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Role of preputial washing in reducing microbial load and improving bovine semen quality

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ABSTRACT

Quality semen production remains the main focus and objective of semen processing laboratories throughout the world. Bacterial and other microbial contaminants affect the semen quality and hence the fertility, and also lead to reproductive disorders as well as lower conception rates and increased embryonic mortality, abortion and other complications in females. Microbial contamination affects the semen adversely, by exerting direct spermicidal effect, formation of reactive oxygen species, toxin production, adherence with spermatozoa, deriving nutrients and oxygen from the medium and thus competing with spermatozoa for the factors of growth and normal functioning. Despite hygienic measures, several ubiquitous and opportunistic microbes find their ways into semen during collection, processing, and storage of semen, and survive even during freezing. Stringent sanitary precautions are therefore required at every step of collecting semen and its processing. Preputial cavity is considered as main source of semen contaminating microorganisms. Flushing the preputial cavity with normal saline or any suitable liquid combination with antimicrobial activity, prior to semen collection reduces the microbial load and thereby improves the semen quality.

1. Introduction

Semen cryopreservation and artificial insemination (AI) as a biotechnological tool in animal reproduction has gained momentum over years due to its undue advantages in terms of better managemental practices through reduced bull maintenance cost, maximum number of females being served from semen of a single elite bull, thus leading to faster dissemination of superior germplasm, ease of transporting semen over long distances, preventing the spread of venereal diseases, and keeping better maintenance of breeding records, *etc.* However, quality of semen being used to inseminate female animals determines the success of this unique biotechnological tool to a very large extent, and thus

necessitates the harvest of quality ejaculate from males. Semen, while passing through male reproductive tract, has every chance of being contaminated with micro flora. It is now known that microbial contaminants of semen deteriorate the semen quality[1] and also affect the subsequent fertility[2,3]. Microbial contamination is considered as a major concern for most semen production centers. Microorganisms usually gain entry through preputial or penile orifice. Infection in animals, environment, preputial sheath/cavity, entry of organisms during semen collection, processing and packaging, contribute to the microbial load of semen. Preputial cavity is said to be one of the major sources of microbial contamination

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in semen samples[4,5]. The principal parts of the contamination are saprophytic or opportunistic pathogens from the prepuce and in some cases from more highly suited parts of the male genital organs[6]. Increased microbial load leads to deterioration of sperm motility and viability[7], and also facilitates transmission of infection to females, both during natural service as well as AI. The bacterial contamination in semen can be reduced by regular rinsing of the preputial cavity and keeping bulls healthy and clean[8]. Perfect AI practices and managerial interventions like preputial washing thus can be helpful in improving the hygiene status of semen, which in turn is important for vitality of spermatozoa and for the fertility of inseminated females.

2. Microbial contamination and semen quality

Semen quality is regarded as a measure of fertility in male animals and is an indicative of fertilizing potential of semen. Semen production, being a quantitative trait, is affected by genetic as well as nongenetic factors. The major factors affecting semen quality include age, breed, nutrition, genetics, management, temperature and season, scrotal circumference, disease of testis, epididymis and accessory glands, etc. Apart from these, microbial load of the semen has profound effect on the quality of semen. Semen quality is adversely affected by bacterial contamination[1], which in turn leads to negative effects on subsequent fertility[2,3]. Presence of bacteria, fungi and viruses has been detected in semen samples that deteriorate semen quality as well as transmit the pathogens to next generation. The role of specific microbes in semen leading to reproductive disorder among dairy animals is well established. Despite sanitary precautions, several ubiquitous and opportunistic microbes find their ways into semen during harvesting, processing, and storage of semen[9] and survive even during freezing. Increased bacterial load leads to deterioration of sperm motility and viability[7]. Microorganisms from bull semen make their ways to genital system of female animals by means of artificial breeding or natural service. The organisms may be bacterial, chlamydial, mycotic or viral and classed as pathogenic, potentially pathogenic or non-pathogenic.

