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Germline Integration Assessment in Preclinical Gene Therapy Studies

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INTRODUCTION

In the last few years, many gene therapy products have advanced in development from preclinical to clinical, with increased chances of approval for curing genetic disorders[1]. It is critical to determine that the gene product will not be transmitted to the offspring of treated patients[2]. Thus, new approaches have been developed to investigate the potential for germline integration. Essentially, this is to prove a negative in terms of DNA integration and a risk for germline modification. In preclinical studies using sexually mature nonhuman primates, genomic analysis is performed on oocytes (females) and semen (males). In male animals, semen is collected at several time points during the study. In females, ovaries are harvested at necropsy for oocyte isolation. The studies typically are 6-12 months of observation after the gene product has been administered to the animals. To facilitate qPCR analysis in female animals, the ovaries collected at necropsy are subjected to manual disruption with subsequent mechanical denudation to remove extraneous cellular/tissue material for 'cleaning' of the oocytes. Under microscopy, an average of 60 follicular oocytes per ovary were isolated. Between 50 and 100 oocytes were established to be adequate to extract DNA for genomic analysis. In male animals, the collected semen samples were subjected to wash steps using various combinations of buffers. Results show that DNA isolation from semen samples yielded 120-2808 ng. In conclusion, the *in vivo* methods and DNA isolation techniques will facilitate analysis to evaluate the potential for germline integration.

ANIMALS AND ANIMAL CARE

Test system: Macaca fascicularis (cynomolgus monkey), male and female; Canis familiaris (beagle), female; and Sus domesticus (pig), female.

Approval for research: All animal-related procedures were approved by the IACUC.

Environmental conditions: Primary enclosure complied with the Animal Welfare Act and recommendations outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council 2011).

MATERIALS AND METHODS

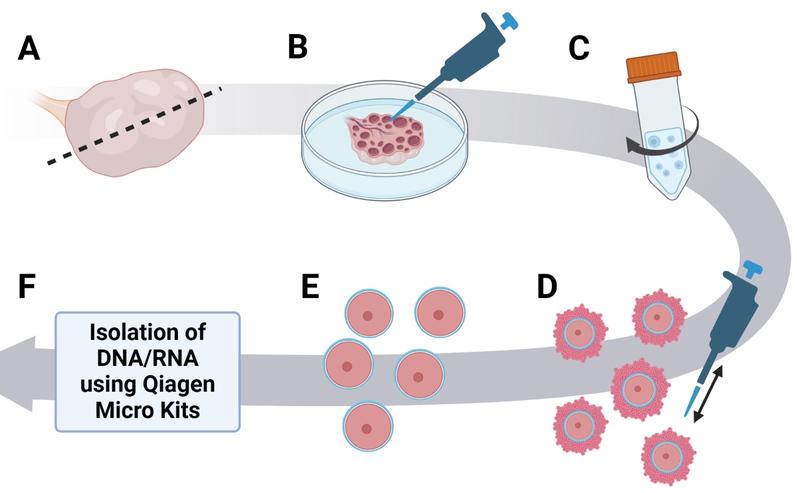

Figure 1. Oocyte collection and processing. A) Previously frozen ovaries are thawed at room temperature and cut longitudinally. B) PBS is added to wash the stroma and release oocytes. C) Oocytes are collected, spun, and washed with PBS. D) Oocytes are mechanically denuded of surrounding cumulus cells by pipetting up and down. E) Denuded oocytes are frozen at -80ºC in residual PBS or lysis buffer. F) DNA is isolated using the QIAmp DNA Micro Kit. RNA is isolated using the RNeasy Micro Kit.

