinct third gene product and its heterotetramers can be easily assembled *in vitro*. Lactate dehydrogenase isozymes are ubiquitous in vertebrates, tissue and cell specific, developmentally controlled and are multifunctional according to various biological, biochemical and genetic studies (Goldberg *et al* 2010) ^[5]. The structure of mouse LDH-C4 was determined by crystallography (Goldberg 1972, Musick and Rossmann, 1979) ^[105, 106] and full sequences for LDH-C4 were made available (Pan *et al* 1983) ^[107]. According to these structural studies, LDH-C was found to be homologous to LDH-A and LDH-B subunits in mice and humans, exhibiting 72.5% and 75.3% similarity with LDH-A and 64.5% and 69.8% identity with LDH-B, respectively. The three-dimensional structure of LDH-C4 exhibited changes and alterations in the amino acid sequence, which endows LDH-C4 with special structural (Hogrefe *et al* 1987) ^[108] and functional characteristics (Blanco *et al* 1976, Li *et al* 1983a) ^[109, 110].

Using gel electrophoresis (Goldberg and Hawtrey 1967) and immunohistochemistry (Hintz and Goldberg 1977) the presence of LDH-C protein in the testis was demonstrated. The protein was first identified in preleptotene spermatocytes and was widely distributed in spermatids and spermatozoa (Li *et al* 1989). Pachytene spermatocytes also contained LDH-A; however, the majority of LDH activity in male germ cells originated from LDH-C4 (Erickson *et al* 1975, Li *et al* 1989; Odet *et al* 2008) ^[28]. Heterotetramers containing A and C subunits were not found in human or murine testis (Goldberg and Hawtrey 1967, Li *et al* 1989) ^[111, 112]. According to Millan *et al* (1987) ^[104], the relative hydrophobicity of LDH-C raised the possibility that it is situated close to the plasma membrane. LDH-C was discovered in the plasma membrane fraction by Alvarez and Storey (1984) ^[1] after separating several subcellular

fractions of rabbit spermatozoa. The finding was supported by the discovery of LDH-C at the spermatozoa's surface (Erickson *et al* 1975, Beyler *et al* 1985) ^[28, 5]. At first, it was thought that LDH-C4 was unique to male germ cells (Blanco and Zinkham 1963, Goldberg 1963) ^[8, 7] and LDH-B was the major LDH isozyme in oocytes (Roller *et al* 1989) ^[114]. But in 2006 Coonrod and his co-workers demonstrated that the LDH-C protein is also present in the preimplantation blastocyst in oocytes and fertilized eggs at the germinal vesicle stage. The enzymatic activity of LDH-C4 could not be detected in egg extracts because of its low level. The amount of LDH-C4 transcript in oocytes (102 per million transcripts) is significantly less than that in the entire testis (2844 per million transcripts). The role of LDH-C4 in oogenesis, oocyte maturation or early development is still unclear but it is well documented that *Ldhc* null females are fertile.

Goldberg *et al* (2001) ^[115] used a chimeric peptide containing T-cell epitope of tetanus toxin and a B-cell epitope of LDH-C4 to immunize four sexually mature male baboons (*Papio sp.*). The animals were vaccinated on day 0 and then given booster shots on days 29, 61 and 344. Antibodies were raised against LDH-C4 and it considerably lowered fertility in female monkey. The investigation showed that sperm from these immunized males had a reduced zona binding capacity and the LDH-C4 epitope did not induce autoimmune illness. Their findings imply that production of anti-sperm antibodies can pave the way for the development of a safe male immunocontraceptive. To evaluate whether LDH-C is required for spermatogenesis, oogenesis and their functions an experiment was conducted in which LDH-C gene was disrupted. Fertility was severely affected in target disrupted LDH-C male mice whereas no effect was observed in case of females. Testis, sperm morphology and spermatogenesis appeared normal. However, compared to wild type sperm, total LDH enzymatic activity was much lower in LDH-C disrupted sperm, demonstrating that the LDH-C homotetramer (LDH-C4) is responsible for the majority of LDH activity in sperm. When isolated, the LDH-C disrupted sperm were motile at first, but their ATP levels and motility declined quickly, compared to wild-type sperm. Furthermore, LDH-C disrupted sperm were unable to achieve hyperactivated motility, enter the zona pellucida *in vitro* or undergo the phosphorylation processes associated with capacitation. The study found that LDH-C is critical for maintaining glycolysis and ATP synthesis in the flagellum, which is essential for male fertility and sperm function (Odet *et al* 2008) ^[116]. Later in 2011, Odet and his co-workers conducted another study to show that male infertility can be caused by disruption of germ cell specific lactate dehydrogenase C gene. Defects in sperm function, quick decline in sperm ATP levels, reduced motility and inability to achieve hyperactivated motility were the possible explanations of infertility. experiment hypothesized that lack of LDH-C would disrupt glycolysis via feedback inhibition, either by producing a malfunction in the replenishment of the nicotinamide adenine dinucleotide (NAD⁺) cofactor required for the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or by pyruvate accumulation. In sperms without LDH-C, glucose intake was disturbed, NAD:NADH ratio and pyruvate levels were also impacted, with pyruvate being quickly converted to lactate. The findings supported the hypothesis that the

residual LDH activity in sperm without LDH-C is attributable to LDH-A that is found in the major portion of flagellum and that LDH-C also have a function in sperm energy metabolism maintenance. Coimmunoprecipitation combined with mass spectrometry found 27 proteins linked with LDH-C, majority of which are involved in ATP production, transport and sequestration (Odet *et al* 2011) [117].

