Spring 2020 – Systems Biology of Reproduction
Discussion Outline – Assisted Reproduction/Contraception
Michael K. Skinner – Biol 475/575
CUE 418, 10:35-11:50 am, Tuesday & Thursday
April 30, 2020
Week 16

Assisted Reproduction/Contraception

Primary Papers:

1. Oritz-Rodriguez, et al. (2019) Plos One 0213420, 1-24.
2. Ayoub, et al. (2017) Andrology 5(2):278-285.
3. Khilwani, et al. (2020) Basic & Clinical Andrology 30:2, 1-12.

Discussion

Student 15: Reference 1 above

- What is the assisted reproductive technology (ART) investigated?
- What is the experimental design and technology used?
- What is the conclusion on the use of this ART technology?

Student 16: Reference 2 above

- What is the potential target for a male contraceptive?
- What reduction in hormones was observed and will this be a good contraceptive?
- What future studies are needed and anticipated limitations?

Student 17: Reference 3 above

- What current assisted reproductive technology does this replace?
- How does this new technology work?
- Is this a contraceptive and what clinical issues should be investigated?

PLOS ONE

RESEARCH ARTICLE

Transcriptome analysis reveals that fertilization with cryopreserved sperm downregulates genes relevant for early embryo development in the horse

José M. Ortiz-Rodriguez[1], Cristina Ortega-Ferrusola[1], María C. Gil[1], Francisco E. Martín-Cano[1], Gemma Gaitskell-Phillips[1], Heriberto Rodríguez-Martínez[2], Katrin Hinrichs[3], Alberto Álvarez-Barrientos[4], Ángel Román[5], Fernando J. Peña[1]*

1 Laboratory of Equine Reproduction and Equine Spermatology, Veterinary Teaching Hospital, University of Extremadura, Cáceres, Spain, **2** Department of Clinical and Experimental Medicine, Faculty of Medicine and Health Sciences, Linköping University, Linköping, Sweden, **3** Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, College Station, Texas, **4** STAB, University of Extremadura, Badajoz, Spain, **5** Department of Biochemistry and Molecular Biology, University of Extremadura, Badajoz, Spain

* fjuanpvega@unex.es

Citation: Ortiz-Rodriguez JM, Ortega-Ferrusola C, Gil MC, Martín-Cano FE, Gaitskell-Phillips G, Rodríguez-Martínez H, et al. (2019) Transcriptome analysis reveals that fertilization with cryopreserved sperm downregulates genes relevant for early embryo development in the horse. PLoS ONE 14 (6): e0213420. https://doi.org/10.1371/journal.pone.0213420

Editor: Christine Wrenzycki, Justus Liebig Universitat Giessen, GERMANY

Received: February 14, 2019

Accepted: June 11, 2019

Published: June 25, 2019

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: FJP received financial support for this study from the Ministerio de Economía y Competitividad-FEDER, Madrid, Spain, grant AGL2017-83149-R. FJP also received support from the Junta de Extremadura-FEDER (IB16030 and GR18008). HRM received support from the

Abstract

Artificial insemination with cryopreserved spermatozoa is a major assisted reproductive technology in many species. In horses, as in humans, insemination with cryopreserved sperm is associated with lower pregnancy rates than those for fresh sperm, however, direct effects of sperm cryopreservation on the development of resulting embryos are largely unexplored. The aim of this study was to investigate differences in gene expression between embryos resulting from fertilization with fresh or cryopreserved sperm. Embryos were obtained at 8, 10 or 12 days after ovulation from mares inseminated post-ovulation on successive cycles with either fresh sperm or frozen-thawed sperm from the same stallion, providing matched embryo pairs at each day. RNA was isolated from two matched pairs (4 embryos) for each day, and cDNA libraries were built and sequenced. Significant differences in transcripts per kilobase million (TPM) were determined using (i) genes for which the expression difference between treatments was higher than 99% of that in the random case ($P < 0.01$), and (ii) genes for which the fold change was ≥ 2, to avoid expression bias in selection of the candidate genes. Molecular pathways were explored using the DAVID webserver, followed by network analyses using STRING, with a threshold of 0.700 for positive interactions. The transcriptional profile of embryos obtained with frozen-thawed sperm differed significantly from that for embryos derived from fresh sperm on all days, showing significant down-regulation of genes involved in biological pathways related to oxidative phosphorylation, DNA binding, DNA replication, and immune response. Many genes with reduced expression were orthologs of genes known to be embryonic lethal in mice. This study, for the first time, provides evidence of altered transcription in embryos resulting from fertilization with cryopreserved spermatozoa in any species. As sperm cryopreservation is

Swedish Research councils VR, (Grant 521-2011-6553) and FORMAS (Grant 2017-00946), Stockholm. JMOR holds a Predoctoral grant from the Valhondo Calaaf Foundation, Cáceres, Spain. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

commonly used in many species, including human, the effect of this intervention on expression of developmentally important genes in resulting embryos warrants attention.