Biological contaminants of semen affect its quality in a number of ways. Roberts[10] observed deleterious effect of bacterial load on fertility of semen. Microbial contamination affects motility, morphology and various semen quality parameters[11]. Sperm cells are said to be adversely affected by bacteria due to their direct spermicidal effect. To cite an example, early studies concluded concentration-dependent spermicidal effects of *Escherichia coli* (*E. coli*). Bacteria like *E. coli* significantly decrease sperm motility and lead to increased agglutination of spermatozoa. Additionally, *E. coli* has been determined to adhere to the sperm membrane/surface via mannose binding sites[12,13]. *E. coli* binding results in ultra-structural damage to the plasmalemma of sperm cells[1]. Trichomonads too adhere to spermatozoa leading to agglutination of sperm cells, reduced sperm motility, and phagocytosis of spermatozoa[14]. Competition with cells (spermatozoa) for nutrients, oxygen and other such factors required for growth and normal functioning, has also been suggested as one of the mechanisms by which microbial

contamination affects the semen quality[15]. Sperm functioning and potential to fertilize are being compromised by microbial contamination owing to production of reactive oxygen species (ROS) by macrophages and polymorphonuclear granulocytes. ROS at higher levels may affect sperm quality by compromising membrane integrity, chromatin integrity, blocking oxidative metabolism, and reducing chances of fertilization and thereby subsequent conception[16]. Microbial contaminants lead to decrease in motility due to adherence with sperm cells. Acrosome intact spermatozoa percentage may be reduced in semen as microorganisms affect the acrosome directly through toxin production[17].

As the total microbial number varies in semen samples, so does the type of microorganisms. Many types of bacteria have been reported to occur even in frozen semen[18]. Yaniz *et al*[19] reported the presence of aerobic bacteria in almost all the harvested samples of semen. The microbial flora of semen may cause reproductive disorders in females, increase embryonic mortality, lower conception rates, and cause abortion and other complications at a large scale, as AI of cows and heifers with frozen semen is widely practiced.

3. Prepuce and preputial cavity (anatomy)

The prepuce or sheath is a tubular structure covering the cranial free portion of the penis in non-erect state. The penis is retracted into the sheath when the bull is quiescent. Donaldson and Aubrey[20] described the anatomical structures of the prepuce of the bull, noting that the parietal layer of the prepuce is attached loosely by an extensive connective tissue to the underlying tissue. Preputial cavity is 35 cm to 40 cm long and narrow cavity, comprising of two parts: the external, and the internal layer. Sheath, the external layer is skin covered with hair and extends from the scrotum to within 6 cm of the umbilicus where the external layer is reflected vertically and laterally, forming the thick margin of the preputial orifice. The preputial orifice forms the opening of the prepuce. It is about 5 cm behind the umbilicus and is about 2.5 cm in diameter but capable of dilatation, faces downward and a little forward and remains hidden by number of long hairs. Coarse hair up to 10 cm in length marks the preputial orifice which is large enough to admit a finger readily. Dorsally the external layer is in continuity with the wall of abdomen.

In the mature bull, the mucous membrane of the internal parietal layer of the prepuce extends from the preputial orifice about 35 cm posterior (caudal), and then turns forward on the penis as the visceral layer terminating as the epithelium of the penis approximately 12 cm distal to the cranial end of the penis[21]. Cavity of the prepuce is rather small but capable of considerable dilatation. The glans is in the caudal half of the cavity.

The visceral layer is loosely attached to the body of the penis but it is closely attached to the glans penis, and is glandless. According to Sisson and Grossman[21], the internal parietal layer of the prepuce is covered with squamous, stratified epithelium and coiled tubular glands which are involuntary and have a serous-mucoid secretion that serves as a lubricant for the preputial and penile tissue.

Penile extension necessary for breeding comes from the sigmoid flexure of the penis located just posterior to the scrotum. This

flexure is effaced during erection. As the penis is extended past the preputial orifice and the prepuce unfolds until at full extension, the tissue covering the erect penis is taut. Young, inexperienced bulls pastured together will attempt to mount each other and also engage in masturbation, increasing the possibility of increased microbial load and trauma to the preputial tissue.

There are two pairs of preputial muscles which are derivatives of the cutaneous muscle. The anterior preputial muscles, or protractors, are two flat bands, 5 cm or more in width which arise closely in the xiphoid region about 20 cm in front of the preputial orifice. They diverge around the umbilicus and then unite behind the preputial orifice and act to draw the prepuce forward. The posterior preputial muscles, or retractors, arise in the inguinal region and converge on the anterior portion of the prepuce. The pudic nerve pursues a flexuous course along the dorsum penis and ramifies in the glans penis and the penile layer of the prepuce[21].

