THE EFFECT OF LOW-DOSE INSEMINATION, SEMEN DEPOSITION SITES, INSEMINATION FREQUENCY AND AI TECHNICIANS ON BLUE FOXES

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ABSTRACT

Artificial insemination (AI) technology is important to the economic benefits of blue fox vixens (*Alopex lagopus*). We studied the pregnancy results after AI with doses of 30, 50 and 70 million spermatozoa, the effect of semen deposition sites, insemination frequency and AI technicians in blue foxes. Compared with normal dose (70 million spermatozoa), low-dose inseminations (30 to 50 million spermatozoa) can achieve similar pregnancy rate, birth rate and mean litter size. However, deposition sites, insemination frequency and AI technicians influence the fertility of vixens. To achieve acceptable conception rate, we should deposit the semen into the uterine body, a minimum of two doses should be available each cycle. In conclusion, with excellent animal management, a dose of 30 million spermatozoa is enough to achieve satisfying pregnancy rates in blue fox vixens. We can use multiple intra-uterine inseminations in low-dose inseminations to achieve acceptable pregnancy results. Further investigation is needed to declare the minimum threshold number of spermatozoa for the best fertilization result.

Key words: Artificial Insemination; Low-dose Insemination; Pregnancy Rate; Blue Foxes

INTRODUCTION

In recent years, with living quality and consumption levels improving, the demand for fur animal products is increasing. Blue fox has gradually become the main fur variety in China and its breeding has developed rapidly. Belonging to seasonal estrus animals, blue foxes are monstrous; they are in estrus from mid February to mid April. The pregnancy rate directly affects the raising number and economic benefits and has an important impact on blue fox breeding farms.

Application of artificial insemination (AI) in animal breeding strategies can quickly spread genes from the best available males to improve economic benefits. In recent years, there has been a significant increase of AI in fox farms. AI has replaced natural mating, because it can reduce selection efficiency and cut down both capital and labour costs. In China, AI is used in nearly all fox farms (Zhang and Tang,2006).

Farmers and AI companies need sufficient effectual fertile spermatozoa. The number of spermatozoa instead of the volume of semen was important in deciding conception (Morris, 2004). An insemination dose of 70 x 10^6 effectual fertile spermatozoa deposited intracervically two or three times during estrus was accepted as a standard to maximize pregnancy rates (Zhang *et al.*, 2003). Under these standard conditions, only a limited dilution is possible, thus constraining the efficient use of males, which may waste the superior fox genetic genes and raise the production cost of AI. As the demand for semen from genetically superior foxes has grown, AI in blue foxes has increased rapidly in recent years.

To improve the fox fertility without reducing the conception rates, low-dose insemination should be of great benefit especially in the case of semen from superior foxes or for sanitary purposes (Vazquez *et al.*, 2005). It would reduce the introduction and raising cost, increase the intensity of selection and allow the effective use of males, thus producing greater economic and social benefits.

Low-dose artificial insemination (AI) has been successfully used in heifers (Kurykin *et al.*, 2003), cows (Kurykin *et al.*, 2006; An *et al.*, 2010),sheep (de Graaf *et al.*, 2007), Sika (Gao *et al.*, 2009), sows (Krueger *et al.*,1999; Roca *et al.*, 2003; Martinez *et al.*, 2006), mares (Leipold *et al.*, 1998; Lindsey *et al.*, 2001), fowl (Lake and Ravie, 1987) and buffalo (Presicce *et al.*, 2005). Little research work in fur animals has been carried out in this area yet. To study factors affecting blue fox artificial insemination, field studies were performed as follows.

MATERIALS AND METHODS

Experimental time and site: We conducted field studies at Liaocheng City Golden Bridge Fox Raising Farm Co., Ltd. from February to June, 2012.

Animal selection, grouping and breeding: A total of 2000, 10 to 36months old foxes were randomly selected.

Female foxes with body weight of 6-8kg were fed with a proper diet twice daily. Water was provided ad libitum 2h after feeding. They were kept on a regime of 14 h light/24 h and were randomly assigned to the studies.

Ten sexually mature male foxes with satisfactory characteristics of pedigree, performance, development, progeny test and fur quality were selected for semen collection. They were housed indoors in individual pens within a confined environment (22±2).

