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CRITICAL CONTROL POINTS FOR QUALITY SEMEN PRODUCTION AND UTILIZATION: AN OVERVIEW

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Bull contributes more than fifty percent towards the success of any herd improvement programme; they also contribute towards lower pregnancy rate equally as female. Appropriate and careful bull management is therefore essential, considering critical points step by step starting from collection phase of semen to their storage. Shifting of subjective to objective assessment of semen like computer assisted semen analysis (CASA) & flow cytometry at bull station is essential component which aids in quality semen production. More innovative techniques are used at artificial breeding center like hemi zona assay, comet assay, sperm chromatin structure assay (SCSA), sperm fluorescence *in situ* hybridization analysis (FISH) etc. to aid quality semen production and processing in well organized unit. Researches reveal that selection of sperm and processing interventions like antioxidant fortification is crucial points in quality semen production. Moreover a complete protocol is required, which contain different critical points starting from health of bulls to handling, processing and packaging semen from bulls. Ensuring these stringent measures would of course require technical inputs to the existing system in form of complete package.

Key words: Bull, semen, vaccination, stress

The impact of bull on overall production of cattle system as they exist today is extremely significant. It begins with bull born in system is therefore is one of the important critical steps. The success or failure is dependent on total out come which is combination of interaction among genetics, health, production, quality control etc. Threats associated with any system which could put it in danger must be attended before they have a chance to place an impact on system. The global targets for controlling hazards set by government should be achieved to attain quality production and safety. The important factors in effective handling of semen like the proper cleaning and sterilization of wares, especially important under field condition, is certain key issues that have not been addressed adequately. The procedures for cleaning and sterilization have often been followed in a casual way with little emphasis being given to the control points. However if bull is not properly monitored and screened for pathological problems related to handling and processing especially unhygienic, the semen would produce and spread the infection rapidly. A count of 500 non-pathogenic bacteria (International Organization of Biological standard, 1947) per dose of semen straw is permissible. Accordingly it is recommended that prepucial washing (a maximum of 50, 000 CFUs/ml) and in neat semen (1000-5000 count CFUs/ml) is permissible, more over no count for artificial insemination (A.I.) equipments and dilutors has been suggested as they are to be used after strict sterilization. Quite a few attempts have been made to improve the quality of semen. The improvement can be brought about by adopting strict hygienic measures including sterilization and precaution. The quality control while preserving semen should be done at each and every step starting from preparation of artificial vagina (A.V.), glass wares, plastic wares or preparation of media or extender. Thus for standardization of best method establishment of control points are

essential. The processing protocol varies in each station, depending on varying conditions. Thus it is clearly observed that each artificial breeding station is following a protocol, which is standard for their respective situation. However it is essential to revalidate semen collection and processing protocol at regular interval to achieve the standard.

There are substantial differences in fertility among individual bulls. Fertility is more important in a bull than individual cow, as one bull may be used to breed up to 40 female with natural service or thousands via artificial insemination. Sub-fertile bulls delay conception, prolong calving season resulting in economic losses. Individual bull should be monitored for identification sub-fertile and infertile bulls. Abnormal semen morphology can be identified however do not consistently identify subfetile bulls with apparently normal semen (Gadea et al., 2004). Fertility of bulls affected by different factors, therefore no single diagnostic test can precisely predict fertility, although a combination of test can be more informative (Kastelic and Thundathil, 2008). Post insemination sperm-oviduct interaction would significantly increase the ability to predict fertility (Flowers, 2013). Seminal plasma proteins apparently have potential as fertility predictors and more than four hundred proteins have been identified in a bull sperm plasma-membrane (Byrne et al., 2012).

Control of pathogens in frozen semen:

The major pathogens such as *Brucella abortus*, *Trichmonas fetus*, *Campylobacter sp.*, can be avoided through using ongoing disease surveillance and maintenance of specific pathogen free herds.

Other potential and non pathogenic microorganism for example Pseudomonas and Staphylococcus should also be taken seriously.

Consumers are not sentient of pathogenicity of microbes, which may be source of disease. There fore semen produced should be of standard, clean and free of contamination as possible.

Strict Bio-security protocol should be put in place across all activities.

Sources of bacterial contamination in frozen semen:

Collection phase: Prepucial sheath, AV liner and cone, lubricants, test tube, disposable collector's gloves, dummy animals and cork over semen collective tubes.

Laboratory phase: Water bath, water, extenders, glass wares, filling, sealing, packaging and laboratory hygiene.

Up to Insemination phase: Common pathogenic and non pathogenic organism can survive well in liquid nitrogen.

Fundamental principles of critical hazard analysis: [Buncic, 2006]

Identification of threat or risks (control points), Identification of critical control points (CCPs), Establishment of limit for each CCP, Establishment of a monitoring system for each CCP, Establishment of corrective action if CCP is out of control limit, Checking of efficiency of protocol and Proper documentation and record keeping.