However, anatomical differences are found with relation to size and character of sheath and prepuce, between indigenous bulls of India and exotic cattle bulls and even within the bulls of same breed. Sheath character is an important trait in breeding bulls. *Bos indicus* and cattle with *Bos indicus* breeding (e.g., Brahman, Santa Gertrudis) can have a very loose and very pendulous sheath. These bulls also tend to have large preputial orifices and an excessive amount of preputial mucosa. Bulls have loose and pendulous sheaths which may vary from small to extremely large size. For example, while judging for the breed characteristics, if sheath is very small, bulls are not considered Sahiwal[22]. Extremely loose and pendulous sheaths are more prone to injury than less pendulous sheaths. Preference should be for bulls with lesser loose sheaths as such bulls are at a lesser risk for sheath and penile injuries during travel and mating. Prolapse of the prepuce is a problem in Sahiwal bulls and bulls differ a lot in this regard, and it gets worse with the age of animal[22].

4. Preputial cavity and microbial contamination

Many studies have confirmed the presence of same microbial species in prepuce as well as semen, suggesting that preputial cavity must be contributing majorly to the microbial load of semen. Microflora from preputial cavity contaminate the semen[23], by making their way into the semen during collection process. Bacteria most commonly reported in semen of healthy bulls include *Coliforms*, *Corynebacteria* and *Diphtheroids*, *Micrococci*, *Bacillus* spp., *Proteus* spp., etc. Many more organisms have been isolated but comparatively at lower frequencies, and are believed to be transient contaminants from bedding, soil, air, manure and other environmental factors. These include species of *Pseudomonas*, *Streptococci*, *Staphylococci*, *Actinomyces*, *Aerobacter*, *Alcaligenes*, *Brevibacterium*, *Chromobacterium*, *Enterococci*, *Flavobacterium*, *Klebsiella*, *Serratia*, *Yeasts*, etc. Different micro-organisms identified in frozen semen samples include species of *Staphylococci*, *Micrococci*, *Corynebacterium*, *Coliforms*, *Pseudomonas*, *Proteus*, *Klebsiella*, *Bacillus*, other than *Bacillus anthracis*. However, most commonly found microbes in the semen are *Diphtheroids*, *Pseudomonas*, *Streptococci*, *Staphylococci*, *Micrococci*, *Bacilli*, *Actinomyces*, *E.coli*, *Campylobacter*, *Corynebacterium pyogens*,

Trichomonas and *Brucella*[4]. Several of these bacteria have been identified in association with breeding failure in cattle and warrants precautionary and preventive measures for successful breeding program[24]. Semen samples most frequently are found to be contaminated with *Staphylococci*, *Coliforms*, *Streptococci*, etc[25] which negatively affect the motility and viability of bovine semen.

A variety of microorganisms have been isolated from semen and the prepuce. Preputial sac has been found to harbour saprophytic microflora and other pathogens. In a study, Flastscher and Holzmann[26] identified similar microflora in semen and preputial cavity, including 27 different kinds of bacteria, blastomycetes and fungi, in 337 samples of semen and 139 preputial washes.

Several bacteria like *Campylobacter fetus* (*C. fetus*) ssp. *venerealis* and *C. fetus* ssp. *fetus*, establish a chronic infection with absence of any characteristic sign, and are found to be located in penis, prepuce and urethra of bulls[27]. *Tritrichomonas foetus* (*T. foetus*) and *C. fetus venerealis*, as such do not cause disease in the bulls but are transmitted to females and cause diseases in them[28,29]. In infected males, these organisms become located on epithelium of the cavity of prepuce. As the age advances, microbes get a microaerophilic environment, due to increased depth of epithelial crypts of the prepuce. This supports their replication and survival. These organisms are found in close association with proximal part of prepuce and glans penis, and thus are very likely to contaminate semen. *T. foetus* and *C. fetus venerealis* withstand the process of semen cryopreservation[29]. Transmission of *T. foetus* or *C. fetus* to the female can result in inflammatory conditions of vagina, cervix, and uterus, and therefore pave way for reproductive disorders, infertility, irregularities in estrus cycle, embryonic mortality, and abortion, although rarely, (up to 4 months of gestation in case of *T. foetus*; 4–7 months of gestation in case of *C. fetus*)[28,29]. Fungal species, both in semen and preputial washings, have been reported to occur. These include the species belonging to *Alternaria*, *Aspergillus*, *Candida*, *Cladosporium*, *Penicilium*, *Thamnidium* and other such genera[30,31].