Semen collection and treatment: Semen was collected once every two days using the gloved hand manual method. Once collected, semen was immediately transported to the laboratory and diluted with prewarmed Fox Artificial Insemination Dilution (Institute of Special Animal and Plant Sciences of CAAS, 1:1, v/v). We evaluate the gross spermatozoa motility at 400x magnification with a phase-contrast microscope (Olympus Optical Co., Ltd., Tokyo, JP). Concentration was assessed with an IMV Micro-Reader I spectrophotometer (IMV International, Inc., Paris, FR). Semen with characteristics equal to or greater than 90% morphologically normal sperm (heads, tails and droplets) and estimated progressive motility was further processed to the final concentration. Each ejaculate is about 0.3-0.5ml. Immediately after collection, ejaculates of ten male foxes with acceptable characteristics were mixed together and centrifuged at 300g for 10 min and semen pellet was left at the bottom of the microtubes. Supernatant was discarded, and semen pellet was resuspended with prewarmed Fox Artificial Insemination Dilution to reach the final concentration based on the measured sperm concentration. Then it was maintained at about 20-25 before insemination.

Estrus detection and artificial insemination: Estrus was detected twice daily at 06:00 and 18:00 h. Those vixens showing a standing reflex with backpressure and less vulva swelling hardness were considered to be in estrus. Estrus detection continued until the standing reflex subsided. Artificial insemination (AI) was performed by professional personnel. Inseminations were conducted with fresh semen extended to 30, 50 or 70 million spermatozoa/ml respectively. Semen was taken into the cervix or uterine body after detection of standing reflex and less vulva swelling hardness. The first AI was done 24h after detection of estrus. Other inseminations were conducted at 24h intervals if needed. The semen was expelled by a syringe. Experimental insemination doses were randomly distributed to three technicians during the entire experiment.

Pregnancy detection: All vixens were monitored twice daily from day 30 after insemination for signs of pregnancy, including estrus ending, frequent urination, sleepy, fatigue, breast swelling, belly enlargement, changes of taste. Pregnant vixen number, pregnancy rates, birth rate and mean litter size (total born and alive) were recorded afterwards. During the calving period, vixens were monitored closely and cubs were matched with their mother.

Experimental design: Some features, including the dosage, deposition sites, insemination frequency and AI technicians, were analyzed with the pregnancy rates after artificial insemination (AI).

Trial 1: Number of vixens inseminated, number of pregnant foxes, pregnancy rate, birth rate and mean litter size, were repeated in each trial and compared among different inseminated semen dosages in Table 1. After detecting spontaneous estrus, insemination doses, varying from 30×10^6 to 70×10^6 sperm/dose, was deposited into uterine body at 24h intervals. Different insemination doses were distributed at random among 820 experimental vixens and three AI technicians. Vixens were inseminated with the same dose respectively.

Trial 2: Deposition sites of cervix and uterine body were compared in Table 2. 406 vixens were inseminated with 30×10^6 effectual fertile fresh semen three times by three professional veterinarians. The number of inseminated vixens per technician varied from 126 to 148, with an average of 135.

Trial 3: Insemination frequency was studied in Table 3 with 30×10^6 effectual fertile fresh semen. 154 vixens were inseminated once, 252 vixens were inseminated twice and 265 vixens were inseminated three times.

Trial 4: The effect of AI technicians using the insemination dose of 30 million spermatozoa deposited twice into the uterine body is summarized in Table 4. The number of inseminated vixens per technician varied from 258 to 286, with an average of 273.

In each field trial, treatment services and vixens were randomly assigned to the technicians.

Statistical Analysis: The differences in insemination doses, sites of deposition, insemination frequency and AI technicians were served to explain the variation in pregnancy. All features were tested for normal distribution. Pregnancy rate and birth rate in the table are all given as percent, mean litter size are shown as mean±SD. Percent features were analyzed by U test and the average values were tested by Z test. Difference was considered statistically significant at P< 0.05 and extremely significant at P<0.01.

To reduce difference caused by experimental conditions and sources of experimental materials, the same batch, the same semen source and the same experimental conditions were repeated each time.

RESULTS

Low dose insemination with 50 and 30 million spermatozoa gives similar pregnancy rates and birth rates compared with standard AI using 70 million spermatozoa. No significant difference was observed in the mean litter size (total born and weaned alive) between groups either. Decreasing the number of effectual fertile spermatozoa inseminated from around 70×10^6 to 30×10^6 did not result in pregnancy rate, birth rate and litter size decrease. It was concluded that insemination with 30×10^6 dose was enough to achieve acceptable fertility.

Pregnancy rates, birth rates and total born mean litter sizes were significantly different between cervix and uterine body inseminations. Yet weaned alive mean litter sizes were comparable between groups and no significant difference was found. When inseminating vixens with 30×10^6 effectual fertile fresh semen, uterine body is the preferred deposition site of insemination.

With insemination frequency increasing in each estrus, higher pregnancy rates and birth rates were obtained. The insemination frequency didn't significantly influence the litter size. The pregnancy rate and birth rate of one-time insemination group were extremely significantly lower than those inseminated twice or three times. However, there was no significant difference between groups inseminated twice and three times, suggesting that not less than a second insemination was required.

