Boar semen bacterial contamination in Italy and antibiotic efficacy in a modified extender

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Abstract

The aims of the study were to identify microbial flora in boar semen under field conditions in northern Italy, to investigate antibiotic resistance and sensitivity of isolated bacteria, and to evaluate elimination of bacteria after storage in two types of extenders added with different antibiotics (amikacin vs gentamicin). A total of 60 boars were collected in 13 pig farms. Bacteriological and mycological investigations were performed immediately on raw semen samples, then at 48 and 120 h of storage on extended semen doses. The analysis of variance of factors affecting bacterial contamination levels was significant for the breed. Antibiotic resistance of these bacteria has been observed among isolates from boar semen against antibiotics commonly used as preservative antimicrobials in commercial porcine semen extenders (Althouse and Lu, 2005). The aims of the present paper were to investigate the microbiological content of boar semen in northern Italy, to test the sensitivity and resistance of isolated bacteria to several antibiotics, and to evaluate elimination of bacteria after storage in two type of extenders added with different antibiotics (amikacin vs gentamicin), and if possible, to point out the importance of periodic microbiological screening of semen/doses performed in swine industry to avoid the use of semen with low sperm quality.

Introduction

Bacterial contamination is a serious and very real deterrent to profitable semen production in swine. It is routinely observed in raw and extended semen produced for artificial insemination (AI) when semen is collected by the gloved-hand technique. A study has reported that 62.5% of raw ejaculates and 79% of extended semen doses investigated yielded bacterial contamination (Tamuli et al., 1984). Nonetheless, bacteriological surveillance is still seldom adopted in swine management even if semen doses are stored at 15-20°C to avoid cold shock of boar spermatozoa (Paulenz et al., 2000; Pursel et al., 1973). Storage temperature favours the survival of bacterial contaminants. Bacterial contamination of porcine semen has been associated with deleterious effects on semen quality (Althouse et al., 2008), affecting motility and structure of the sperm and causing sperm agglutination and shortening of viability (Monga and Roberts, 1994). This reduces fertility, conception rates, and litter size at birth (Engblom et al., 2007) and shortens shelf life of semen doses (Althouse et al., 2000). Contaminating microorganisms can come from different sources of animal or non-animal origin (Althouse et al., 2005). Mammalian origin includes microorganisms from preputial fluids, urogenital system, skin, hair, respiratory secretions, feces, poor hygiene conditions, and personnel contamination (Althouse, 2008). Environmental contamination comes from contaminated water, feed, bedding, equipment, and air ventilation systems (Althouse and Lu, 2005; Althouse et al., 1998). Many bacterial species have been isolated from semen, most frequently Escherichia coli, Pseudomonas spp., Staphylococcus spp., Proteus spp., Enterobacter spp., Klebsiella spp, and occasionally also Clostridium spp. (Althouse and Lu, 2005; Arredondo et al., 2001; Bussalleu et al., 2013). For stallions, the environment in which they are housed has been reported to be the main cause of the presence of organisms on the external genitalia (Varner et al., 1998); this may be true for boars as well. Hygienic semen collection and proper processing techniques with stringent laboratory procedures are the first and primary lines of defense to successfully reduce contamination. Also, antibiotics are added to semen extenders as a preventive measure to reduce bacterial contamination (Gadea, 2003; Johnson et al., 2000). New generation antibiotics such as apramycin and cefitiofur have been used, although conclusive results are not yet available (Althouse, 1997). Drug resistance has been observed among isolates from boar semen against antibiotics commonly used as preservative antimicrobials in commercial porcine semen extenders (Althouse and Lu, 2005). The aims of the present paper were to investigate the microbiological content of boar semen in northern Italy, to test the sensitivity and resistance of isolated bacteria to several antibiotics, and to evaluate elimination of bacteria after storage in two type of extenders added with different antibiotics (amikacin vs gentamicin), and if possible, to point out the importance of periodic microbiological screening of semen/doses performed in swine industry to avoid the use of semen with low sperm quality.

