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Replacement of serum with ocular fluid for cryopreservation of immature testes

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ABSTRACT

Cryopreservation of immature testis is a feasible approach for germplasm preservation of male animals. Combinations of dimethyl sulfoxide (DMSO) and foetal bovine serum (FBS) are used for testis cryopreservation. However, an alternative to FBS is needed, because FBS is expensive. Buffalo ocular fluid (BuOF), a slaughter house by-product, could be an economical option. The objective of the present study was to assess whether BuOF can replace FBS for cryopreservation of immature mouse (*Mus musculus*), rat (*Rattus norvegicus*), and buffalo (*Bubalus bubalis*) testes. Results showed that rodent and buffalo testes frozen in DMSO (10% for rodents and 20% for buffalo) with 20% FBS or BuOF had similar numbers of viable and DNA-damaged cells ($P > 0.05$). The expression of cell proliferation- (PCNA) and apoptosis-specific proteins (Annexin V and BAX/BCL2 ratio) were also comparable in mouse and buffalo testes frozen in DMSO with FBS or BuOF ($P > 0.05$). Interestingly, rat testis frozen in DMSO with BuOF had lower expression of Annexin V protein than testis frozen in DMSO with FBS ($P < 0.05$). The percentage of meiotic germ cells (pachytene-stage spermatocytes) in xenografts from testis frozen either in DMSO with BuOF or FBS did not significantly differ in rats or buffalo ($P > 0.05$). These findings provide evidence that BuOF has potential to replace FBS for cryopreservation of immature rodent and buffalo testis. Further investigation is needed to explore whether BuOF can replace FBS for testis cryopreservation of other species.

1. Introduction

Cryopreservation of testis is a promising technique for preserving the germplasm of male animals that die before attaining sexual maturity; it is especially important for preservation of germplasm of valuable, rare, and endangered animals such as black-footed ferrets, black bucks, lions, leopards, and Sumatran serows, which have high neonatal/juvenile mortality [31]. Testicular tissue has a complex tissue structure which includes cells of varying size and membrane permeability. To date, testes from laboratory and domestic animals, including pigs [1,14,47,49], goats [14], sheep [32], cattle [43,44], buffalo [7], chickens [39], Japanese quail [22], rabbits [38], rats [41], and mice [6,11,23], have been cryopreserved. Cryopreserved immature testis from monkeys [16,28], humans [29,45,46], and cats [25] survived and established spermatogenesis with differentiation of spermatogonia into spermatocytes when xenografted onto mice. The xenografted tissue from pigs [14,17], goats [14], mice [10,14,38], rabbits [38], and sheep [32] showed completion of spermatogenesis with production of haploid spermatids. Spermatozoa retrieved from cryopreserved xenografted testis have led to successful production of live offspring in mice, rabbits [38], and pigs [17] after intracytoplasmic sperm injection. However, there have been no reports on xenografting of cryopreserved buffalo and rat testes.

In an earlier report, it was demonstrated that cryomedium (CM) containing 5–10% dimethyl sulphoxide (DMSO) with 20% foetal bovine serum (FBS) can efficiently preserve rat testis using the uncontrolled slow freezing (USF) protocol [41]. In a recent report, we showed that immature buffalo testicular tissue can be optimally cryopreserved in CM containing combination of 20% DMSO and 20%

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FBS using the USF protocol [7]. DMSO has also been reported as an optimal cryoprotectant for USF of testicular tissue from monkeys [16], humans [4], mice [10], and sheep [32]. FBS contains a rich variety of proteins and growth factors, and is the most widely used supplement for cell culture [12]. Because FBS supports cell survival, it is a commonly used non-permeable cryoprotective agent that is combined with DMSO for testicular tissue cryopreservation. However, FBS is expensive and not produced in developing countries. Thus, there is a need for identification of an alternative cryopreservative that is as efficient as FBS yet economical for testis cryo-banking.

In several previous studies, FBS has been replaced with human serum albumin (HSA) for cryopreservation of testis [18,20,36,45,46]. However, the extremely high cost of HSA limits its use, especially in developing nations. Recently, we demonstrated that CMs containing buffalo ocular fluid (BuOF) efficiently cryopreserved adherent and primary suspension cells [42]. Collection of BuOF is feasible in India, because buffalo eyes are readily available as slaughter house by-products. The cost of BuOF is approximately 10- to 15-fold lower than that of FBS in India. In addition, BuOF remains sterile post-slaughter, because eyes are enclosed organs, and, on average, 15 ml filter-sterilized BuOF can be obtained from an adult buffalo. Moreover, biochemical and proteomic analyses of BuOF revealed the presence of several components that could be cryoprotective [42].

The objective of the present study was to assess if BuOF can replace FBS for cryopreservation of testes of immature buffalo, mice, and rats. The cryopreserved testes were evaluated both *in vitro* and *in vivo* to assess the efficiency of CMs that contained FBS or BuOF.

2. Material and methods

2.1. Animal research

The Institute Animal Ethics Committee (IAEC) of the Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India approved all animal procedures.