Factors which influence fertility of sire herd: Fertility of bull should be up to the mark to produce quality semen like proper nutrition and herd health, structural correctness: eyes, feet, legs and skeleton, proper functioning of genitalia, adequate scrotal circumference, high grade semen, age at puberty, calving ease: maintenance of proper birth weight in calf, libido and serving capacity, social interactions and ranking, proteins present in the semen and fertility associated antigen

Control points in frozen semen productions:

Semen harvesting and its evaluation: It includes routine and periodical evaluation of semen. Routine evaluation of semen includes volume, colour, pH, concentration and progressive motility. Periodical evaluations at the interval of 3 months are required for evaluating morphology, intactness of acrosome and test for integrity of membrane i.e., HOS (Hypo osmotic swelling) test.

Bulls' selection: The bull should have high genetic merits for economic characters i.e., dam production or progeny tested having high fertility, high producing ability, good growth and prominent sexual behavior. Standard scrotal circumference and weight

gain index for various breed shall be fixed by initiating age wise recording of scrotal circumference with respect to age. Significant ($P<0.04$) positive correlation ($r = 0.72$) was observed between scrotal circumference and volume and concentration of sperm in crossbred bulls (Latif et al., 2009). Overall breeding soundness evaluation (BSE) plays an important step in selection of bull for quality semen production. Minimum standard of bull establishing pregnancy include sperm motility (≥ 30% progressive motility), sperm morphology (≥ 70% normal & ≤ 20% defective heads) and added scrotal circumference. Scrotal circumference is highly correlated with weight of testicular mass, which in turn positively correlated with daily sperm production and semen quality. Bulls with large testes have female siblings and daughters with earlier puberty and better fertility. Heritability of scrotal circumference in young bulls is approximately around 0.5 therefore respond well to selection (Barth, 2007).

Compensable Vs Un-compensable sperm abnormalities:

Compensable abnormalities can be overcome by increasing the dose used for artificial insemination in other words these abnormalities are associated with sperm reaching the zona pellucida, are associated with motility, acrosome intactness and plasma membrane integrity. Chromosomal abnormalities, protamine status and abnormality of m-RNA are common un-compensable defect. In addition, laboratory processing further enhances the sperm defects. The threshold quality for a particular bull or ejaculate can vary widely and depends on severity and ratio of compensable and un-compensable sperm defects (Amann and DeJarnette, 2012). Increase in fertility with increasing numbers of sperm is supposed to have compensable sperm defects, whereas those that quickly reach a plateau are considered to have more un-compensable sperm abnormalities. Most fertility curves reach their threshold when 70 percent of sperm have compensable defects (Flower, 2013).

Calving ease: The calving ease could be believed as a reproductive trait in bulls since birth weight is known to usually be the main cause of parturition difficulty. Female that experience difficult birth has reduced calf survivability and have lower the chances of rebreeding. Cows that do become pregnant, those that experience calving difficulty will usually display longer post-partum time to breed back (Cleere and Craig, 2010).

Proper pre-stimulation, false mount and restraint: Protocols of semen collection is either, Twice a week semen collection or once a week with two ejaculates per collection is adopted. As a result 85-90 collections per year are harvested from each bull. The sexual preparation should be standardized for individual bull by means of false mounts and restraints. Emphasis should be given on techniques rather taking collection mechanically.

Washing of bull and use of bull aprons: The bull apron is used to tie on heart girth of dummy. The apron should fit properly to the bull. The penis of the bull will not be rubbed against hind quarter of the teaser and get contaminated.

Prepucial washing: Infections usually passes through penile urethra. The sheath and prepuce are easily contaminated because of their contact with soil, dung, urine and other contaminants. The presence of bacteria in the prepuce has been correlated with contamination of semen. A significant decline in bacterial count was found after prepucial washing and there by improvement in semen quality and fertility was observed successively. Studies concluded that collection technique has greatest impact on bacterial contamination of semen collected by the gloved hand technique. Pathogens like *Escherichia coli* and *Staphylococcus aureus* were isolated from prepucial washing supposed to be the cause of low reproductive performance of herd (Moreira et al., 2013).

Twenty percent of semen sample were found positive for bacterial isolates and fungi in a random semen straw testing of Gujarat state of India using standard plate count method (Patel et al., 2011).

Testicular temperature: The temperature of scrotum must be 2-6^{o}C cooler than core body temperature for producing fertile semen ejaculates. Consequently increased

testicular temperature, regardless of cause reduces the semen qualities (Waites, 1970) especially with defective heads (Barth and Oko, 1989). Moreover, increased testicular temperature is a common underlying cause of infertility in bulls (Kastelic, 2013). When scrotal temperature increases sperm morphology is unaffected initially (interval equal to epididymal transit time) although subsequently decline (Barth and Oko, 1989). However in some of the experiment it was observed that sperm morphology may changes immediately after scrotum heating (Wildeus and Entwistle, 1983). Sperm morphology usually returns to normal after six week of cessation of thermal insult (Vogler et al., 1991). Conversely a prolonged increase in testicular temperature will increase the interval for recovery i.e., decrease semen quality is related to the severity and duration of thermal insult.

