The use of unfrozen semen for *in vitro* fertilisation of *in vitro* matured bovine oocytes

A.D.SEATON, J.W.CATT, S.L.RHODES, M.F.McDONALD[1], AND R.A.S. WELCH.

Grasslands Division, DSIR, Private Bag, Palmerston North, New Zealand.

ABSTRACT

The use of unfrozen, diluted bovine semen for *in vitro* fertilisation (IVF) was investigated. *In vitro* matured bovine oocytes were incubated with various (0.125 - 4.0 x 10^6/ml) concentrations of washed sperm. Penetration rates ranged from 21% at low sperm concentrations to 80 - 100% above 1 x 10^6 sperm/ml (P<0.01). Unfrozen sperm gave better penetration rates than frozen-thawed sperm. Oocytes were incubated with washed sperm (1 x 10^6/ml) in increasing dilutions of caprogen extender. Sperm penetration was significantly inhibited at dilutions of 10-fold or less (P<0.01). Oocytes were inseminated with washed sperm (1 x 10^6/ml) at various concentrations (0 - 50 μg/ml) of heparin. No significant differences were found.

Keywords Bovine, IVF, sperm, extender, heparin.

INTRODUCTION

The techniques of *in vitro* maturation and fertilisation (IVM and IVF) of bovine oocytes have the potential to benefit both research and practical agriculture in a variety of ways. Application of these techniques can produce large quantities of fertilised oocytes for experimental manipulation or for embryo transfer programmes.

Unfrozen semen is often utilised in bovine IVF experiments, but only a few workers (Critser *et al.*, 1984; Bondioli and Wright, 1983; Wheeler and Seidel, 1986) have carried out research on the fertilisation parameters involved. Unfrozen semen may be preferable to the more convenient frozen-thawed semen for *in vitro* work because the sperm have no freezing damage and therefore tend to have greater viability.

A series of trials was designed to explore the use of unfrozen semen in IVF experiments. Sperm concentration (known to influence fertilisation rates and later embryo development in A.I. and in IVF trials - Bratton *et al.*, 1954; First and Parrish, 1987), and the effects of caprogen (the extender used with unfrozen semen by the New Zealand Dairy Board - Shannon 1965; Shannon and Curson, 1984) and heparin (a glycosaminoglycan known to facilitate sperm

capacitation - Parrish *et al.*, 1985) were investigated.

MATERIALS AND METHODS

Oocyte Collection and Maturation

Ovaries were collected from unidentified cattle at the freezing works approximately 20 minutes after slaughter. A pre-warmed (39°C) thermos was used to transport the ovaries back to the laboratory, where they were trimmed of excess fat and tissue and washed three times in 39°C PBS. Follicles on and under the surface of the ovaries were aspirated with an 18-gauge needle attached to a 10 ml syringe. The aspirated follicular fluid was expelled into plastic petri dishes and searched for oocytes using a dissecting microscope. Oocytes were transferred into a dish containing modified Hepes 199 medium (H199) supplemented with foetal calf serum (FCS). The dish was kept in a bench incubator (39°C) until all ovaries had been aspirated. Non-atretic oocytes with several intact surrounding layers of cumulus cells were then selected to undergo maturation in petri dishes containing modified bicarbonate 199 medium with 10% FCS, 10% oestrous cow serum and approximately 100 000 granulosa cells. Oocytes were matured at 39°C in a gas atmosphere of 5% CO_2 in air with 100% humidity for

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[1] Department of Animal Science, Massey University, Palmerston North, New Zealand.

24 hours.

Time from slaughter to start of maturation was approximately 2-3 hours.

Sperm Preparation

A simple wash technique was employed.

1) *Frozen-thawed semen.* A straw of frozen semen was thawed in 37 °C water for 15 seconds and the semen released into a 15 ml centrifuge tube. The sperm were centrifuged twice for 7 minutes at 50xg, using a modified calcium-free Tyrode's solution (TALP) as the wash medium. After the second centrifugation all but 100 µl of the supernatant was removed and the sperm pellet was resuspended by tapping the tube. 5 µl of the suspension was removed for sperm counting.

2) *Unfrozen semen.* The procedure was as for frozen semen, except that 3 washes were required; the first 2 for 5 minutes at 185xg and the third for 7 minutes at 50xg.

The first ejaculate from each of the two bulls used was split and utilised in the first trial as frozen-thawed or unfrozen diluted sperm. Unfrozen semen was collected twice a week and sperm from each collection was used on 2 consecutive days.

Fertilisation

The washed sperm were added to fertilisation medium to give the desired concentration. The fertilisation medium was modified TALP supplemented with caffeine (1.39 mM) and heparin (10 µg/ml). Drops (45 µl) were placed in a petri dish and covered with warm paraffin oil. Matured, cumulus-expanded oocytes were partially stripped of their supporting cells and transferred to the fertilisation droplets (10-20/drop), which were then incubated (39°C, 5% CO_2 in air, 100% humidity) for 18 hours.