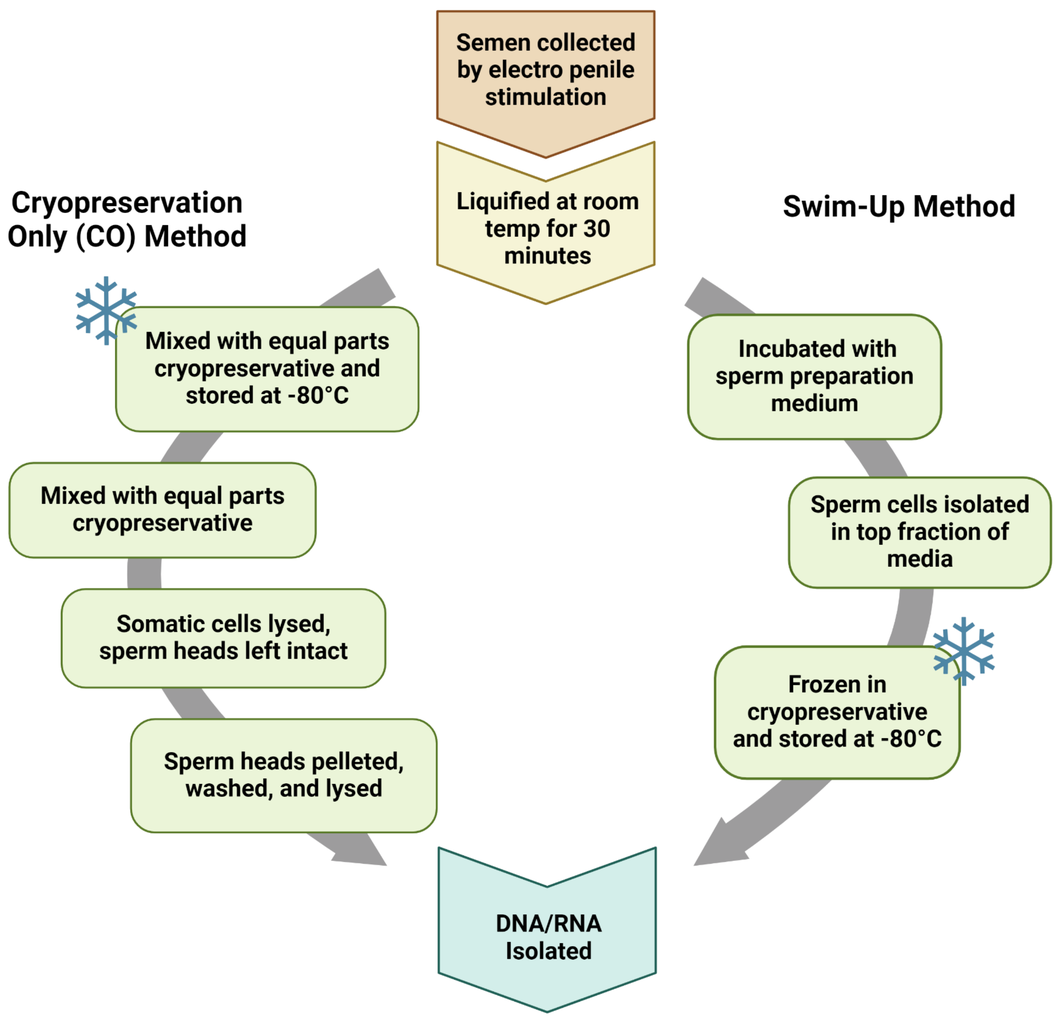

Figure 2. Sperm purification and processing. Sperm is isolated via 2 methods: the cryopreservation-only (CO) method and the swim-up method. In the CO method, the whole semen sample is mixed with equal parts of cryopreservation buffer and frozen in liquid nitrogen for downstream processing and analysis. A series of washes and lysis buffers rid the sample of contaminants, leaving intact sperm heads for analysis. In the swim-up method, semen is incubated with a sperm preparation medium, which draws motile sperm to the top layer. The motile sperm fraction is then mixed with cryopreservation buffer and frozen in liquid nitrogen for downstream processing and analysis.