Scrotal skin is thin with less hair and extensive subcutaneous vasculature that promote heat loss by radiation. A long distinct scrotal neck and pendulous scrotum reduce testicular temperature by increasing the area for heat loss and enabling the testes to hang away from body when ambient temperatures are increased. Testicular vascular cone made up of pampniform plexus (Cook et al., 1994) of testicular artery helps in controlling temperature by transfusing heat from artery to vein. Scrotum surface is rich in sweat gland keeps the testis cool. Temperature gradient were 1.6, 0.4 and -0.2 for scrotal surface, subcutaneous and intra-testicular respectively (Kastelic et al., 1995). *Bos taurus* bulls are susceptible more to heat stress as compared to *Bos indicus* bull (Skinner and Louw, 1966) further heat stress in crossbred bull is less as compared to purebred taurus bull (Johnston et al., 1963). Heat stress leads to hypoxia to testicular cells however testicular temperature rise is route cause of semen deterioration rather hypoxia (Kastelic et al., 2008). Heating affect sertoli, leydig cell, further germ cells are most sensitive to heat (Waites and Setchell, 1990). Spermatocytes in meiotic prophase are killed by heat, where as spermatozoa that are more grown-up usually have metabolic and structural abnormalities (Setchell et al., 1971). The interval from cessation of heating to restoration of normal spermatozoa in the ejaculate corresponds to the interval from the beginning of differentiation of ejaculates, even though sperm morphology has returned to normal their utilization may decrease fertilization rates and increase the incidence of embryonic death (Burfening and Ulberg, 1968).

Bio-security of system: Bio-security of system includes site selection, prevailing wind and proximity to other organization which may affect this system. It also include external source of contamination specifically birds, rodents and wild animals. Control at this stage includes chain link fencing, bird netting, bait boxes and insect control especially fly and mosquitoes. The point of bio-security control is also applicable on resident bull as new comer put a direct effect on health status of existing herd. Isolation including health monitoring, vaccination and acclimation is critical and must be rigidly controlled to insure the continued health status. The status of source animal as well as current population in herd will determine the vaccination and testing programme, as well as the length of isolation. The entry of visitors should be restricted and controlled. Standards for equipments should also be fixed for maintenance of quality.

Feed delivery using truck should be properly disinfected after each trip, the ration need to guarantee that there is not potential contamination or disease introduction through the individual components. In case of outbreak of disease medicated feed and waters are required.

Quarantine: A Quarantine period of at least 60 days for new comer bulls is compulsory. Preferable separation of 5 km. is required with resident bulls. This quarantine period can be used to observe for feet, leg, back, eye problems and for infectious diseases.

Testing of disease and culling of bulls: Bull should be tested and culled if found positive for following diseases like TB, JD, Campylobactriosis, brucellosis and trichomoniasis. Bovine respiratory syncytial virus was also a prominent cause of lowered sperm quality in young bulls (Alm et al., 2009). Semen infected with Infectious bovine rhinotracheitis (IBR) and Bovine

viral diarrhea (BVD) are transmitted through semen should be culled (Pena-Joya et al., 2011). Infection is one of the main causes of infertility in buffalo (Perumal et al., 2012).

Bull shed: The bull shall have spacious individual pens with adequate loafing area with proper facility of food and water. Special arrangement of cooling system in summer is required like sprinkler fan and mist cooling. Disinfectant like formalin and phenyl based compound should not be used in bull sheds. Gluteraldehyde shall be used in place. Weekly spraying of sodium carbonate 4 percent solution may be used.

Vaccination stress and its amelioration: Vaccination is common stress factor that affects semen quality. It is well known that there is an elevation of body temperature during post-vaccination period and also the temperature of testes which causes derangement in spermatogenesis. Viral vaccination produces more deleterious effect than that with bacterial vaccines. Foot and Mouth Diseases (FMD) vaccination adversely affects the semen quality of exotic and crossbred bulls. The effect of vaccination was observed in KF crossbred bull and Murrah buffalo bull, which affect the semen profile significantly (Bhakat et al., 2010).The author recommended the suspension of semen collection and preservation till re-storage normal semen profile to avoid the failure of conception. However there is need to explore more about these issues to minimize the stressful conditions to the bulls. Since the practice of routine preventive vaccination of bulls against diseases like FMD, HS and BQ is in vogue in the Artificial Insemination Centers and Breeding Farm, this work is necessary with a view to find out the possible harmful effect of vaccination of spermatogenesis in bulls. Vaccination increased percentage of sperm cells with fragmented DNA by 10 fold on average (from 6.5±7.9 to 63.4±24.2) which restored after 40 day of vaccination (Gosalvez, et al., 2008).