Inseminators are also important. In our research, there was statistically significant difference (P<0.05) between inseminator 1 and 2, significant difference was also found between inseminator 1 and 3, but no significant difference was found between inseminator 2 and 3.

Table1. Effect of insemination doses on pregnancy rate after artificial insemination

Dosage of inseminated semen	Number of vixens inseminated	Number of pregnant vixens	Pregnancy rate (%)	Birth rate (%)	Mean litter size	
					Total born (mean ±SD)	Weaned alive (mean ±SD)
70×10^6	243	227	93.4	90.1	7.54±1.55	5.73±1.53
50×10^6	281	262	93.2	90.0	7.54±1.65	5.74±1.46
30×10^6	296	276	93.2	90.2	7.55±1.96	5.76±1.54

Note: In the same row, values with same small letter superscripts mean significant difference (P<0.05), and with same capital letter superscripts are extremely significant difference (P<0.01), while with no letter superscripts mean no significant difference (P>0.05) or no extremely significant difference (P>0.01).The same below.

Table 2. Effect of deposition sites on pregnancy rate of vixens

Deposition site	Number of vixens inseminated	Number of pregnant vixens	Pregnancy rate (%)	Birth rate (%)	Mean litter sizes	
					Total born (mean ±SD)	Weaned alive (mean ±SD)
cervix	114	98	86.0[a]	84.5[b]	7.11±0.75[c]	5.52±0.99
uterine body	292	273	93.5[a]	91.2[b]	7.52±1.91[c]	5.73±1.43

Table 3. Analysis of mating times on pregnancy rate after artificial insemination

Insemination frequency	Inseminated vixen numbers	Number of pregnancy	Pregnancy rate (%)	Birth rate (%)	Mean litter sizes	
					Total born (mean ±SD)	Weaned alive (mean ±SD)
Once	154	102	66.2[A,B]	62.3[C,D]	7.50±1.16	5.69±1.21
Twice	252	227	90.1[A]	87.0[C]	7.49±1.38	5.69±1.24
Three times	265	248	93.6[B]	91.2[D]	7.47±1.72	5.71±1.41

Table 4. Effects of AI technicians on artificial insemination of blue foxes

Technicians	Inseminated vixen numbers	Number of pregnancy	Pregnancy rate (%)	Birth rate (%)	Mean litter sizes	
					Total born (mean ±SD)	Weaned alive (mean ±SD)
Technician 1	232	198	85.3[a,b]	81.9[c,d]	7.50±1.25	5.71±1.27
Technician 2	268	246	91.8[a]	88.8[c]	7.52±1.37	5.72±1.34
Technician 3	239	220	92.1[b]	89.1[d]	7.52±1.42	5.72±1.39

DISCUSSION

According to Table 1, low dose insemination (50 and 30 million spermatozoa) had no difference on the conception rate of vixens with normal dose (70 million spermatozoa) and thus can save the sperm count. The result of this study confirm that 30 million good quality fresh semen is enough to achieve satisfactory fertility in blue fox insemination which enables the effective use of good quality semen. These results allow us to conserve spermatozoa, use frozen-thawed semen, and commercialize sex-preselection technology (Morris, 2004). This practical procedure will reduce the commercial cost and offer great economic and social benefits to the fur animal industry.

Although low dose insemination had produced similar or superior fertility in many breeds (Lake and Ravie, 1987; Leipold *et al.*, 1998; Krueger *et al.*, 1999; Lindsey *et al.*, 2001; Kurykin *et al.*, 2003; Roca *et al.*, 2003; Presicce *et al.*, 2005; Kurykin *et al.*, 2006; Martinez *et al.*, 2006; de Graaf *et al.*, 2007; Gao *et al.*, 2009; An *et al.*, 2010), some people hold adverse views (Bracken *et al.*, 2003; Andersson *et al.*, 2004; Andersson *et al.*, 2006; Peippo *et al.*, 2009), which may due to the diversity of breeds, herd management and insemination technique.

There is little published evidence of pregnancy loss or lower litter size correlated with low dose insemination. We showed there is no significant difference in the mean litter size between low dose (50 and 30 million spermatozoa) and normal dose insemination groups. However, early pregnancy loss was found in sows after low dose, deep uterine artificial insemination (Bathgate *et al.*,2008). There is a lower litter size in spontaneously ovulating sows non-surgical deep intrauterine inseminated with 1.5×10^8 spermatozoa (Martinez *et al.*,2006) and a negative effect on both the conception rate and mean litter size was observed with decreasing frozen silver fox (*Vulpes vulpes*) sperm numbers in blue fox vixens (Farstad *et al.*, 1992). Causes influencing AI, including the number and motility of spermatozoa, estrus detection and timing of AI as well as the animals' physical condition can serve to explain the difference.

