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Addressing Stray Animal Population Through Alteration of Sex Ratio

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ABSTRACT

Livestock plays a significant role in rural economy and livelihood by providing milk, meat, skin, drought power, etc. India's livestock sector is one of the largest in the world with a holding of 11.6% of the world's livestock population. But due to economic reasons, dairy farmers have a strong preference for female calves for milk production and hence there is a very limited demand for male calves. Furthermore, excessive focus on crossbreeding in the past few decades, increased mechanisation and the national policy to ban on cow slaughter have further added to the problem. In the common parlance, stray cattle include low-yield cows, bulls or calves that are abandoned and free to roam during dawn to dark because they are unproductive and creating a traffic nuisance in cities, they also attack crops in villages. Therefore, techniques for gender selection and skewing of sex ratio towards female are demanded of the hour. Flow cytometric sorting of spermatozoa is one of the best approaches to select the sexed semen for desire sex of calf, but its cost and patented technology is a definite disadvantage of this technology. In the present review, we will discuss in detail about the techniques available for skewing of sex ratio to address the stay animal population.

Introduction

India houses largest livestock population in the world (535.78 million) as per the 20th livestock census with a recorded increase of 4.6% over the previous livestock census of 2012. The total cattle inventory in the country is 305.5 million, which is the largest in the world followed by Brazil and China. Due to economic reasons, dairy farmers have a strong preference for female calves for milk production and hence there is a very limited demand for male calves. Rearing of male calf is an economic burden on dairy farmers. Hence a large number of male calves and unproductive animals are abandoned and are left as stray animals. India has over five million stray cattle, as per the 20th Livestock Census, which is a matter of concern for the country's livestock economy. All the major states with large livestock population, including Uttar Pradesh (17.34%), Madhya Pradesh (95.01%) and Punjab (38.69%)

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have seen a quantum leap in the stray cattle population in the 20th livestock census as compared to 19th livestock census. Therefore, techniques for gender selection and skewing of sex ratio towards female are demanded of the hour. Sexed semen has been available extensively since last one decade and offers potential for increasing the affordability of dam selection and heifer replacement (DeVries, 2008), increasing efficacy of progeny testing programme (Weigel, 2004) and increasing the profitability of dairy cattle production (DeVries, 2008). Sex sorting of spermatozoa with flow cytometric sorting offers an accuracy of more than 90% (Tubman et al., 2004) but higher cost of sex sorted semen and lower conception rate are the major limitations in acceptability of this technology in the field level. Therefore, alternate, potentially effective and economic techniques, for alteration of sex ratio need to be explored. Some of these technologies which hold promise for the future have been discussed in the following section:

Preconception maternal manipulations

Manipulations of maternal reproduction before conception to alter the sex ratio have been sporadically researched and many economic and potentially effective techniques, if improvised for field application, holds the promise for the future. Some of the important techniques are as under:

Time of artificial insemination: Alteration of sex ratio by varying the time of insemination has been attempted by many authors in the past (Pursley et al., 1998; Martinez et al., 2004). It has been suggested that inseminations far before ovulation during early part of oestrous will result in birth of more number of male calves; which might be due to differences in capacitation timing and longevity of spermatozoa containing X- and Y-chromosome in the female reproductive tract (Martinez et al., 2004). The majority of studies in this area used different insemination times based on visually detected oestrous (Martinez et al., 2004) and intra-vaginal conductivity (Wehner et al., 1997), which are unlikely to reliably predict ovulation time. As a result, this method yields contradicting results. When the cows were inseminated according to the conductivity of the cervico-vaginal mucus, the results were fairly convincing: inseminations performed during declining conductivity resulted in more female calves, while inseminations performed during rebounding conductivity resulted in more male calves (Wehner et al., 1997).

Oestrus lasts 18 hours on average in cattle, and ovulation occurs 10-12 hours after end of oestrus, bringing the total duration from commencement of oestrus to ovulation 30-32 hours (Looper et al., 1998; Jainudeen et al., 2000). Artificial insemination (AI) within the first 18 hours after the commencement of the oestrus can enhance the percentage of female offspring, whereas postponing AI beyond 30 hours after the onset of the oestrus dramatically increases the percentage of males (Martinez et al., 2004).