Significant rise in concentration, motility and normal sperm was reported after supplementation vitamin C at dose rate of 1000 mg twice a day per bull (Akmal et al., 2006). Dietary vitamin E may be beneficial to improve semen characteristics (Eskanazi et al., 2005; Biswas et al., 2007) especially after vaccination in bull (Rao, 2009). Vaccination stress was also partially ameliorated by injection of Levamisole (Rao, 2009). The adverse effect of Gossypol feeding can be overcome by vitamin E supplementation to bull (Velasquez-Pereira et al., 1998). Dietary intake of antioxidants such as Vitamin E and C has been demonstrated to be critically important for normal semen quality and reproductive functions, as it protect cells from destructive oxidation of poly unsaturated fatty acid (PUFA, vitamin A) of plasma membrane. Vitamin A associated with spermatogenesis (Martins et al., 2009).

Effect of De-worming on semen qualities: Doramectin has no adverse effect on spermatogenesis, fertility or reproductive performance of males especially the dog (Wiebe and Howard, 2009). Ivermectin has a significant effect on reproductive potential of breeding ram. By observation up to eight week post Ivermectin injection, the semen volume increased, motility increased and other parameters of semen were unaffected (Tanyildizi and Bozkurt, 2002). Ivermectin when used in combination with "Diminazene aceturate" showed adverse effect on sperm profile and hormones like testosterone and Follicle stimulating hormone in Red sokoto buck (Onakpa et al., 2010). Ivermectin was found superior over albendazole and levamisole in preserving semen quality of Friesian bulls (Tag-El-Dien et al., 2011).

After injection of Ivermectin to horse at dose rate of 0.2mg/kg body weight, there was significant improvement in the quality of semen declared after eight weeks of observation. Semen volume was increased, post thaw motility was improved, sperm abnormality was reduced and sperm concentration was increased. There was no effect on testis and epididymis confirmed by necropsy.

Effect of silage feeding on semen qualities: A diet containing 70 percent olive-pulp silage and 30 percent alfalfa hay did not have a detrimental effect on sperm quality, increase percentage of unsaturated fatty acids in the sperm membrane and is also cheap (Farji et al., 2011).

Collector: Technically sound collector is desired for quality semen production. It is reported that there is differences in bull behavior and semen profile as a result of change in semen collectors. The semen collector should be neat and clean wearing light colour dress with white clean apron. The overall colour especially bright may affect the bull to produce quality semen.

Personnel's hygiene: Change room, sterilized aprons, caps and sterilized hands are essential.

Sterilization of glass wares and calibration of instruments: Glass wares like conical flask, measuring jars, thermometers should be sterilized before using them for semen dilution, measuring the semen extender, etc., calibrated at least once in a year to have accuracy in measurements. The thermometers also calibrated by sending them to standard equipment laboratory.

Environment control: Dust and contamination free processing room is required together with provision of fumigation, air condition and curtains. Environmental control not only required at processing level but also at the level of external environment. Semen quality is affected by several factor of which season is one of the major factors. It exerts its effect on reproductive performance through macro and micro climatic factors like temperature, humidity, rainfall and photoperiods (Bhakat et al., 2009).

Significant effect of aging on motility and concentration of spermatozoa was reported by different authors (Eskandar et al., 2012).

Artificial Vagina (AV): AV should be properly assembled with liner, cones and collection tube and well covered with wool lined leather jacket. Disinfection of AV includes disinfection of rubber ware and glass ware used for the purpose. Rubber wares being thermo-labile is sterilized by chemical sterilization, where as, glass ware are sterilized either by autoclaving or by microwave. Microwave sterilization is easier to perform, economical and reproducible than autoclaving. After 3 minutes in microwave oven the glass ware become free of contaminants. Size of AV is also important factor for proper ejaculation from a bull. Optimum size AV should be used for the purpose. AV should be properly sterilized after previous day of collection and overnight incubation at 42-45^{o}C. The liners and cones used for AV should be kept in polyethylene. Collection tube made up of borosilicate glass should be properly jacketed to maintain a temperature of 35-37^{o}C to avoid shock to the spermatozoa. Temperature of warm water (42-45^{o}C) loaded in AV should be checked before pouring into the AV to maintain the temperature inside. Temperature of AV is important factor for proper ejaculation from bull. If temperature is above normal bull will mount with pelvic thrust but ejaculation is not possible and if temperature is below optimum bull will mount without pelvic thrust therefore no ejaculation observed.

Initial assessment: Checking for contamination, temperature of water in water bath, warm stage and glass slide. Regular calibration of instruments, volume, consistency, concentration, colour and initial motility must be assessed.

Semen processing: Quality control at the level of semen processing consists of following phases like water treatment and storage, temperature of processing room, osmolarity and semen extenders.

