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Gene Therapy Studies and Germline Integration Assessment in Nonhuman Primates

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ABSTRACT

Background and purpose: 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. It is critical to determine that the gene product will not be transmitted to the offspring of treated patients. Thus, new approaches have been developed to investigate the potential for germline integration. The objective is to prove a negative regarding DNA integration and a risk for germline modification.

Methods: Preclinical studies investigating germline integration are conducted on sexually mature nonhuman primates (NHPs), with a 6-12-month observation period following gene product administration. Genomic analysis is performed in male animals on semen collected at several time points during the study (**Figure 2**). In this study, the purification of sperm cells from semen was compared between 1) whole cryopreserved semen samples subjected to differential lysis treatments (whole cryopreservation method) and 2) fresh semen incubated with a 'swim-up' buffer from which a sperm fraction is cryopreserved for downstream analysis (swim-up method). In female animals, ovaries collected at necropsy are subjected to manual disruption with subsequent mechanical denudation under microscopy to clear oocytes of extraneous cellular/tissue material. DNA and RNA are isolated from the oocytes using Qiagen column isolations (**Figure 1**).

Results: Semen yielded an average of 10^8 purified sperm cells, yielding 120-4404 ng of DNA for analysis. Additionally, of the methods evaluated, the whole cryopreservation method resulted in higher sperm cell input and subsequent DNA yields than the swim-up method. Far fewer gametic cells were obtained from females, averaging 72 follicular oocytes per ovary. Thus, lower yields ranging from 140-892 ng of DNA and 72-800 ng of RNA were obtained.

Conclusions: The purification of gametic cells and DNA isolation techniques reported herein and in ongoing studies will facilitate analysis and provide gold standards for the industry to evaluate the potential for the evaluation of germline integration.

MATERIAL AND METHODS

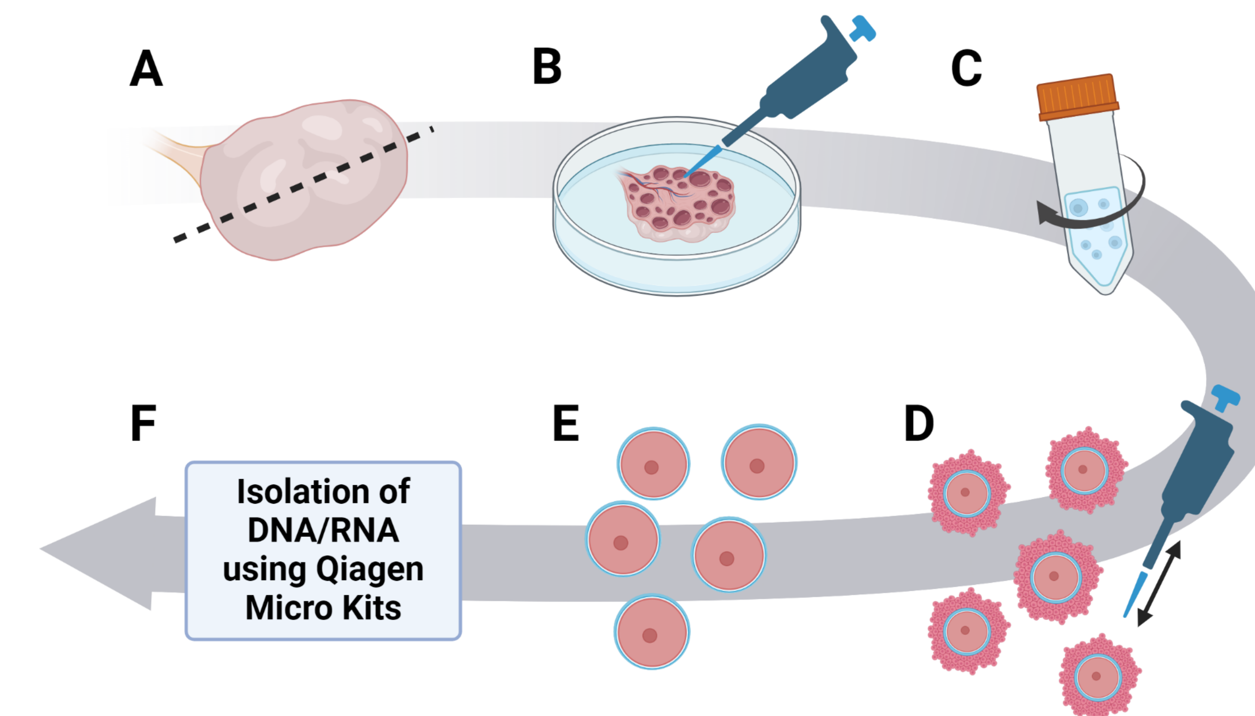

Figure 1. Oocyte Collection and Processing. A) Previously frozen ovaries are thawed at room temperature and cut longitudinally. B) PBS is added to wash the stroma and release oocytes. C) Oocytes are collected, spun, and washed with PBS. D) Oocytes are mechanically denuded of surrounding cumulus cells 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.