Materials and methods

Samples

During September and October 2012, 60 samples of raw semen from 60 different mature boars reared on 13 pig farms were collected. Thirty-five samples came from 2 farms specialized in semen dose production, processing, and delivery all over Italy; the other 25 were from 11 farms practicing integral pig production. The farms chosen were located in northern Italy (Provinces of Reggio Emilia, Modena, Mantova, Cremona, Brescia, and Verona), where more than 75% of Italian pigs
are housed (Moreno et al., 2010). The boars included in this study belonged to different breeds (Large White, Landrace, Pietrain, Duroc) and synthetic populations (Goland, Hypor, Maxgrow, PIC, TopD) and were all housed in an individual box and were of proved fertility. All the semen presented good quality standards. Frequency of collection was 2/3 time a week by specialized farm personnel. Semen was collected by gloved-hand technique with a dummy into an insulated bottle (500 mL), with the opening covered with gauze to separate the gel particles; only the second and third phases were considered. All the boars used as semen donors were healthy.

Semen evaluation and dilution

Evaluation of semen characteristics (volume, pH, colour, smell, motility, concentration of the sperm) was performed immediately after collection. Only ejaculates with sperm motility higher than 75% immediately after collection and at least 90% of spermatozoa showing normal morphology were considered. After evaluation, raw semen aliquots were randomly diluted in one of two types of extender at a 1:9 dilution (100 mL 3×10⁶ sperm/mL): 35 samples were diluted in a new modified extender (ME-S) for short-term storage containing amikacin sulphate and fructose (Bresciani et al., 2013) and 25 samples were diluted with a commercial extender CRONOSTM (Medinova™, Reggio Emilia, Italy). The composition of ME-S is reported in Table 1, while CRONOSTM contains gentamicin (0.33 g/L) and glucose, as declared by Medinova™. The sperm characteristics and motility during storage were evaluated in vitro as reported in a previous study (Bresciani et al., 2013).

Bacteriological and mycological investigations

The raw samples were immediately sent to the laboratory and cultured for bacteriological and mycological investigations. The same investigations were performed on diluted semen after 48 and 120 h of storage at 15±1°C. Culture swabs were plated on bovine blood agar medium (tryptose agar - Beckton Dickinson, Sparks, Maryland, USA containing 5% bovine erythrocytes) (Quinn et al., 1994; Carter et al., 1995) and MacConkey agar (Beckton Dickinson, Sparks, Maryland, USA). For each specimen cultures were then incubated aerobically and anaerobically (AnaeroGen™, Oxoid, UK) for 24-48 h at 37°C. For mycological investigations, Sabouraud agar (Beckton Dickinson™, Sparks, MD, USA) plates were inoculated and incubated aerobically for 48 h at 35°C. Bacterial isolates were identified using standard microbiological procedures, i.e. growth and colonial characteristics, Gram staining, cellular morphology, catalase and oxidase reactions, coagulase test and haemolysis production. Species identification was carried out using the API biochemical test systems (bioMérieux™, Marcy-l’Étoile, France) as well as conventional biochemical tests (Quinn et al., 1994). Antibiotic sensitivity tests were performed with the standard Kirby- Bauer disk-fusion susceptibility methods (Quinn et al., 1994), evaluated on the basis of the criteria employed by the Clinical and Laboratory Standard Institute (CLSI), formerly the National Committee for Clinical and Laboratory Standards (NCCLS) (CLSI, 2008). In the present study 25 antibiotics were tested on 7 bacterial species isolated from fresh boar semen for a total of 43 strains (Table 2). We were specifically interested in amikacin and gentamicin activity because they are present in the two extenders compared in this investigation.

Data collection and analysis

Data were submitted to statistical analysis to assess the frequency of contaminated samples and the kind and degree of contamination. A χ² test was performed to assess differences between observed and expected frequencies. An analysis of variance was then performed on data from raw and diluted samples to evaluate the effects of type of extender, breed of boar (nested within herd and type of extender), and kind of farm (nested within type of extender) on the degree of contamination.