Effect of water treatment and storage: Water used for semen dilution is either triple glass distilled or micro-filtered. Micro-filtered water was superior over triple distilled water as micro-filter act mechanically as well as through electrostatic adsorption of particle to their surface. Storage of purified water deteriorates its quality due to leaching of heavy metals from glass wares vials.

Room temperature: The temperature of room should be maintained between 20-25^{o}C, as it is optimum for semen processing.

Osmolarity of Extenders: Best percentage of motile spermatozoa was found in Osmolarity between 240-260 mOsmol [Pankaj et al., 2009]. At 5^{o}C the sperm survival is highly dependent on Osmolarity being maximum at 250-300 mOsmol. Also it is better to be on hypotonic side than on hypertonic side for extenders due to "solute

loading" phenomenon and intracellular "water outpouring" phenomenon.

Quality of Semen diluents: Good semen extender helps to maintain sperm integrity through pH of diluents, provision of metabolites, protect from ultra low temperature, prevent cell water in forming ice crystal and provide buffer action.

A study was designed to compare the powdered and fresh egg yolk extender and it was reported that powdered egg yolk was better in preservability of Zebu bulls semen as compared to fresh egg yolk (Ansari et al., 2010).

Quality of chemical, water and egg used in extender: Chemical should be of high quality analytical grade. Water should be double/triple distilled or micro-pore filtered. Resin based water purification system was superior over triple distilled water and water obtained by this system should not be stored for more than three days [Pankaj et al., 2009]. Egg used for diluents preparation should be fresh and undamaged and of standard shape and size. Bradykinin (a polypeptide of nine amino acids forms from a blood plasma globulin) added at the dose rate of 2 ng/ml in egg yolk tris glycerol extender showed significant effect on motility, viability and HOS percent in both pre-freez and post-thaw (Shukla and Misra, 2007).

Use of antibiotics in diluents: It is essential control points for controlling the growth of pathogenic bacteria in the extender. Broad spectrum antibiotics are added to extender for keeping growth of pathogenic bacteria under limit. GTLS (Combination of gentamicin, tylocin, lincomycin and streptomycin) was more capable than SP (streptomycin penicillin) for bacterial control of buffalo bull semen, moreover, GTLS and SP are equally efficient in preserving spermatozoa quality of extended buffalo bull semen for 3 days at refrigeration temperature (Akhter et al., 2008). Even with use of streptomycin and penicillin, the five samples out of twenty samples collected from different A.I. stations of Gujarat was found positive (> 500 CFU/ml) for various bacterial isolate and fungi (Patel et al., 2011).

Animal based extender vis a vis Plant based extender: It is pre requisite for an extender to retain the functional integrity of bovine spermatozoa for cryopreservation. In Conventional preservation technique diluents contain up to 20 percent egg yolk and hence higher the risk of bacteria or xeno-biotic contamination. However, egg may be a potential source of virus infections or allergic reactions. Presence of abnormal microflora in the semen beyond certain level may reduce the survivability of spermatozoa due to endotoxin production and fertility in cows. As a direct consequence, most countries fear the risk of introducing exotic diseases through the transportation of milk or egg based products. Presence of yolk globules interfere with the microscopic assessment of semen and it increases the viscosity of extender. The presence of particulate debris in extender could be the cause of reduced fertility. Also the semen extenders containing animal products components such as egg yolk and skim milk are difficult to standardize. Hence pathogen free substitute of non animal origin for egg yolk would obviously be a viable option. Soybean contains lecithin, a high molecular weight lipoprotein substitute for egg yolk, to prevent or repair damage to the sperm plasma membrane during cryopreservation. Therefore, there is need to develop economically efficient plant based extender for bovine semen that would yield optimal freezability and fertility rates in the present climatic condition. The soya milk based extender was developed and standardized for Murrah buffalo at 25 percent soyamilk level. Extender with 25 percent soya milk was superior over 10, 15, 20 and 30 percent soya level with respect to individual motility, viability, membrane integrity and acrosomal intactness (Singh et al., 2012). Soyabean lecithin based extenders like L1G7 and Bioxcell were also found suitable for ram semen, preservability is similar to egg yolk based extender (Sharafi et al., 2009).

Semen packaging: Standard semen packaging protocol should be standardized for each lab includes quality of straw used, hygienic handling of straw, vacuum pressure of filling, complete filling of cooled straws at 5^{o}C, sealing, checking of laboratory seal.

Daily cleaning of filling machine, needle used. Printing of straw should be legible long lasting. Printing on straw include breed of bull, bull number and Date of freezing.