The percentage of males grows 1.85 percent every hour from the commencement of oestrus, according to regression analysis. This could be because Y-chromosome-bearing spermatozoa progress through cervical mucus faster than X-chromosome-bearing spermatozoa, and they will reach the utero-tubal junction and undergo adequate capacitation stage sooner than X-chromosome-bearing spermatozoa, and will be released from the utero-tubal junction to reach the fertilisation site before X-chromosome-bearing spermatozoa (Rodriguez-Martinez et al., 2001; Hunter et al., 2001).

This is due to the fact that Y-chromosome-bearing sperm reach capacitation earlier than X-chromosome-bearing spermatozoa, and are released from the oviductal epithelium and arrive at the site of fertilisation well before ovulation. Most of these spermatozoa would die before the egg arrived at the fertilisation site because they had been capacitated. The X-chromosome bearing spermatozoa, on the other hand, would undergo capacitation later and have a longer lifespan, so they would arrive at the fertilisation site at the right time and so be more likely to fertilise the ovum (Wehner et al., 1997).

If inseminations were delayed from the commencement of oestrous to 30 hours, Y-chromosome bearing spermatozoa would have a better chance of fertilising the ovum since they would arrive around the time of ovulation and before X-chromosome bearing sperm. The failure to precisely identify the timing of commencement of oestrus is, however, a shortcoming of this approach. Wehner et al. (1997) utilising an electronic instrument that measures the conductivity of cervical mucus and hence can precisely assess the condition of the oestrous cycle, found that altering the time of insemination increased sex selection efficacy by over 90%. These researchers discovered that 93 percent of females inseminated 20 hours before ovulation (22 hours from the onset of oestrus).

Electrical resistance of cervical mucus: The length of the follicular phase was also responsible for alteration of sex ratio as more number of female calves were born to dams showing longer follicular phases (Martin et al., 1997). Moreover, Martin et al. (1997) hypothesized that the difference in length of follicular phase results in difference in properties of cervico-vaginal mucus and thus facilitating better survival and transport of sperm bearing a particular chromosome over the other. In cows, Wehner et al.

(1997) observed that significantly more heifer calves were born when inseminations were performed approximately 20 hours prior to ovulation at low impedance (35-45) of cervico-vaginal mucus and significantly more bull calves when inseminations were performed approximately 10 hours prior to ovulation at high impedance values (50-70) of cervico-vaginal mucus.

Maternal testosterone: Various authors have established the relationship of testosterone levels in the mother before conception and the offspring's sex (Grant and Irwin, 2005; Garcia-Herreros et al., 2010). A study involving Nubian Ibexes revealed that the dominant females have greater faecal testosterone and produced a male biased sex ratio compared to subordinate females. Also, it was recorded that higher glucose and testosterone concentration in the dam resulted in more male offspring (Helle et al., 2008). In human females, testosterone titre in follicular fluid was much higher than its concentration in blood (10000-30000 times more). It was observed that ova developing in follicular fluid having high testosterone concentration had a selective preference for Y-chromosome bearing spermatozoa and hence their probability of being fertilized by a Y- bearing spermatozoa is more. This is particularly true if the high testosterone concentration is simply not because of testosterone to oestradiol aromatization below par (Grant et al., 2008). It is suggested that during a crucial time period, the molecular composition of the zona pellucida may be regulated by high follicular testosterone and hence it affects the probability of oocyte for fertilization by a Y-bearing spermatozoa. Moreover, Garcia-Herreros et al. (2010) reported that oocytes from the follicles with testosterone concentration more than 300 nM when fertilized, subsequently resulted in production of higher number of male foetuses (33 versus 13) compared to female foetuses. Though the possible explanation for the fact is lacking, but it is speculated that at the time of antral development of follicles, two of the three Zona pellucida surface proteins are produced. The high testosterone concentration might alter the nature or expression of these proteins during antral stage and hence may facilitate preferential fertilization by Y-bearing spermatozoa. Interaction between cumulus cells and spermatozoa is also altered in a subtle way by high testosterone concentration (Grant and Chamley, 2010).