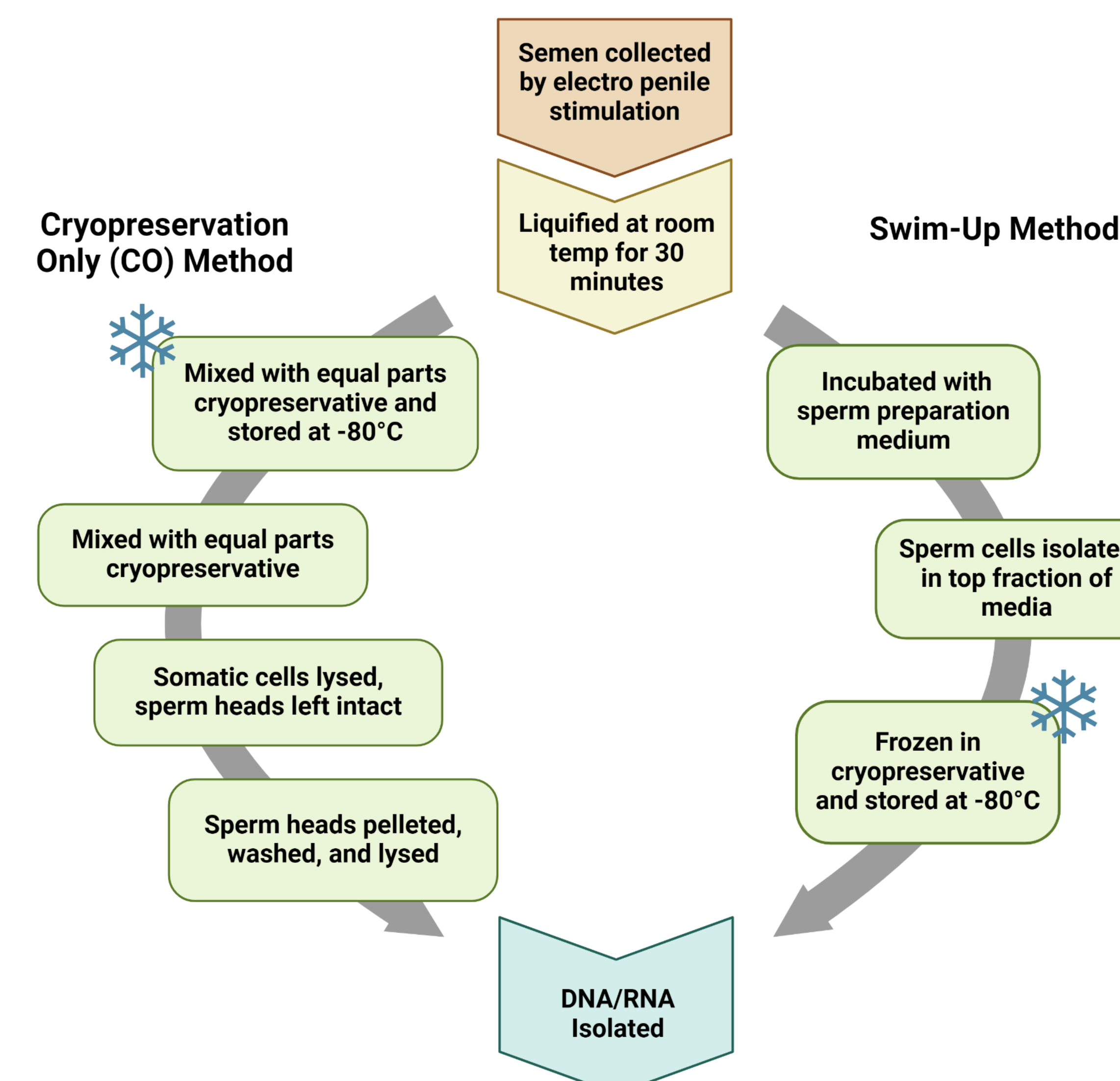

Figure 2. Sperm Purification and Processing. Sperm is isolated via two methods: the cryopreservation-only (CO) method and the swim-up method. In the CO method, the whole semen sample is mixed with equal parts of cryopreservation buffer and frozen in liquid nitrogen for downstream processing and analysis. A series of washes and lysis buffers rid the sample. 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 AND DISCUSSION