It has been suggested after a study on 45 donor bulls that mycoplasma establish in the distal part of urethra and also in prepuce. Therefore, it would be advisable to wash and disinfect these sites before semen collection[32]. Parts of male reproductive system like prepuce and the urethra normally inhabit *Ureaplasma diversum*, while as testicles and accessory glands rarely do[33]. Species of *Acholeplasma* have been reported from 32% of preputial washes and 12% of semen samples; however, there seem to be not much indications of its relation with infertility problems[32]. Clamidia was found in 9.2%, 10.7% and 18% of semen samples, preputial washes and faecal samples, respectively in a study involving 120 male bovines in 6 German federal states[34]. In the transmission of venereal vibriosis, the major role of bulls has been suggested by many epidemiological studies[35]. Further, isolation of *C. fetus* subsp. *fetus* from preputial fluids by making use of selective media made the bull animal of choice for the herd diagnosis of the disease[36].

A study[37] reported differences in the microbial population in preputial cavity of cattle and buffalo breeding bulls and corresponding differences of microbial load in semen. Microbial load was seen lesser in buffalo compared to cattle breeding bulls.

Various types of bacteria present in soil, bedding, and manure gain entry through preputial orifice into the preputial cavity of bulls[38]. Probable reason for higher bacterial count in semen of Gir bulls was attributed to their predisposition to preputial prolapse and thus exposure to infection from surroundings[39].

Bovine herpesvirus 1 has been reported to replicate in certain parts of male reproductive system notably preputial mucosa, penis, and distal part of urethra, and it affects the genital tract of males causing infectious pustular balanoposthitis[40]. So, apart from bacteria and protozoa, viruses too have been found to be associated with preputial cavity and subsequently the semen.

5. Effect of preputial washing on semen quality

Preputial cavity significantly contributes to the microflora most commonly reported in semen[4,5]. Bacteria adapt to a wide and varying set of conditions. As such semen extenders do not specifically nurture and prolong *in vitro* sperm cell viability, but also facilitate growth and survival of contaminant microflora[41]. Zamjanis[42] flushed the preputial cavity of bull just before semen collection to reduce the bacterial count in bull semen. Prasad and Pachauri[43] washed the preputial cavity with one of the four solutions of antibiotics (benzylpenicillin and/or streptomycin, oxytetracycline) just before collection of semen, which resulted in reduction in bacterial number in semen by 61% to 77%. Meredith[44] found the mean bacterial population per mL of ejaculate was 40.4 10³ for seven control bulls and 365 10³ for the eight bulls seen to evert preputial epithelium. Reddy *et al*[4] found SPC ($\times 10^3$) 258, 834 and 1 177 in preputial washings and 43, 97 and 101 in semen of bulls of less than 4 years, 4-8 years and greater than 8 years age, respectively. Gangadhar *et al*[45] observed 442 ± 100 , $10\ 559 \pm 3\ 059$ and 61 ± 14 bacterial load per mL in frozen semen of buffalo bulls at the first, second and third centres, respectively, in South India. Kher and Dholakia[46] found the mean bacterial load per mL were 53 229, 63 153 and 26 200 in washings and 5 373, 3 575 and 2 456 in neat semen of Murrah bulls in summer, winter and monsoon seasons, respectively.

It was observed that the corresponding bacterial load in frozen semen was significantly low, when compared with that of neat semen. In the process of freezing, when the neat semen is diluted and equilibrated, there was interaction between the antibiotics added to the diluent and the organisms and eventually there was some bacteriostatic and bactericidal effect, and during the first stage there is some reduction in the load of organisms. Secondly, during freezing, some organisms could not have been able to withstand sudden reduction of temperature, and thus the bacterial load was further reduced. The presence of organisms has spermicidal effect and also reduces the fertility of bulls[47]. Ramaswamy *et al*[48] found the range of microbial count varied between 7.0×10^3 and 540.0×10^3 organisms/mL of the frozen semen. Kumar *et al*[49] revealed that the mean bacterial load in the first ejaculate was 1 100 organisms per mL as against 4 860 organisms per mL in the second ejaculate. More than the four fold increase in the bacterial load of the second ejaculate can be attributed to a greater contamination of the artificial

vagina with the perpetual discharge. Bindra *et al*[50] observed the better motility percentage and live sperm count in both fresh and post thawed semen of preputial washed bulls, and attributed it to pre-freeze quality improvement and reduction in bacterial contamination.