Synchronized and spontaneous estrus was not compared in our study which needs further research.

In this field study, a volume of 1 ml was used for insemination in the present experiment, we achieved nearly 90% pregnancy rate. This rate is similar to 90.7% (Xu *et al.*, 2000) and is higher than 73% (Yang *et al.*, 1999) and 78.5-88.5% (Yue *et al.*, 2001). Vixen fertility may be increased by improving several causes, such as careful selection of male foxes for AI, accurate estrus detection, the exact time and location of insemination as well as optimizing semen processing, handling and insemination technique (Bodmer *et al.*, 2005). The

conception rates were also affected by technicians (Table 4). To effectively improve its pregnancy rate, the experience of the inseminator may be special important. The technician staff should master the techniques of artificial insemination and estrus determination of vixens.

However, to make great progress, we should take further research, including large-scale insemination trials using low-dose inseminations to confirm our observation, determination of the minimum effective dose and more precise timing of insemination to get satisfactory fertility results (Vazquez *et al.*, 2005).

While using lower insemination doses, semen deposition closer to the site of fertilization has been shown to improve pregnancy rates (Verberckmoes *et al.*, 2005). Compared with standard AI, the number of fresh sperm can be reduced three times in post-cervical insemination to achieve acceptable pregnancy rate (Vazquez *et al.*, 2008). Although It was reported that semen deposition sites did not influence pregnancy rates (Andersson *et al.*, 2004). In this study, we achieved about 93.5% pregnancy rate from vixens inseminated into the uterine body versus 86.0% into the cervix in the control group, which also makes the birth rate and total born mean litter size differences. These results showed that to achieve acceptable pregnancy rate, the semen is better taken into the uterine body than the cervix. It seems that deep insemination into the uterine body can save the number of spermatozoa each cycle. Therefore, intrauterine insemination technique can broaden the use of low dose insemination in foxes. Our results were consistent with previous studies (Morris,2004; Kurykin *et al.*, 2007). This theory, however, has to be statistically confirmed in future studies using a larger sample sizes. The interaction of insemination location (cervix versus uterine body) and insemination time (about ovulation) needs to be tested in future studies too.

The main advantage of uterine body insemination procedure is decreasing sperm losses by phagocytosis, avoiding backflow loss from 25 to 45% (or more) by the cervical folds (Mezalira *et al.*, 2005; Vazquez *et al.*, 2008) and reducing the distance the spermatozoa have to travel before fertilization. Therefore, we should pay careful attention to the proper insemination technology to minimize the incidence of cervical and uterine damage (Vazquez *et al.*, 2008). More investigations are needed to declare the mechanisms related to phagocytosis and backflow loss to increase the fertilization rate especially when ovulation is delayed.

Besides deep insemination (into the uterine body), multiple inseminations were considered helpful especially with low dose sperm.

Efficient and accurate detection of estrus and proper timing of insemination are also important to ensure fertilization (An *et al.*, 2010). As the growth of follicles and the time of ovulation cannot be diagnosed exactly by rectal palpation, only secondary signs of estrus

are used as signs, it's not easy to determine precisely the correct time of insemination to achieve acceptable fertilization. Therefore, at least one dose should be available each cycle (Rath, 2002). Multiple inseminations can ensure plenty sperm were available at the fertilization site for the oocytes.

Based on our results, two doses should be inseminated each cycle; a third dose may be required to cover the time span of multiple ovulations or delayed ovulation. Yet no difference between mares inseminated once and multiple doses was found before (Loomis and Squires,2005). To minimize insemination frequency and discover the best time for AI, more reliable methods for predicting the time of ovulation are required and ultrasonography may be a solution in the future.

No statistics was performed on male fox effect alone, due to the experimental design, in which the effects of male fox could not be distinguished.

We also saw a significant variation between cubs weaned alive and total born cubs, that's because the offspring of Finland foxes showed a poorer quality of maternal behavior than that of local breed. Maternal behavior can affect development and survival of offspring, the cub will be strong, have high disease and trouble resistance ability if its mother has normal maternal behavior (Dwyer, 2008). To explore the neuroendocrine mechanisms those underpin the differences between diverse breeds and differences between vixens of primiparous and multiparous deserves future work.

In conclusion, we have achieved an acceptable conception and birth rate with low dose insemination (30 to 50 million spermatozoa). Multiple intra-uterine inseminations with low doses are good for satisfactory pregnancy results. In addition, excellent animal management is critical to achieve these results under field conditions.

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