Semen freezing and standardization of freezing protocol: Semen could be frozen using conventional freezing method or freezing with biological freezer. In conventional system height of rack from LN_2 (liquid nitrogen) level, no. of racks simultaneously kept for freezing. Vapor temperature of liquid nitrogen and time of exposure (freezing rate) should be standardized for a particular laboratory. The cryo-preserved samples to be collected quickly in pre cooled goblets and immediately plunged in LN_2. Freezing rate, adequate time between freezing cycles for setting of vapor should be standardized. The rate at which the samples are cooled can be regulated very precisely using programmable freezers. The protocol of freezing should be standardized for different breeds of bulls. Freezing of bull semen using biological programmable freezer with induced ice nucleation (seeding) was superior over conventional technique in crossbred bulls (Rao et al., 2012). Freezing protocol should be standardized for each animal separately using programmable freezer is need of the time [Barbas and Mascarenhas, 2009]. Scientist reported superiority of fast freezing over slow freezing rate.

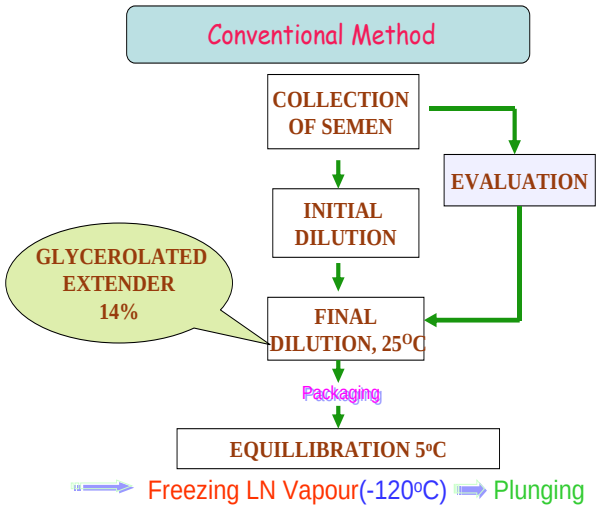

Fig-1: Steps in conventional freezing

Semen storage: The storage of semen for quality maintenance is even more important as compared to its production. It includes quality of cryo-vessels, level of liquid nitrogen in vessel and quarantine the semen in separate container for bull under testing scheme.

Semen transfer: Starting from the collection to processing and freezing semen needs handling by trained personnel. Once the semen leaves the laboratory handling, transportation, storage and insemination become highly variable and are critical limiting factor to the ultimate fertility potential of the cryo-preserved semen. There is drop of around 2-5 percent in overall viability of sperms during straw storage and distribution is common. Decrease in motility mainly attributed to rough handling of semen straw during storage and transfer of straw in the containers.

Fig- 2: Bio-freezer Cryocell 1205, SYLAB

Thawing of straw: Optimum thawing protocol should be standardized as it interact semen processing steps. The recommended thawing temperature-time combination in lab is 37°C water bath for 15-30 seconds.

Post thaw protection: Adequate thermal protection to the semen straw post-thaw is essential to ensure optimum viability is maintained until the semen deposited in the reproductive tract of the cow. Exposing the thawed straw to ambient air temperature can result in irreparable damage to sperm

motility and acrosome integrity. Ideally straws should be thawed and insemination guns loaded in a relatively warm dust free area. If possible, thawing of semen and loading of guns in a warm protected area that is in close proximity to the animals to be inseminated, should be followed.

Fig-3: Bio-freezer with inbuilt seeding rod

Overall hygienic condition: The uterus of cow is relatively sterile environment with few pathogens or bacteria present. However, the uterus is also excellent place to grow bacteria, should it be contaminated during the insemination process. Clean paper, towel and warm waters for washing hands and equipment are required between cows. A semen thaw bath, especially one that is thermostatically controlled, is an ideal location for growing bacteria. The water should be dumped in thaw bath and wipe the unit dries after every day's use. Thus the overall hygienic condition include: Hygienic design, construction and operation of equipments, plan for maintenance, cleaning and sanitation**,** training, health and personal hygiene, standard operating procedure (SOP) should be followed and Identification as well as traceability.

Handling of Low grade semen:

Large numbers of bulls (around 70%) were rejected from semen production due to poor semen quality and unacceptable freezability. Un-acceptable freezable semen include oligo-asthenozoospermic, astheno-normozoospermic and normozoo-spermic but rejected due to unsatisfactory semen freezability (Mandal et al., 2012).

Processing of low grade semen including filtration (Rao et al., 2012) and antioxidants fortification is very important for preserving the quality attributes of sperms. Extender supplemented with "trehalose" reduced the oxidative stress induced by freez-thaw and improved measures of bovine semen quality. The antioxidant abilities of trehalose related to its effectiveness in membrane cryopreservation (Hu et al., 2010). Fortification of semen with Vitamin E and C improves the preservability of spermatozoa (Rao, 2009). Suitable management strategies are being searched for and incorporated like laboratory procedures i.e., the use of filtration technique so as to maximize quality semen production without discarding too many poor quality ejaculates and rescheduling of vaccination protocol to unfavorable season in terms of semen production (Bhakat et al., 2009).

Transportation and handling semen at field level: The semen should be transported and maintained in the breeding area in a Styrofoam box or cooler to prevent additional cooling and protect it from ultraviolet light which are spermicidal. If semen is thawed, it should be inseminated as quickly as possible and not returned to the semen storage unit for use later.