Fixing and Staining

After insemination the oocytes were removed from the droplets, placed in H199 and stripped of their cumulus layers by gentle pipetting. Oocytes were mounted on slides and fixed in acid-alcohol (25% acetic acid in 75% alcohol) for at least 24 hours. They were cleared in ethanol for 30 minutes before being stained with either lacmoid or orcein (1% in acetic acid).

Assessment

The oocytes were classified by their stage of development and whether or not they were penetrated. Oocytes were assumed to be penetrated if they possessed one or more pronuclei with either a detached sperm tail or evidence of decondensing sperm in the cytoplasm. Unpenetrated oocytes were classified according to whether or not they had reached the metaphase II stage of nuclear maturation. Immature oocytes were excluded from the results.

Statistical Analysis

Analyses of variance were used to determine penetration rate differences between treatments, bulls and semen ages.

Trial 1
Sperm Concentrations

The concentration of the washed sperm was determined using a haemocytometer count and then sperm were diluted to the desired concentrations of 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 x 10^6/ml. Oocytes were then added to microdroplets of each concentration. 214 oocytes were inseminated with frozen-thawed sperm from each bull at the 4 higher concentrations. 60 oocytes were incubated without sperm as a control for parthenogenesis.
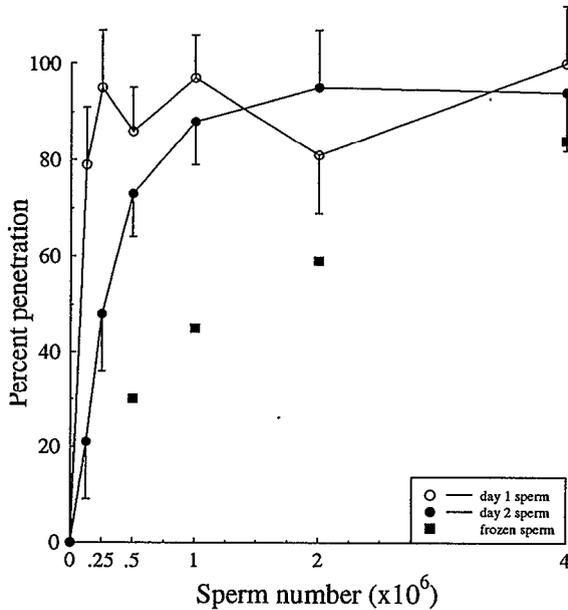
Trial 2
Caprogen Dilutions

Washed sperm were added to tubes containing caprogen diluted with fertilisation medium to the concentrations required: 1:0 (neat caprogen), 1:1, 1:10, 1:100, 1:300, 1:1000. The 1:1000 treatment (essentially washed sperm) acted as the control for the trial. The final sperm concentration used was 1 x 10^6/ml.

Trial 3
Heparin Concentrations

Heparin was added to the fertilisation medium to give the various concentrations of 0, 1, 5, 10, 20, 30 and 50

μg/ml. The final sperm concentration was 1×10^6/ml. Only 1 bull was used in this trial, the semen characteristics of the second bull having become unsatisfactory.

RESULTS
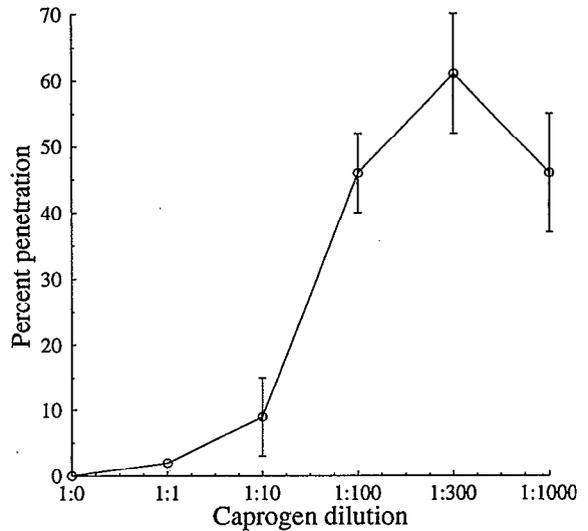

data pooled over replicates and semen ages

FIG 1 Penetration rates vs sperm number for 1- and 2-day old sperm.