Maternal oestradiol concentration: Oestradiol when administered to the dairy cows 24 hours after $PGF_2\alpha$ treatment induces LH surge (Lopes et al., 2000) and could be used instead of GnRH in the Ovsynch protocol to induce ovulation in preovulatory follicles (Pancarci et al., 2002). Oestradiol hormone is responsible for oestrus expression (Reames et al., 2011), moreover duration of oestrus expression has a dose dependent relationship with blood oestradiol concentration (Reames et al., 2011). Therefore, oestradiol treated cows have longer duration of oestrus expression and hence enhanced oestrus detection rates and conception rates (Cerri et al., 2004; Jinks et al., 2013). Increased dry matter intake and metabolic rates causes high hepatic clearance rate, which in turns results in high hepatic clearance of ovarian steroids (Wiltbank et al., 2006), thus high producing dairy cows have low circulating oestradiol concentration (Chagas et al., 2007). Furthermore, low preovulatory oestradiol concentrations result in premature luteolysis in the subsequent oestrous cycle (Mann et al., 2000) because low concentrations of preovulatory oestradiol could lead to lesser expression of endometrial progesterone receptors (Bridges et al., 2012), greater expression of endometrial oxytocin receptors (Zollers et al., 1993) and greater oxytocin binding activity (Mann et al., 2000). Oestradiol treated cows has higher concentration of preovulatory oestradiol (Lopes et al., 2000), which reduces chances of premature luteolysis, hence leading to a higher conception rate of estradiol treated cows compared to untreated cows.

Incubation with high oestradiol concentration has been reported to result in male-biased sex ratio *in vitro* produced embryos in murine (Zhang et al., 2006). Furthermore, Holstein dairy cows treated with oestradiol before insemination produces more male embryos compared to untreated contemporaries (Emadi et al., 2014). Oestradiol is thought to affect sex ratio *via* oocyte-related mechanisms (Zhang et al., 2006) or by altering the duration between ovulation and fertilisation (Martinez et al., 2004). Depending on the amount given, estradiol has been shown to either accelerate or postpone oviductal transit of ova in laboratory animals (Greenwald, 1967), advancing or postponing the moment when an egg meets spermatozoa. The interval between the time of ovulation and fertilization influences the sex ratio of offspring in bovine (Wehner et al. 1997), deer (Verme and Ozoga, 1981) and rodents (Krackow et al., 1997).

Vaginal electrical resistance: Sex ratio can be skewed if insemination timed with reference to ovulation (Wehner et al., 1997). Moreover, resistance of vaginal secretion has an inverse relation with the blood estrogen level i.e. electrical resistance was at maximum, when blood estrogen reaches at peak (Rezac, 2008). Resistance of vaginal secretion starts increasing when estrogen level decline following gonadotrophin surge. It is possible to predict the time of onset of oestrus and gonadotrophin surge by measuring and plotting resistance of vaginal and cervical secretions (Rorie, 1999). Furthermore, ewes inseminated at oestrus when resistance of vaginal and cervical secretion

was 18 and > 30 with minimum probe readings (18-23) cases skewing sex ratio with birth of 59.4 per cent males. Indeed, X bearing spermatozoa has more chance to fertilize oocytes under low oestrogen concentration. Thus, more female offerings can be produced if females are inseminated during the early phase of estrus.

Skewing of sex ratio using assisted reproductive technologies

Although the chromosomal sex was determined at the time of fertilization, the sexual differentiation occurs at a later stage. If the sex of embryo can control with timing of insemination, it will extremely beneficial for the livestock industry. Until now, many factors *viz.* embryo culture conditions (Kimura et al., 2005), maturational stage of oocytes (Agung et al., 2006; Iwata, 2012) and day of blastocyst appearance (Avery et al., 1993) have been shown to affect the sex ratio of bovine embryos produced *in vitro*. Therefore, these facts can be explored to cause desired alterations in the sex ratio.

Methods of sperm preparation: Though flow cytometer-based sorting is the only commercially available option for sperm sorting but it has limitations like longer time required for sorting and sperm damage during the process of sorting; these limitations can be circumvented with other techniques *viz.* serum albumin layering, swim-up and Percoll density gradient centrifugation (Ericsson et al., 1973; Madrid-Burry et al., 2003; Hossepian de Lima, 2007).