Results and discussion

Bacterial species were present in 38 samples out of the 60 examined (63%). Thirty-three samples (86.85%) were positive for pure culture of a single bacterial species (16 E. coli, 6 Serratia marcescens, 5 Staphylococcus epidermidis, 1 Streptococcus spp., 2 Proteus spp., 2 Pseudomonas spp.). Five samples (13.16%) were positive for co-infected cultures of two bacterial species (2 E. coli and Proteus spp., 1 E. coli and Staphylococcus epidermidis, 1 Staphylococcus epidermidis and Streptococcus spp., 1 E. coli and Serratia marcescens). A total of 43 isolates were detected: E. coli (n=20), Staphylococcus epidermidis (n=8), Serratia marcescens (n=6), Proteus spp. (n=4), Staphylococcus aureus (n=2), Streptococcus spp. (n=2), Pseudomonas spp. (n=1). E. coli was the most frequent contaminant bacterium (46.5%), followed by Staphylococcus epidermidis (18.6%), Serratia marcescens (13.95%), Proteus spp. (9.30%), Staphylococcus aureus and Streptococcus spp. (4.65% each), and Pseudomonas spp. (2.3%) (Table 2). Each isolate was characterized as either obligate aerobic or facultative anaerobic. No strictly anaerobic isolate was detected. All tested samples of raw and diluted semen were negative for mycological investigation. The antibiotic resistance data regarding bacterial isolates are also shown in Table 2. Significant differences (P<0.05) between observed and expected frequencies of bacterial isolates resistant or not to the antibiotics contained in the extenders were found. Values of χ² ranged from 5.67 to 200 (data not tabulated). In particular, E. coli isolates showed 50% resistance to amikacin and 70% resistance to gentamicin; Staphylococcus epidermidis was 12.5% resistant to amikacin and 50% to gentamicin; Serratia marcescens showed a resistance of 66.6% to amikacin and 50% to gentamicin; Proteus spp. isolates were 25% resistant to amikacin and 50% to gentamicin; Streptococcus spp. isolates were 0% resistant to amikacin and 50% to gentamicin; Staphylococcus aureus isolates were totally sensitive to amikacin (0% resistance) and 100% resistant to gentamicin. Some other antibiotics, like ceftriaxone, enrofloxacin and marbofloxacin, proved to be effective in several cases and will be useful for further investigations. Table 3 shows the efficacy of the two extenders for the reduction of bacteria concentration. After 48 h of storage in ME-S extender, E. coli strains were reduced from 12 to 3 (75%) while in CRONOSTM group dropped from 8 to 4 (50%); at 120 h no changes were observed compared to 48 h. Serratia marcescens was inhibited in amikacin group at 48 h and in the gentamicin group it was reduced by 50%; Proteus spp. was present only in CRONOSTM extended sample and was reduced by 25% after 48 h of storage. During storage, amikacin completely inhibited the growth of Serratia marcescens, Pseudomonas spp., Staphylococcus
Staphylococcus aureus, Staphylococcus epidermidis, and Streptococcus spp. The analysis of variance of factors affecting contamination levels in raw and diluted sperm samples (Table 4) showed a significant effect of the farm of origin (P<0.05) and no significant effect of the breed of boar. The type of extender tended to be significant (P<0.10) for aerobic contamination of diluted semen. The least squares means of contamination degree (Table 5) showed a reduction of aerobic contamination after ME-S dilution by 85.3% and after CRONOSTM by 63.8%. For anaerobic isolates the reduction was 100 and 91.8%, respectively. No difference in contamination degree was found between 48 and 120 h. Mean percentages of progressively motile sperm at 48 h and 120 h were not significantly different (P>0.05) between ME-S and CRONOSTM (70.0±5.2 vs 76.0±1.9 and 59.2±9.1 vs 55.0±5.3).

Table 2. Bacterial contaminants and antibiotic resistance in raw semen.