2.2. Buffalo ocular fluid collection

Intact eyeballs from healthy Murrah male calves (n = 12; age, 6–8 months) were collected from the municipal slaughterhouse of Hyderabad, India. Only healthy calves with good nutritional status based on physical evaluation were chosen for organ collection. The collected eyeballs were transported on ice in phosphate buffer saline (PBS; Invitrogen). The eyeballs were trimmed and washed several times with PBS before processing. Ocular fluid was collected from buffalo eyes as previously described [42]. Collected ocular fluid was pooled from animals in a given trial (n = 3), centrifuged at 775 × *g* (15 min at 4 °C), and then filtered through 0.45-μm and 0.22-μm filters. The sterile BuOF was aliquoted into cryovials (Nunc; Thermo Scientific) and stored at −30 °C until use.

2.3. Testis collection

Immature mouse (*Mus musculus;* Balb/c; n = 32) and rat (*Rattus norvegicus*; Wistar; n = 74) pups that were 5–6-days-old were obtained from breeding colonies of the CCMB Animal House. The animals were euthanised by CO_2 inhalation. After collection, the epididymis was removed and testes were weighed. The average weight of a mouse testis was 4.2 ± 1.2 mg and that of a rat testis was 6.1 ± 2.2 mg. Testes from sexually immature Murrah male buffalo calves (*Bubalus bubalis;* n = 14; age, 2 months; average testis weight, 4300.0 ± 400.0 mg) were collected from the municipal slaughterhouse. Within 1 h of slaughter, the collected testes were transported on ice to the laboratory in PBS, and the tunica albuginea was removed. The testis was cut into fine pieces of approximately 5–7 mg thickness and pooled for each trial. Fresh rodent testes and buffalo testicular tissues were cultured for 4 h before analysis to initiate cell proliferation and gene expression, as previously described [7]. Briefly, four testis/testicular tissue pieces were cultured as explants in wells of 6-well tissue culture dishes (TPP) in DMEM/F12 supplemented with 10 μg/ml insulin, 10 μg/ml apo-transferrin, 2 mM glutamine, 100 IU/ml penicillin–streptomycin +40 μg/ml gentamycin, single strength non-essential amino acid solution (Invitrogen), 1 mM pyruvate, and 10% FBS (Invitrogen) for 4 h in a humidified chamber with 5% CO_2 at 34 °C (designated as the fresh cultured control, FCC, from here onwards). An outline of the study design is provided in Fig. 1.

2.4. Cryopreservation and thawing of testis

Cryopreservation of testis was done as previously described using the USF protocol [7]. The basal CM contained DMEM/F12-hepes along with DMSO Hybri-Max™ (Sigma) and FBS (Invitrogen) or BuOF. As reported in previous studies, the optimal CM, 10% DMSO, was evaluated for cryopreservation of mouse and rat testis in our study [10,24,36]. The CM was supplemented with 20% FBS (D10S20) or 20% BuOF (D10O20) to evaluate FBS and BuOF efficiency in testis cryopreservation. For buffalo testis, CM that contained 20% DMSO with 20% FBS (D20S20) was evaluated, because this was the optimal CM for buffalo testis in our previous study [7]. FBS was replaced with similar concentrations of BuOF, such as 20% DMSO with 20% BuOF (D20O20), to evaluate BuOF and FBS efficiency in testis cryopreservation. For controls, 10% DMSO (D10) and 20% DMSO (D20) without FBS or BuOF were also evaluated to determine cryopreservation efficiency for rodent and buffalo testis, respectively. The testes were cryopreserved and thawed after 2 months, as previously described [7]. Briefly, four rodent testes or buffalo testicular tissues were equilibrated in 1.8-ml CM in a 2-ml cryovial (Nunc; Thermo Scientific) in an ice cold isopropyl alcohol container (Mr Frosty Freezing Container; Thermo Scientific) for 30 min. After equilibration, the container was transferred to a −80 °C freezer (Thermo Scientific). An uncontrolled cooling rate of approximately 1 °C/min was achieved, as mentioned in the product catalogue. The cryovials from the −80 °C freezer were then plunged into liquid nitrogen after 24 h for long-term storage. Cryopreserved testes were thawed by swirling the cryovials in a 34 °C water bath until the contents completely melted. The thawed testes/testicular tissues were transferred to a 15-ml centrifuge tube (TPP) and washed by adding five volumes of DMEM/F12-hepes supplemented with 10% FBS + 100 IU/ml penicillin–streptomycin +40 μg/ml gentamycin with gentle mixing. After three washes, the cryopreserved–thawed testes/testicular tissues were cultured as explants, similar to fresh testes/testicular tissues, under conditions described above (designated as cryopreserved–thawed culture, CTC, from here onwards).

2.5. Evaluating effects of cryopreservation on testis

FCC and CTC testes were analysed and xenografted to evaluate the effect of cryopreservation. Mouse tissues were excluded from the transplantation experiment, because only testicular tissue xenografting was evaluated in the present study.

2.5.1. Cell isolation and cell viability estimation

Two-step enzymatic dissociation was performed to isolate cells from FCC and CTC testes/testicular tissues to estimate cell viability as previously described [9]. Cell viability was estimated by trypan