RESULTS

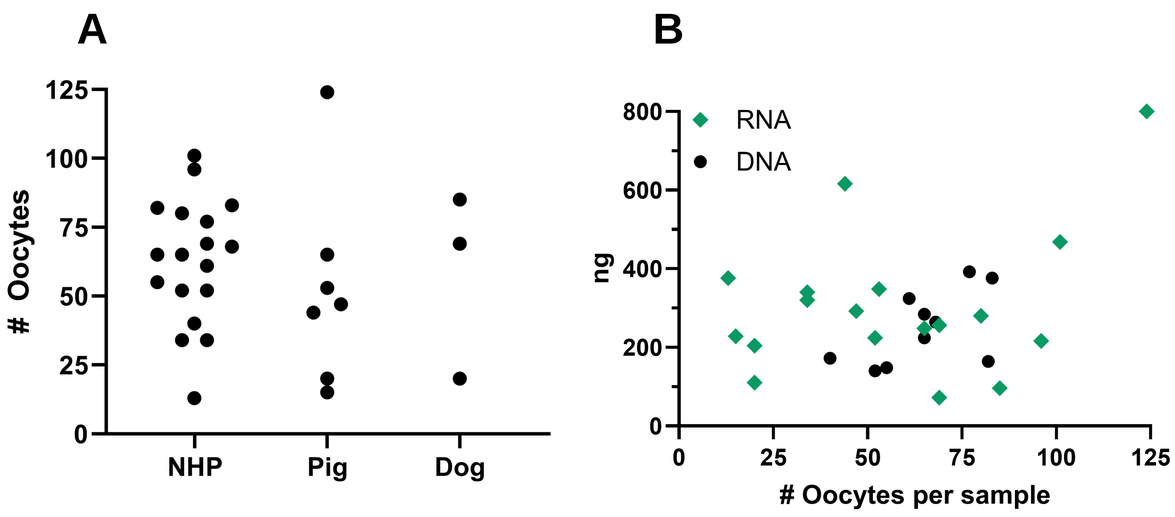

Figure 3. RNA and DNA isolation from oocytes. A) Oocytes were collected from flash-frozen NHP, pig and dog ovaries stored at -80˚ C for up to 150 days. A total of 28 oocyte samples was obtained from 11 NHPs, 6 pigs, and 2 dogs, averaging 60 oocytes per ovary. Each time point represents oocytes collected from a single ovary. **B)** RNA is isolated from all oocyte samples as few as 13 oocytes, while DNA was isolated from as few as 40 oocytes. Isolated RNA and DNA concentrations were determined on a NanoDrop One Spectrophotometer. Total yield ranged from 72-800ng (RNA) and 140-392ng (DNA). Similar yields of RNA and DNA are observed for samples less than 100 oocytes, and no differences were observed between animal sources. suggesting low nucleic acid concentrations at the limit of detection, however still present.

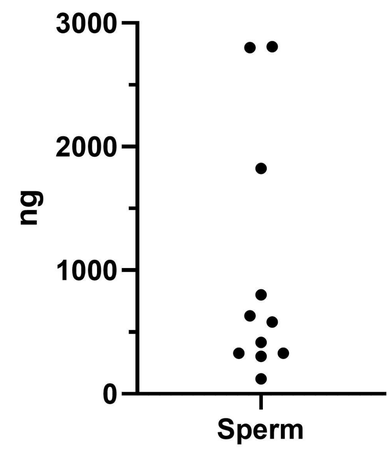

Figure 4. DNA yield from isolated sperm. Sperm was collected from 7 male NHPs over several days, resulting in 11 individual cryopreserved samples. All samples were isolated using the CO method described in Figure 2. DNA yield from isolated sperm ranged from 120-2808ng, as determined with the NanoDrop One Spectrophotometer.

CONCLUSION

The *in vivo* methods and DNA isolation techniques represented will facilitate analysis to evaluate the potential for germline integration. Data from the different preservation methods proposed will inform optimum collection standards that will result in quality data.

References

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3. Figures 1, 2 created with Biorender.com.