Percoll density gradient and sperm swim up: Percoll density centrifugation technique has been used for separation of X and Y in many species (Hossepian de Lima, 2007). Moreover, Percoll density centrifugation enhances spermatozoa motility (Parrish et al., 1995; Lucio et al., 2008), membrane and acrosome integrity (Oliveira et al., 2012) and reduces morphological abnormalities (Prakash et al., 1998) and separate X bearing live sperms in order of 70 per cent (Hossepian de Lima, 2007) with no untoward effect on sperm acrosomal intactness (Resende et al., 2010) and plasma membrane (Oliveira et al., 2012) in cost effective manner. X and Y sperm can be separated on the basis of their swimming speed (Sperm swim up technique; Rodriguez-martinez et al., 1997). The same can also be utilized for sex pre selection of spermatozoa (Cesari et al., 2006; Yan et al., 2006; Joshi et al., 2021). Centrifugation of thawed semen using Percoll density gradients yields 59.6 per cent females (Resende et al., 2010) for *in vitro* produced embryos. Skewing of sex ratio varying from 55.7 to to 74.3 per cent in favour of females have been reported previously (Hossepian de Lima et al., 2011).

On the contrary sperm swim up method has been reported to cause skew of sex ratio of upto 60.80% in favour of male (Lucio et al., 2008). The possible reason for the skew may be the fact that spermatozoa with Y chromosome is faster than the X-bearing spermatozoa because of lesser DNA content and thus is associated with different velocity (Yan et al., 2006). Therefore, the supernatant of swim up procedure was reported to contain more Y-bearing spermatozoa (Check and Katsoff, 1993; Check et al., 1994).

Serum albumin layering: Layering of human sperm on a liquid albumin column yields approximately 85 per cent of Y chromosome bearing spermatozoa on the lowest portion of the column (Ericsson et al., 1973). Likewise, skewing of sex ratio also reported in sheep and cattle (Rathje et al., 2019). Conflicting reports of no alteration of sex ratio following serum albumin gradient are also on records in cattle (White et al., 1984).

Maturational status of oocytes: The sex ratio of embryos has been shown to be affected by the maturational condition of oocytes at the moment of insemination (Dominko and first, 1997; Agung et al., 2006). The proportion of female embryos increased when oocytes were inseminated immediately after the first polar body was extruded, whereas the proportion of male embryos increased when inseminations were delayed for 8 hours (after polar body extrusion) (Agung et al., 2006). This suggests that maturational status of oocytes affect the processing of X and Y-chromosome bearing spermatozoa. In other words, delaying inseminations may allow M II arrested oocytes to process Y-chromosome bearing spermatozoa more effectively compared to X-chromosome bearing spermatozoa. Delaying insemination permits a higher proportion of oocytes to reach M II before insemination, however the oocytes age as a result of the prolonged maturation culture.

After 22 hours of culture, the proportion of oocytes developed to M II achieved its highest level, and the proportion did not differ between oocytes cultured for 22, 28, or 34 hours. However, compared to oocytes cultured for 22 hours, the proportion of blastocysts generated from oocytes cultured for more than 28 hours dropped. Before fertilisation, aged oocytes had aberrant morphological traits such as the removal of the microfilament-rich region above the mitotic spindles, disruption of spindle position, and chromatin disorder. It has been discovered that older oocytes have a greater ability to be triggered, resulting in a higher rate of fragmentation and lower maturation promoting factor activity. Furthermore, when the meiotic arrest time is prolonged, cytoplasmic alterations impacting oocyte quality (e.g., lower ability to achieve fertilisation

and decreased capability for development). As a result, morphological and cytoplasmic alterations in the oocytes may be the cause of markedly decreased developmental competence in oocytes incubated for more than 28 hours. In another study (Agung et al., 2006), blastocysts formed from oocyte fertilisation after 16 hours of maturity culture alter the sex ratio in favour of female embryos, but longer maturation culture (>28 hours) increased the number of male embryos. On the other hand, no variation of a 1:1 the sex ratio was found after 22 hours of maturation culture.

Conclusion

Though the techniques like flow cytometric sorting of spermatozoa is still considered as golden standards in the sexing of sperm, but its cost is a definite disadvantage. This is coupled with the limitations of the compromised conception rate because of DNA damage, exposure to fluorescent dye and time taken for sorting of spermatozoa. Therefore, it is necessary to devise economic and effective methods for skewing the sex ratio. Some techniques like manipulating the time of insemination, sperm preparation methods in *in vitro* embryo production and manipulating *in vitro* maturation and gamete co-incubation timings in *in vitro* production set up, holds some promise and needs to be validated further.

Conflict of interest

Authors declare none

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