Trial 1

744 oocytes were assessed and the results are shown in Figure 1. Penetration rates at a sperm concentration of 0.125×10^6/ml were significantly lower than those obtained at all other concentrations (P<0.01). Penetration rates achieved with 1- and 2- day-old semen were significantly different (P<0.01). There were no differences between concentrations when day-old semen was used, with penetration rates consistently exceeding 80%. A significant difference was found with 2-day-old semen between concentrations below 0.5×10^6/ml (P<0.01). There were no differences between the two bulls. The 60 oocytes not inseminated did not show signs of parthenogenesis.
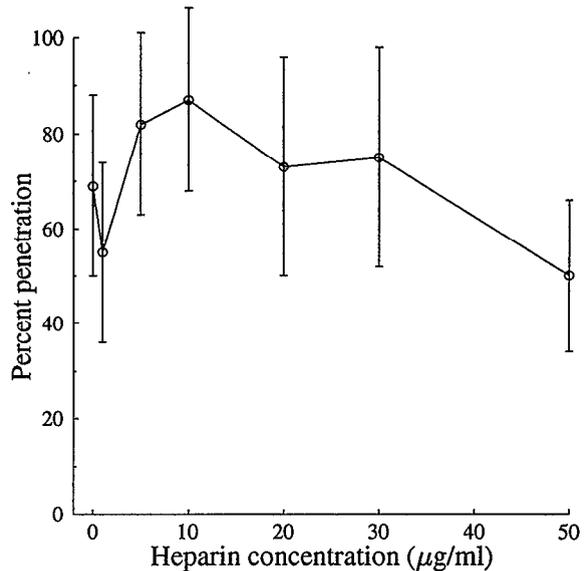
Trial 2

447 oocytes were inseminated and the results are shown in Figure 2. Highly significant differences (P<0.001) existed between caprogen levels, with dilutions of less than 1:100 giving very low penetration rates. No bull or semen age differences were found.

data pooled over replicates, bulls, semen ages

FIG 2 Penetration rates at various caprogen dilutions.

data pooled over replicates and semen ages

FIG 3 Penetration rates at various heparin concentrations.

Trial 3
671 oocytes were assessed and the results are shown in Figure 3. No significant differences were found between the treatments.

DISCUSSION

Trial 1 showed differences in penetration rates between 1- and 2-day-old sperm, with the latter giving less penetration at sperm concentrations under 0.5×10^6/ml. This suggests that 2-day-old sperm has a reduced viability which is masked at higher sperm concentrations. The reduced viability could be attributed to several factors. One possibility concerns the nitrogen saturation of the caprogen. Before sealing, the flask containing the caprogen is saturated with nitrogen to exclude oxygen, thereby reducing the activity and metabolic output of spermatozoa. In our work the seal was broken on day 1 and it is possible that the semen to be used on day 2 was not sufficiently resaturated with nitrogen.

Trial 1 clearly demonstrates that unfrozen sperm gives greater penetration than frozen-thawed sperm. This is probably due to the reduced viability and fertility of sperm that have been subjected to freezing, a process which can cause membrane damage.

There were no significant differences between the two bulls in these trials. Several groups (Leibfried-Rutledge *et al.*, 1989; Parrish *et al.*, 1986; Shi *et al.*, 1990) have found that individual bulls vary widely in the ability of their sperm to penetrate oocytes *in vitro*. Others (Miller and Hunter, 1987) contend that there is more variation between replicates (ejaculates) than there is between bulls, and these trials support this idea. Both bulls were NZDB sire-tested and were selected for this experiment on the basis of their similar high semen quality. This would have reduced the variability between them.

Trial 2 showed that penetration was reduced by the presence of more than 10% caprogen in the fertilisation medium. One of the main purposes of the caprogen is to promote sperm longevity. It is therefore nutrient-rich, containing caproic acid and egg yolk. The results of this trial indicate that some ingredient in caprogen prevents fertilisation. This inhibition could be a direct effect on sperm, either by acting as a decapacitating agent or by stabilising sperm membranes and thus inhibiting capacitation. Alternatively the

inhibition could be oocyte-directed, preventing the sperm from binding to the zona surface. Another possibility is that high concentrations of caprogen may be toxic to oocytes. Elucidation of these possibilities needs further experimentation.

Penetration rates for the various concentrations of heparin in trial 3 were uniformly high and not statistically different. This implies that heparin was not a necessary component of the fertilisation medium, a finding that contrasts with the work of Parrish *et al.* (1985, 1986). Our medium also included caffeine, a substance that both enhances and prolongs sperm motility (Garbers *et al.*, 1971, Niwa and Ohgoda, 1988). Sperm motility has not been conclusively linked with capacitation (Sidhu and Guraya; 1989). Caffeine can act synergistically with heparin on frozen-thawed semen. Niwa and Ohgoda (1988) obtained 48% penetration with heparin and caffeine, 0% with caffeine and no heparin, and 35% with heparin and no caffeine. We obtained good penetration (69%) in the presence of caffeine without heparin. Preliminary data from another trial using unfrozen and frozen-thawed semen from 5 bulls support this finding. It appears from these results that caffeine can enable sperm to capacitate in the absence of heparin. This further underlines the fact that capacitation is a complex, multi-step procedure.

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