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Gene Therapy Studies and Germline Integration Assessment in Nonhuman Primates

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ABSTRACT

Background and purpose: Gene therapies targeting rare genetic conditions and disorders are on the rise. While somatic or tumor cell targets are often the aim of these therapies, risk of germline integration of the vector target sequence in gametic cells is of recent safety and regulatory awareness. With the intent to confirm zero target germline integration, *in vivo* sample collection must be performed with precision, and optimal nucleic acid isolations are required.

Methods: Preclinical gene therapy studies are conducted on sexually mature laboratory animals, including nonhuman primates (NHPs), canines, and minipigs. A 6–12-month observation period follows gene therapy administration. Semen is collected at various study timepoints for downstream nucleic acid isolations. Two sperm cell separation techniques were compared for feasibility and preservation of nucleic acids present: (1) whole cryopreservation method, where whole semen samples were collected, flash frozen, and then thawed prior to washes and nucleic acid isolation; and the (2) swim-up method, where fresh semen was incubated with a 'swim-up' buffer where the sperm cells are separated from the extraneous cells contained in semen; the sperm fraction is then flash frozen and preserved for nucleic acid isolations. For females, ovaries are collected at necropsy, and the tissue is manually disrupted under a microscope to isolate individual oocytes; extraneous cellular/tissue debris is removed, and 'clean' oocytes are collected and flash frozen for downstream isolation procedures. DNA and RNA are isolated from both frozen sperm and oocytes.

Results: Semen yielded an average of 10^8 purified sperm cells per collection, which yielded between 120 and 4404 ng of DNA. Of the two methods evaluated, the whole cryopreservation method resulted in higher sperm cell isolation and subsequent DNA yields as compared to the swim-up method. Notably fewer gametic cells were obtained from female ovaries, averaging 72 oocytes per ovary. Accordingly, lower DNA yields of 140-892 ng and RNA yields of 72-800 ng were obtained.

Conclusions: Successful and meticulous collection of gametes from *in vivo* test models will be applied to ongoing and future gene therapy studies to assess potential vector target germline integration. These collections and isolation procedures pave the way for and impact preclinical industry standards for sufficient assessments of gene therapies with regard to the risk of potential germline integration.

MATERIAL AND METHODS

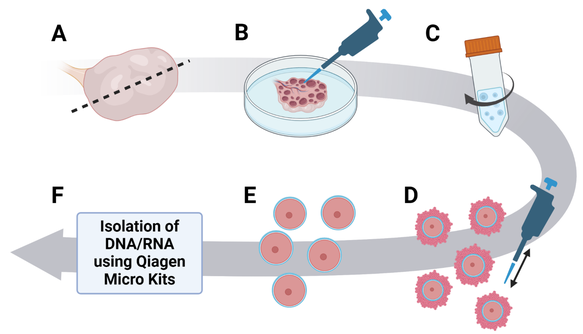

Figure 1. Oocyte Collection and Processing. A) Previously frozen ovaries are thawed at room temperature and cut longitudinally. B) PBS is added to wash the stroma and release oocytes. C) Oocytes are collected, spun, and washed with PBS. D) Oocytes are mechanically denuded of surrounding cumulus cells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

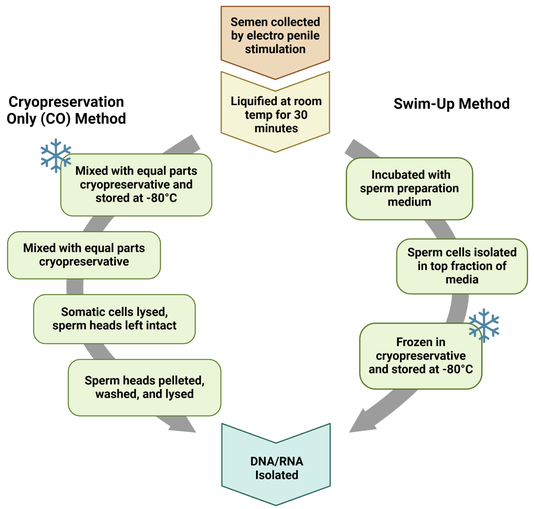

Figure 2. Sperm Purification and Processing. Sperm is isolated via two methods: the cryopreservation-only (CO) method and the swim-up method. In the CO method, the whole semen sample is mixed with equal parts of cryopreservation buffer and frozen in liquid nitrogen for downstream processing and analysis.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

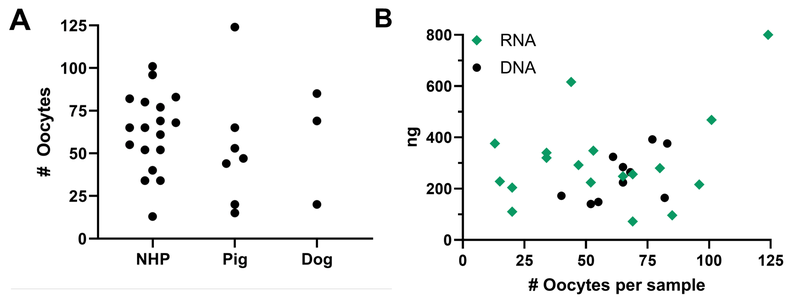

Figure 3. RNA and DNA Isolation From Oocytes. A) Oocytes were collected from flash-frozen NHP, pig, and dog ovaries stored at -80°C for up to 150 days. A total of 28 oocyte samples were obtained from 11 NHPs, six pigs, and two dogs, averaging 60 oocytes per ovary. Each time point represents oocytes collected 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.

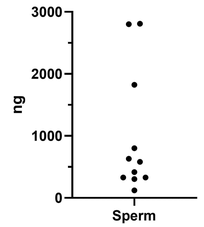

Figure 4. DNA Yield From Isolated Sperm. Sperm was collected from 7 male NHPs over several days, resulting in 11 individual cryopreserved samples. All samples were isolated using the CO method described in **Figure 2**. DNA yield from isolated sperm ranged from 120- 2808ng, as determined with the NanoDrop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

DISCUSSION AND CONCLUSION

In order to accurately assess if, and to what extent, germline integration of a gene therapy target or vector is present, superlative oocyte and sperm cell collections are essential. Successful collections and appropriate storage will facilitate potential target evaluation following biodistribution data acquisition. Data from oocyte collection techniques and sperm preservation methods evaluated will inform *in vivo* study decisions for use in downstream applications. Appropriate DNA and RNA isolation procedures, which ensure intact nucleic acids free of contaminants, are also paramount when considering downstream processes. Our successful isolations from the range of available gamete samples allow us to deliver quality gene target and vector analysis results.

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