| Antibiotic                  | E. coli | Staph. epidermidis | Serratia marcescens | Proteus mirabilis | Streptococcus spp. | Staph. aureus | Pseudomonas spp. |
|-----------------------------|---------|--------------------|---------------------|-------------------|-------------------|---------------|------------------|
| Amikacin (30 µg)            | 10 (50%)| 1 (12.5%)          | 4 (66.6%)           | 1 (25%)           | 0 (0%)            | 0 (0%)        | 0 (0%)           |
| Amox. + Clav. acid (30 µg)  | 11 (55%)| 2 (25%)            | 4 (66.6%)           | 2 (50%)           | 0 (0%)            | 1 (100%)      | 1 (100%)         |
| Ampicillin (25 µg)          | 15 (75%)| 3 (37.5%)          | 5 (83.3%)           | 3 (75%)           | 0 (0%)            | 1 (100%)      | 1 (100%)         |
| Aztreonam (30 µg)           | 11 (55%)| 0 (0%)             | 3 (50%)             | 1 (25%)           | 0 (0%)            | 1 (100%)      | 1 (100%)         |
| Aminosidine (60 µg)         | nd      | 3 (37.5%)          | 4 (66.6%)           | nd                | nd                | 1 (50%)       | nd               |
| Cefapirin (30 µg)           | 11 (55%)| 2 (25%)            | nd                  | nd                | nd                | nd             | 0 (0%)           |
| Cefazolin (30 µg)           | 11 (55%)| 3 (37.5%)          | 5 (83.3%)           | 3 (75%)           | 0 (0%)            | 1 (50%)       | 1 (100%)         |
| Cefoperazone (30 µg)        | 13 (65%)| 1 (12.5%)          | 5 (83.3%)           | 2 (50%)           | 0 (0%)            | 1 (100%)      | N.D.             |
| Cefotaxime (30 µg)          | 4 (20%) | 1 (12.5%)          | 4 (66.6%)           | 0 (0%)            | 1 (100%)          | 1 (100%)      | 1 (100%)         |
| Ceftriaxone (30 µg)         | nd      | 0 (0%)             | nd                  | nd                | 0 (0%)            | nd             | nd               |
| Cefquinome (30 µg)          | 7 (35%) | 3 (37.5%)          | 5 (83.3%)           | 3 (75%)           | 0 (0%)            | 0 (0%)        | 0 (0%)           |
| Colistin (10 µg)            | 19 (95%)| 7 (87.5%)          | 6 (100%)            | 4 (100%)          | 1 (50%)           | 2 (100%)      | 1 (100%)         |
| Doxycycline (20 µg)         | 17 (85%)| 3 (37.5%)          | 5 (83.3%)           | 1 (25%)           | 0 (0%)            | 1 (50%)       | 1 (100%)         |
| Danofloxacin (5 µg)         | 17 (85%)| 3 (37.5%)          | 5 (83.3%)           | 4 (100%)          | 0 (0%)            | 1 (100%)      | 1 (100%)         |
| Enrofloxacin (5 µg)         | 1 (5%)  | 0 (0%)             | 6 (80%)             | 1 (25%)           | 0 (0%)            | 0 (0%)        | 0 (0%)           |
| Florfenicol (30 µg)         | 13 (65%)| 2 (25%)            | 3 (50%)             | 3 (75%)           | 0 (0%)            | 0 (0%)        | 1 (100%)         |
| Flumequine (30 µg)          | 12 (60%)| 2 (25%)            | 5 (83.3%)           | 3 (75%)           | 0 (0%)            | 1 (50%)       | 1 (100%)         |
| Gentamicin (10 µg)          | 14 (70%)| 4 (50%)            | 3 (50%)             | 2 (50%)           | 1 (50%)           | 2 (100%)      | 0 (0%)           |
| Marbofloxicin (5 µg)        | 0 (0%)  | 0 (0%)             | 0 (0%)              | 0 (0%)            | 0 (0%)            | 0 (0%)        | 0 (0%)           |
| Oxytetracycin (30 µg)       | 17 (85%)| 3 (37.5%)          | 5 (83.3%)           | 4 (100%)          | 0 (0%)            | 1 (50%)       | 1 (100%)         |
| Penicillin G (10 µg)        | 17 (85%)| 3 (37.5%)          | 5 (83.3%)           | 4 (100%)          | 0 (0%)            | 1 (50%)       | 1 (100%)         |
| Rifaximin (40 µg)           | 14 (70%)| 3 (37.5%)          | 5 (83.3%)           | 1 (25%)           | 0 (0%)            | 1 (50%)       | 1 (100%)         |
| Streptomycin (10 µg)        | 17 (85%)| 3 (37.5%)          | 5 (83.3%)           | 3 (75%)           | 0 (0%)            | 1 (50%)       | 1 (100%)         |
| Tiamulin (30 µg)            | 20 (100%)| 7 (87.5%)         | 6 (100%)            | 4 (100%)          | 1 (50%)           | 2 (100%)      | 1 (100%)         |
| Tylosin (30 µg)             | 20 (100%)| 7 (87.5%)         | 6 (100%)            | 4 (100%)          | 2 (100%)          | 2 (100%)      | 1 (100%)         |

nd, not detected.