Identification and characterization of sperm specific proteins About 25% of infertile couples experience conception failure primarily due to male infertility. The proteins on the spermatozoa have a role in the causes of male infertility. A study determined whether the spermatozoa of fertile and infertile males expressed different sperm membrane proteins and functions of these proteins. A 57 kDa protein was detected and refined by various chromatographic methods. Immunofluorescence, enzyme linked immunosorbant assay (ELISA), flow cytometry, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis characterised the protein. In vitro research revealed the function of 57 kDa protein in sperm-oocyte interaction. Infertile and normozoospermic patient's sperm extracts displayed 16–18 major and 12–15 minor protein bands. In non-acrosome-reacted spermatozoa (NAR), the protein was localised to the head, whereas in acrosome-reacted (AR) spermatozoa, it was relocated to the equatorial segment. In a dose-dependent way, the antibody against the purified 57 kDa protein prevented human sperm from attaching to zona-free hamster eggs (Rajeev and Reddy 2004).

SDS-PAGE characterized the proteins in the epididymal fluid, epididymal tissue and sperm membranes of mongrel dogs. Dog spermatozoa extracts were subjected to SDS-PAGE under reducing conditions to detect proteins with molecular weight of 173, 135, 94, 72, 65, 46, 36, 23, 17 kDa; 55, 46, 36, 28, 23 kDa; 173, 116, 86, 72, 62, 55, 46, 42, 36, 32, 28, 23, 20, 17 kDa and 173, 116, 86, 72, 62, 55, 46, 42, 36, 32, 28, 23, 20, 17 kDa using Triton X-100, deoxycholate (DOC), sodium dodecyl sulphate (SDS) and lithium diiodosalicylate (LDIS) respectively. The proteins with molecular weight 17 kDa, 23/32 kDa, 36 kDa, and 46 kDa showed correlation with the percentages of motility, hyper-osmotic solution (HOS) positive, motile and live spermatozoa. SDS-PAGE separated around 7–9 proteins, each ranging in size from 17–207 kDa, from the tissue extracts and canine luminal fluid. The luminal fluid contained only 55 and 36 kDa proteins suggesting that they are secretory and functional in nature. The sperm membrane extracts included the 173, 135, 94, 80 and 20 kDa proteins, indicating that these were either modified or acquired during ejaculation or epididymal maturation. The identification of surface proteins linked to spermatozoa maturation during epididymal transit was demonstrated by the presence of 55, 46, 42, 36, 28, 23 and 17 kDa proteins in sperm extracts, luminal fluid and tissue extracts (Cheema *et al* 2011) [12].

An investigation on the electrophoretic profile of sperm membrane proteins in Assam Hill goat (AHG) bucks and their relationship to properties of fresh semen was conducted. Sixty-four ejaculates, eight from each of the eight bucks were collected using an artificial vaginal canal. Two equal portions were separated from each ejaculate. The first portion was utilized to assess fresh semen and the second portion was used to study proteins. The molecular weight of 20 distinct protein bands was identified by SDS-

PAGE ranging from 10 kDa to 75 kDa. Six protein bands out of the twenty were consistently found in all the eight ejaculates (100%). Significant positive correlations were found between specific fresh semen properties and protein bands with molecular weights of 10, 14, 22, 28, 55, 57, and 60 kDa; in contrast, a negative association was observed between fresh semen features and the 47 kDa protein band (Deori *et al* 2016) [23].

Another research applied the use of hetero- and iso-antibodies to identify and characterise antigenic proteins specific to dog sperm. One male dog and two male rabbits received injections of dog sperm proteins isolated with sodium dodecyl sulphate- sperm membrane extract (SDS-SME) and lithium diiodosalicylate- sperm membrane extract (LDIS-SME). A robust precipitation line in immunodiffusion and an increase in the quantity of immunoglobulin (Ig) in the blood serum demonstrated a fast humoral response. Nine antigenic proteins measuring 97, 46, 42, 38, 36, 32, 30, 28 and 20 kDa were found in dog sperm by partially purified iso-Ig and only three protein bands measuring 97, 38 and 36 kDa were discovered by partially isolated hetero-Ig from rabbits. The role of these antigens in sperm maturation during epididymal transit and their circulation in blood was demonstrated by cross-reaction of anti-LDIS-SME and anti-SDS-SME antibodies with 46 and 28 kDa protein bands in epididymal fluid, tissue and blood plasma. Anti-SME in blood serum, tissue or fluid recognised 14 and 116 kDa antigens depicting their non-specific nature. Antigenic proteins were more heavily localised on the acrosomal cap. The ejaculates of two dogs from 45–90 and 90–150 days post immunization (DPI) showed a substantial ($p < 0.05$) decrease in motility, live spermatozoa, HOS positive, sperm count and an increase in sperm abnormalities. Sperm parameters showed an improvement starting at 150 DPI and returned to levels comparable to canine ejaculates obtained before vaccination at 210 DPI (Cheema *et al* 2012) [13].

Therefore, it can be said that sperm membrane extract (SME) contains antigenic proteins with immuno-contraceptive potential. However, their molecular structure, specificity and function need to be studied in order to better characterize and understand their role in gamete interaction.

Conclusion

It is highly desirable that the contraceptive vaccinations that have been proposed so far do not completely prevent fertility in all of the immunized subjects. Despite great anticipation for safer, more effective and longer-acting contraceptives, there are currently no approved contraceptive vaccines in the market. Additionally, there exists heterogeneity in the immunological response generated by immunization; certain individual exhibit elevated antibody titers for an extended duration, whereas others do so for a brief period. Contraceptive vaccines have a place as a nonsurgical method of achieving sterility and a tool for birth spacing. In terms of comfort, affordability, effectiveness, infrequent consequences and likely non selective impact on animal populations as well as people, the future of contraceptive vaccines appears bright. More study is needed in this area, with the ultimate goal of developing a vaccine that is more immunogenically successful.

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