Leucocytospermia: Infection of reproductive tract directly or indirectly causes infertility. Pyospermia is a laboratory finding categorized as abnormal presence of leucocytes in ejaculates, may indicate tract infection.

Sperm agglutination: Protection to sperm antigens are provided by tight junctions of sertoli cells forming the blood testis barrier. Spermatozoa induce immune response when exposed to systemic immune response, in case of disruption of barrier leading to formation of anti sperm antibody (ASA). It has cytotoxic effect on sperm especially on motility. It also produces agglutinated clump of sperm cells, which ultimately hampers the passage of sperm. Immobilized sperm with

side to side shaking motion suggest presence of antisperm antibody either on sperm or in cervical mucus.

Semen quality control using CASA (Computer assisted semen analysis) and flow cytometry [Vincent et al., 2012**]:**

CASA was used since 1980s and several researchers have reported the CASA parameters as predictor of male fertility. Flow cytometer was developed in 1968 by Wolfgang Gohde from Germany, moreover during early mid 1980s Glenn Spaulding first time used in semen of human and rabbit for sorting of viable spermatozoa. The technology was developed to select spermatozoa for further use.

Manual semen analysis using light microscope has been the standard method for analysis in most of the semen production center. However this analysis is very subjective and prone to within and between technician errors (DeJarnette, 2005). Similarly, the use of fluorescence microscopy to assess spermatozoa for acrosome, membrane and DNA integrity is markedly slow and limited due to less number of spermatozoa analyzed from each sample and inability for and extensive multi parametric analysis. Therefore artificial breeding centers are moving away from subjective semen assessment of a few hundred spermatozoa for concentration, motility and morphology using microscopy that is largely uncorrelated to field fertility to objective semen analysis that incorporate CASA and flow cytometry to analyze thousands of cells within seconds for characteristics such as viability, mitochondrial activity, acrosome, DNA and capacitation status.

CASA is a distinctive tool for the objective assessment of sperm motility and is hence now frequently used for evaluating semen quality. It is also able to measure kinematics of sperm motion (Kathiravan et al., 2011). The components of this technology include a microscope to visualize the sample, a digital camera to capture images and a computer with software to critically analyze the movement of spermatozoa. The principle of CASA system is that a series of successive images of motile spermatozoa within a static field of view are acquired by computer software algorithms, which then scan these images sequence to identify individual spermatozoa and trace their progression across the field of view. This involves recognizing the same cell in each image by its position, and inferring its next position by estimating likelihood that it will only have moved a certain maximum distance between frames. CASA also provide information regarding concentration, morphology, viability and index of DNA fragmentation of frozen-thawed sperm. With CASA, several motility parameters describing the specific movements of spermatozoa can be obtained in greater detail than what is possible in subjective assessment. These computerized software based measurements can be useful to assess various sperm characteristics simultaneously and objectively, and are valuable for detection of fine changes in sperm motion that can not be identified by conventional subjective semen analysis (Kathiravan et al., 2011). The parameters typically collected using CASA system are motility, velocity, linear and lateral displacement (ALH) of spermatozoa as they progress along their trajectories in a sample. Progressive motile spermatozoa swim forward in straight line. Spermatozoa which swim with an abnormal path, like tight circles, are not included in the proportion of progressively motile sperm. The software calculates the kinetic values of spermatozoa, which covers the velocity of movement, width of sperm head trajectory and frequency of the change in direction of sperm head. Velocity values evaluated using CASA include curvilinear velocity (VCL), straight line velocity (VSL) and average path velocity (VAP). The amplitude of lateral head displacement and beat cross frequency (BCF) are also measured.

A positive correlation between straight line velocity (VSL) of spermatozoa and field fertility was observed (Kathiravan et al., 2008).

Flow cytometry: Analysis of sperm functions with the help of one or several lasers (3 lasers) through the fluid in which high speed sperm cells are passing. The light emitted by fluorochrome bound cells in captured by photo-multiplier tubes (at least

10 tubes) and converted into electronic signal after that digitalized by software [Martinez-Pastor et al., 2010; Hossain et al., 2011]. Dead cells are identified by "propidium iodide" dye which excited in 488 nm laser [Partyka et al., 2010; Oldenhof et al., 2011,] other ready to use fixable dyes are also available. Acrosome integrity judged by *Pivum sativum* and *Arachis hypogaea* agglutinin labeled with FITC fluoro-chromes is used. Damaged acrosome takes the stain (Carvalho et al., 2010; Anzar et al., 2011; Yi et al., 2012). Mitochondrial activity, DNA integrity, calcium influx are also measured by using this technique (Colas et al., 2009; Guthrie et al., 2011; Kumaresan et al., 2011). Modern multi-parameric approach for quality control at semen production centers is based on combination of CASA and Flow cytometry resulting in increase of rejection as compared to CASA or flow cytometer alone i.e., it provides very high rejection/acceptance decision. Research of new markers to identify high fertility semen needs to be extensive. Incorporation of new markers in multi-parametric approach will lead to a better evaluation of semen quality and fertility. It also helps in characterization of semen with high genetic merit.