Dela Pena *et al*[51] reported that the prepuce of the bulls before and after washing contained a wide population of bacteria. There were lesser types of bacteria present in the fresh buffalo semen compared with those in the prepuce. The frozen buffalo semen contained the least number of bacterial organisms. Rathnamma *et al*[52] found the bacterial count ($\times 10^3$) 7.28–20.90, 0.03–3.34, 5.05–171.40 and 0.81–39.04 in undiluted semen, extended semen, frozen semen and stored (15 days) semen per mL, respectively, in Holstein-Friesian bulls. Jasial *et al*[53] observed zero to more than 50 000 bacterial load per mL of buffalo bull semen. Ahmed *et al*[51] found the bacterial count per mL $253.05 \times 10^3 \pm 37.10 \times 10^3$, $14.70 \times 10^3 \pm 2.50 \times 10^3$, 207.00 ± 29.12 , 2.08 ± 0.85 , 1.25 ± 0.69 and 12.40 ± 4.82 in preputial washings, fresh semen, extender, empty straw washings, sealing powder and artificial vagina, respectively, in a study on Murrah bulls. The maximum coliform count recorded (540×10^3) was far below the detrimental level of 30 million/mL[55]. Prasad and Pachauri[43] reported that preputial washing with antibiotic solution could be able to reduce the bacterial contamination in raw semen by 61% to 77%. Bhakat and Raina[56] observed the mean values of bacterial load in Murrah bulls and found reduction in bacterial count after deep freezing which could be attributed to injury of some bacterial cells, and sensitive to ultra-low temperature ($-196\ ^\circ\text{C}$) used for freezing purpose. The reduction in total bacterial count in frozen semen samples after antibiotic treatment has been recorded. Preputial cavity commonly harbours some bacteria which may contaminate the semen and washing with antibiotic solution could reduce the contamination. It was observed that a combination of Streptomycin, Penicillin and Gentamicin, when employed for preputial washing, reduces the microflora to the maximum extent. The preputial washing coupled with Streptomycin + Penicillin + Gentamicin combination treatment was found to be most efficient for reduction of bacterial load to a greater extent in the bovine semen, in fresh as well as in post-thawed samples without affecting semen quality and preservability.

For better success rate in assisted reproductive technology (ART) centres, before cryopreservation of semen, preputial washing is recommended for reducing the microbial load to achieve better post thaw results[57]. Further, by keeping bulls clean and regular rinsing of the preputial cavity, the number of bacteria in semen can be decreased[8]. However, anatomical differences with relation to size of sheath and prepuce existing between breeds of exotic and indigenous bulls, should be taken care of while deciding amount of liquid (mostly normal saline) to be used for preputial washing. Further, stringent hygienic measures are also required before as well as during semen collection and processing[24].

Preputial washing should preferably be carried out on the day of collection and before collecting semen[58]. The procedure is carried out by infusing liquid (mostly normal saline) into the preputial cavity by use of a syringe and tube. It is also being carried out by introducing physiological saline under gravity into prepuce. Moreover optimum temperature regulation of the liquid to be

infused, pressure and the rate at which the liquid should be infused into the cavity to ensure efficiency of the procedure, needs to be taken care of. Normally the liquid is warmed to body temperature of animal prior to its use. Animals are restrained properly in a crate, preputial hairs clipped to a length of maximum 2 cm; sheath is washed externally to remove any dirt, debris or dung present over there. This is followed by insertion of catheter (usually dry plastic uterine infusion pipette) as far as possible into the prepuce, slowly and gently. The person carrying out preputial washing is advised to use disposable gloves and separate sterilized nozzle/catheter for each bull to avoid transmission of infection[58]. A hand is clamped over preputial orifice to seal around catheter and hold it in place, liquid is infused into the cavity, and with use of other hand massage is done thoroughly along full length of prepuce to scrotum for about one minute. Occasionally bulls urinate during the procedure which necessitates re-infusion of liquid. After preputial washing of all the bulls is over, ground area is washed with running tap water, or water at farm for washing purpose.

6. Conclusion

Preputial washing as a managerial tool is highly valuable for harvesting quality semen and thus maximising the use of good quality sires especially in developing countries like India, where lack of quality semen has been the main hurdle in dairy animal improvement. Proper hygienic measures and sanitary precautions are required at every step of semen collection and processing to achieve success in AI programmes. Preputial washing significantly reduces the microbial load and thus improves the semen quality. This aptly justifies the employment of preputial washing as a routine practice at every semen station and semen processing laboratory.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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