Table 3. Comparison of bacterial contamination in extenders containing amikacin (ME-S) and gentamicin (CRONOSTM) during storage at 15±1°C.

| Bacterial isolates | Semen samples diluted | Semen storage 48 h | Semen storage 120 h |
|--------------------|------------------------|--------------------|---------------------|
|                    | ME-S (n=35)            | CRONOS™ (n=25)     | ME-S (n=35)         | CRONOS™ (n=25)     | ME-S (n=35) | CRONOS™ (n=25) |
| Escherichia coli   | 12                     | 8                  | 3 (25%)             | 4 (50%)            | 3 (25%)      | 4 (50%)       |
| Staphylococcus epidermidis | 5                  | 3                  | 0                   | 0                   | 0           | 0             |
| Serratia marcescens| 2                      | 4                  | 0                   | 2 (50%)            | 0           | 2 (50%)       |
| Proteus spp.       | 0                      | 4                  | 0                   | 1 (25%)            | 0           | 1 (25%)       |
| Streptococcus spp. | 2                      | 0                  | 0                   | 0                   | 0           | 0             |
| Pseudomonas spp.   | 1                      | 0                  | 0                   | 0                   | 0           | 0             |
| Staphylococcus aureus | 1                  | 1                  | 0                   | 1 (100%)           | 0           | 1 (100%)      |
| Total strains      | 26                     | 17                 | 4                   | 7                   | 4           | 7             |

ME-S, modified extender short-term.
sequently on livestock reproductive efficiency (Althouse, 2008), there is constant demand for innovative boar semen extenders to improve the viability of semen doses (Bresciani et al., 2013). The present study reported that bacterial contaminant species in boar semen are also commonly isolated (63%) in Italy though the species isolated are partly different from those reported in the literature. In our study we isolated 7 species (*E. coli*; *Serratia marcescens, Staphylococcus epidermidis, Streptococcus sp., Proteus spp.; *Staphylococcus aureus, Pseudomonas spp.*) while other authors have counted an additional 18 bacterial species in North America, Brazil, Cuba, Korea, and Thailand (Althouse et al., 2000; Bussalleu et al., 2013; Maroto Martín et al., 2010; Park et al., 2008; Suwimonteeraburt et al., 2011). The majority of the contaminants identified in extended semen are Gram-negative bacteria, almost all belonging to the *Enterobacteriaceae* family. It has been scientifically proven that the presence of contaminants damages the viability and fertility of the semen (Althouse, 2008). As reported in humans by Monga and Roberts (1994), *E. coli* induces an agglutinating effect on 40-75% of motile sperm, reported even in pigs (Althouse, 2008; Bussalleu et al., 2011). Bacterial contamination overgrowth yields worse semen quality and longevity (Aurich and Spergser, 2007, So et al., 2011) in a concentration-dependent manner (Althouse and Lu, 2005). If such doses are used in AI programs can increase returns to oestrus, *post*-insemination vulvar discharges, and reduced herd reproductive performances (Sone et al., 1989). The substitution of amikacin in place of gentamicin in our new modified extender has shown to improve the fertility of the semen. In fact, for all the 7 different bacterial species from 43 isolates amikacin had good antibacterial activity, and in a recent field trial the fertility was increased, with a significant rise in the number of piglets (Bresciani et al., 2013). *E. coli* was the most frequently isolated contaminant bacteria [20/38 (prevalence 53%) of semen samples]; a potentially pathogenic agent like *P. aeruginosa* (Pasing et al., 2013) was found only in one semen sample. A previous study reported that microbiological controls in boar farms that complained of reproductive management problems (*i.e.* sperm agglutination, decreased sperm longevity, increased regular returns to oestrus, or even vaginal discharges across parity) showed the presence of bacteria in 66% of semen doses. All contaminant bacteria were found to be resistant to gentamicin and *E. coli* exhibited spermicidal activity even in the absence of acid environment (Althouse et al., 2000). The World Organisation for Animal Health (OIE; Office International des Epizooties) published the standard of 5.0×10³ cfu/mL as a limit for the bacterial contamination of bovine semen samples (OIE, 2001) and Maroto Martín et al. (2010) reported that when boar semen used for AI is contaminated with *E. coli* above a threshold value of 3.5×10³ cfu/mL there is a significant reduction in litter size. According to Bussalleu et al. (2011), significant adverse effects on porcine sperm quality were reduced regular returns to oestrus (AIMA). *E. coli* in the experiment performed at 37°C. In our study, pH was maintained neutral in both extenders. Some authors advised a minimum contamination technique (MCT) protocol to standardize hygiene and sanitation since in field conditions bacteria come from multiple origins but almost all relating to boar preparation, semen processing, and laboratory sanitation (Althouse, 2008; Althouse et al., 2000). Likewise, extenders are very rich media that contain enough nutrients to support bacterial growth (Maroto Martín et al., 2010). Amikacin showed a good activity against isolated bacteria, in our opinion improving semen quality by reducing bacterial contamination. In a previous study, the ME-S amikacin concentration did not show any deleterious effect on sperm viability, and reproductive fertility increased in an AI field trial (Bresciani et al., 2013). In our samples we did not identify *Clostridium* species, which has been reported in swine industry (Baker et al., 2010; Maroto Martín et al., 2010) and in frozen equine semen (Corona and Cherchi, 2009). Varner et al. (1998) demonstrated that amikacin in extender for equine semen was more effective in eliminating bacterial growth than was polymixin B, which showed detrimental effects on sperm motility, while amikacin appeared to provide the best conditions for maintenance of sperm motility. Moreover, Aurich and Spergser (2007) demonstrated that gentamicin was not effective in reducing bacteria detrimental effects in equine semen samples. The presence of *Ureaplasma diversum, Histophilus somni, Chlamydophila abortus, Escherichia coli*, and *Staphylococcus sp.*, has been reported during reproductive tract infection of the cows and *Pseudomonas*, are opportunistic microorganisms of the bull’s genital tract (Schlafer and Miller, 2007). Recently, Kilburn et al. (2013)