Assessment of quality of sperm by hemi-zona assay (HZA) and zona-pellucida binding: The interaction between spermatozoa and zona pellucida is a critical event leading to fertilization and reflect completion of capacitation (displayed by hyper-activation by spermatozoa) as manifestation by ability to bind to zona pellucida and legand induced acrosomal reaction. How ever it has limited use due to its species specific binding ability [Vasan, 2011].

Sperm penetration assay (SPA) or sperm capacitation index or zona free hamster oocyte penetration assay: SPA utilizes the golden hamster egg, zona pellucida removed results in loss of all species specificity to egg penetration.

DNA damage direct test:

Terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay and DNA oxidation measurement.

Comet assay: Comet assay is used to study the DNA fragmentation of sperm. It include following steps: 1. Embeding of sperm in agarose gel, 2. Postive control by H2O2 treatment, 3. Lysis and electrophoresis, 4. Neutralization, DNA precipitation and staining of microgels, 5. Slide analysis by microscopy for head to tail length using eye piece micrometer; lower the tail length better the sperm (Shamsi et al., 2010). Cryopreservation causes a significant decrease in percentage of DNA in comet head; low level of DNA fragmentation after cryopreservation is a characteristic of bull spermatozoa (Slowinska et al., 2008). Neutral comet assay (NCA) and sperm chromatin structure assay (SCSA) can be used in assessing DNA integrity in bovine sperm equally and effectively (Boe-Hansen et al., 2005).

DNA damage indirect test: It include sperm chromatin structure assay (SCSA) [Waterhouse et al., 2010], sperm chromatin dispersion assay [Lymberopoulos and Khalifa, 2010], sperm fluorescence *in situ* hybridization analysis (FISH). Sperm DNA damage is associated with poor pregnancy outcome (Frydman et al., 2008).

Assessment of reactive oxygen species (ROS):

Reactive oxygen species (ROS) also referred to as free radical, formed as byproduct of oxygen metabolism. Contaminating leucocytes is predominant source of ROS in suspension. During freezing and storage of semen, exposure to oxygen and light radiation accelerate the production of ROS and peroxidation of lipid in plasma membrane of sperm [Andrabi, 2009; Garg et al., 2008]. They may be eradicated by enzyme like catalase, glutathione peroxidase, co-enzyme Q10 [Ibrahim et al., 2011] or by non enzymatic antioxidants albumin, glutathione, hypotaurine, tocopherol and ascorbic acid. Small amount of ROS is essential for capacitation and acrosomal reaction. High level of ROS leads to oxidative stress. Infertility is proportional to high ROS in males [Agrawal et al., 2006].

Sperm proteomics: It deals with comparison of proteins of normal and defective spermatozoa. It helps in

identification of molecules responsible for sperm abnormality [Aitken, 2010].

Cow to bull ratio: With respect to utilization of quality semen to produce a calf every year, the cow must be bred within three months. In order to achieve high pregnancy in short breeding season, bull should not be overused. The cow numbers may be increased by about 30 percent if cattle are kept in a confined area or are presented to the bull only when in heat (Blezinger, 1999).

Bull age	Number of cow
One year old	15-20
Two year old	20-30
3 years and above	30-40

Bull contributes fifty percent towards the success of any breeding program, they also contribute at least half towards the risk for a lower than expected pregnancy rate. Appropriate and deligent bull management is therefore a sound practices.

NRR (Non return rate) of cow at 60 days after first insemination under field condition may be a good practice to select quality semen among different ejaculates of individual bull, semen types (chilled vs frozen), quality of semen (poor, good, best) and sources of semen (source of purchase). Assessment and monitoring of these control points will go a long way in making AI programme and livestock improvement a great success (Pankaj et al., 2009).

CONCLUSION

Bull is essential for improvement of herd; as they contribute significantly towards overall production and reproduction. Bull management is therefore essential, for all steps starting from harvesting of semen to their storage. Evaluation of semen complemented with computer assisted semen analysis (CASA), automated sperm morphology analysis (ASMA), sperm quality analyser (SQA) & flow cytometry at artificial breeding unit is essential component and a step ahead in high grade semen production. Advance techniques are used at bull station like sperm fluorescence *in situ* hybridization analysis, comet assay, zona binding etc. to aid quality semen production and processing at organized bull farm. Intervention in semen processing is fundamental points in quality semen production. Ensuring these critical issues, which require technical input to the existing protocol to develop complete package of practices. Successful bull management with objective evaluation of sperm, standardized semen processing, freezing and packaging will efficiently exploit elite bulls for quality semen production and utilization.

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