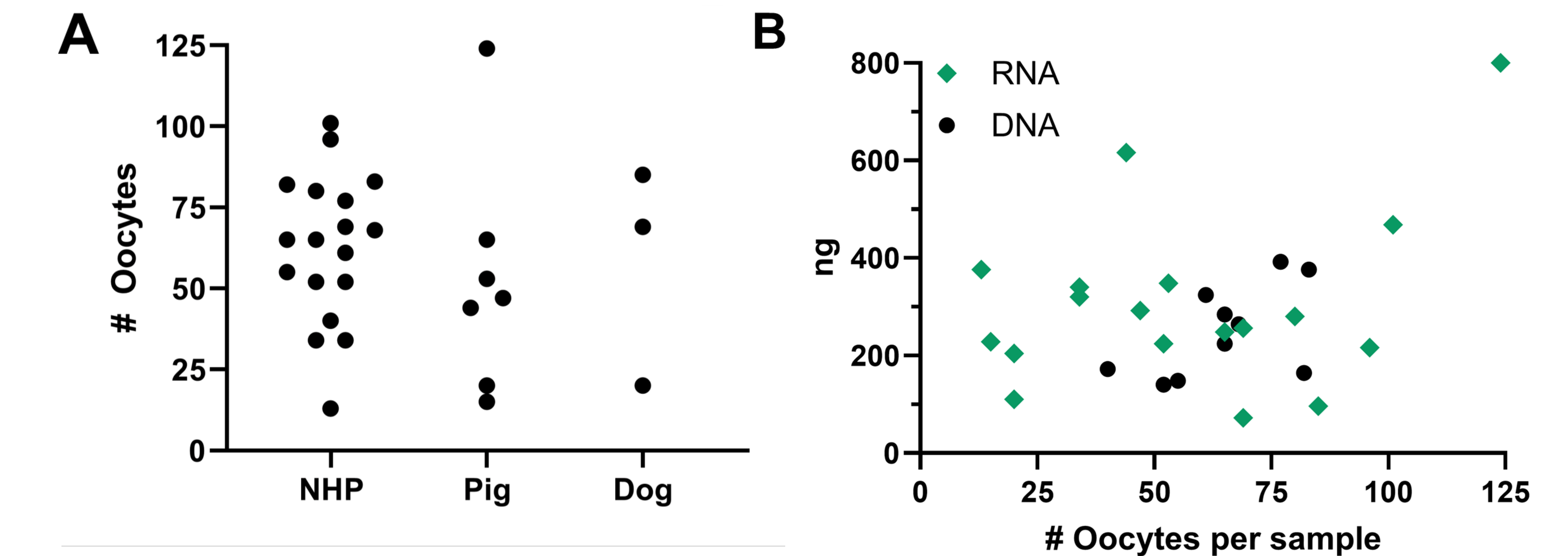

Figure 3. RNA and DNA Isolation From Oocytes. A) Oocytes were collected from flash-frozen NHP, pig, and dog ovaries stored at -80°C for up to 150 days. A total of 28 oocyte samples was obtained from 11 NHPs, six pigs, and two dogs, averaging 60 oocytes per ovary. Each time point represents oocytes collected 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 were observed for samples of less than 100 oocytes, and no differences were observed between animal sources. suggesting low nucleic acid concentrations at the limit of detection; however, they are still present.

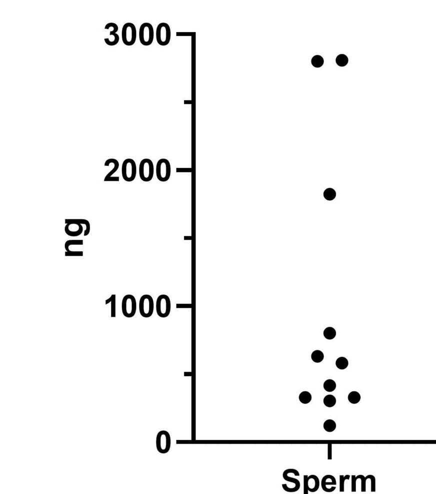

Figure 4. DNA Yield From Isolated Sperm. Sperm was collected from 7 male NHPs over several days, resulting in 11 individual cryopreserved samples. All samples were isolated using the CO method described in **Figure 2**. DNA yield from isolated sperm ranged from 120-2808ng, as determined with the NanoDrop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

DISCUSSION AND 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.

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

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