Table 4. Analysis of variance (P) of factors affecting boar semen contamination.

| Type of extender | Breed of boar | Herd | RSE | R² |
|-----------------|---------------|-----|-----|----|
| Raw semen       |               |     |     |    |
| Aerobic         | ns            | ns  | 0.035 | 0.544 | 0.593 |
| Anaerobic       | ns            | ns  | 0.028 | 0.474 | 0.373 |
| Diluted semen, 48 h | 0.083 | ns  | 0.021 | 0.311 | 0.556 |
| Aerobic         | ns            | ns  | 0.004 | 0.114 | 0.492 |
| Anaerobic       | ns            | ns  | 0.004 | 0.114 | 0.492 |

ns, not significant; RSE, relative selection efficiency. *Facultative anaerobic.

Table 5. Least squares means of degree of contamination as affected by type of extender.

| Group          | ME-S | CRONOS™ | ME-S | CRONOS™ |
|----------------|------|---------|------|---------|
| Aerobic        | 0.641±0.099 | 0.784±0.118 | 0.094±0.057 | 0.284±0.068 |
| Anaerobic      | 0.318±0.087 | 0.553±0.103 | 0.000±0.021 | 0.029±0.025 |

ME-S, Modified extender short-term. Degree of contamination refers to the following parameters: 0=sterile, 1=contaminated by one bacteria species, 2=contaminated by two bacteria species. *Facultative anaerobic. *Means in the same raw with different superscript are significantly different at 0.05-P<0.01.
reported the presence of Gram-negative bacteria Stenotrophomonas spp. Sphingomonas and Pseudomonas, sensitive to gentamicin in bovine semen. Greater scientific understanding of the transfer of antibiotic resistance, especially in pathogenic bacteria, is needed and concerns over the routine use of antibiotics as a substitute for good animal husbandry practices within intensive farming must be addressed (Kilburn et al., 2013).

Conclusions

The results obtained in the present study showed that raw boar semen in northern Italy has a consistent microbiological content. Almost all microorganisms came from collecting procedures (herd environment) or from health conditions of the boar. The germs are different from those isolated by authors working in other countries. Amikacin showed a higher microbial activity compared to gentamicin decreasing germ viability and concentration and thus is a useful component of boar semen extenders. Obviously, contamination control of semen by adding an adequate antibiotic in the medium must be accompanied by optimal hygiene measures during semen collection. In our opinion, performing periodic microbiological screening of boar semen in the swine industry is highly advisable so as to avoid the use of low sperm quality doses.

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