Introduction

Cryopreservation is a common procedure in assisted reproductive technology, in both humans and the animal breeding industry [1–3]. Cryopreserved sperm are routinely used for artificial insemination (AI), in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). However, it is clear that sperm cryopreservation methods are currently sub-optimal, as pregnancy rates with cryopreserved sperm are lower than those with fresh sperm in humans and horses [4–6], among other species. Cryopreservation leads to extensive damage of sperm cell membranes and causes metabolic and functional alteration of sperm [7, 8], particularly of their mitochondria [9–11]. Cryopreservation may alter sperm DNA [12]; recently, specific cryodamage to sperm genes and transcripts have been reported [13, 14], even in samples with good sperm motility post thaw and in the absence of detectable DNA fragmentation. The sperm DNA is epigenetically programmed to regulate embryonic gene expression, and changes to this epigenome cause developmental disregulation [15]. Cryopreservation has been found to significantly change the sperm DNA methylome, as well as to alter expression of epigenetic-related genes such as methyltransferases [12, 16]. Cryopreservation of sperm imposes oxidative stress and redox deregulation in spermatozoa, leading to the presence of toxic adduct-forming compounds such as 4- hydroxynonenal (4-HNE) in sperm membranes [17]. Moreover, mitochondria of spermatozoa surviving cryopreservation show increased production of reactive oxygen species [9, 10, 18]. Signaling pathways crucial to normal embryo development are sensitive to perturbations of endogenous redox state, and are also susceptible to modulation by reactive oxygen species [19]. Thus, fertilization by damaged spermatozoa may impact early embryo development and even have effects that appear later in the life of the offspring [20].

Moreover, appreciation of the contribution of sperm to embryo development has evolved from the concept that the only role of sperm at fertilization is to introduce the male genome into the egg. Sperm carry a myriad of small noncoding RNAs with potential roles in early embryo development [21, 22]. Notably, sperm carry the activating factor PLCζ, which triggers calcium oscillations that induce oocyte activation [23, 24], and it has been shown in mouse and rabbit that alterations in frequency and amplitude of post-fertilization calcium oscillations can affect the phenotype of the resulting embryo into post-implantation development and adulthood [25, 26]. Thus, there are extensive pathways by which cryopreservation of sperm could alter the development of the fertilized oocyte and embryo.

Despite the widespread use of cryopreserved sperm, and the known decrease in pregnancy rates with its use, little direct information is available on the effect of sperm cryopreservation on development of the resulting embryo. Recent advances in transcriptome amplification and next-generation sequencing provide the ability to obtain the full transcriptome of individual embryos [27], thus offering a basis for studies on differences in gene expression associated with fertilization with cryopreserved sperm. In the present study, we analyzed the transcriptome of equine embryos produced with fresh or frozen-thawed sperm, to determine the impact of sperm cryopreservation on gene expression during early equine embryo development.

Material and methods

Animals and experimental design

Animals belonging to and housed in our institution were maintained according to European laws and regulations, and all experimental procedures were reviewed and approved by the Ethical committee of the University of Extremadura, Cáceres, Spain. Six mares were used for this study; they were inseminated with the same stallion of known fertility to reduce genetic variability [28]. Each mare was assigned a day of embryo recovery (8, 10 or 12 days post ovulation) and on successive cycles was assigned to be inseminated with fresh or frozen-thawed sperm from the same stallion, to provide a matched embryo pair for that day of embryo development. The mares were treated with a prostaglandin analogue to shorten the luteal phase and were monitored daily by transrectal ultrasonography. When a follicle of at least 35 mm diameter was detected in the absence of luteal tissue, with marked uterine edema and low cervical tone, mares received 2,500 IU of hCG i.v.. The follicle was monitored by transrectal ultrasonography every 6 h thereafter to detect the time of ovulation. Mares were inseminated immediately once ovulation was detected, with a minimum of 100 million either fresh sperm or frozen-thawed sperm, from the same stallion and ejaculate. For this, semen was collected, and half of the ejaculate was processed as fresh semen for the immediate insemination of the mare. The other half was frozen following the standard protocol in our center [17, 29, 30], and stored in LN for the next insemination of the same mare. Following this protocol, each mare was inseminated with the same ejaculate, first with the fresh extended aliquot and on a second cycle with the frozen-thawed aliquot.

Embryos were obtained by uterine lavage on the designated day after ovulation. For each embryo day, 2 embryos produced with fresh sperm, designated FRSH embryos, and 2 embryos produced with frozen-thawed sperm, designated CRYO embryos were obtained. Embryos were snap-frozen in liquid N_2 and stored at -80˚C until analysis. Previous clinical reports indicated that there is no a significant effect in the rate of embryonic vesicle growth between mares inseminated with fresh or frozen-thawed sperm if both are inseminated post-ovulation [31].

Isolation of RNA

Total RNA was isolated from the embryos using the PicoPure RNA Isolation Kit (Catalog number KIT0204, Thermofisher) following the manufacturer's instructions. RNA concentration and quality were assessed by automatic electrophoresis using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

RNA-seq analysis

cDNA libraries were built using an IonTorrent S5/XL sequencer (Thermo Fisher Scientific, Waltham, MA USA). The raw reads were aligned to a horse transcriptome generated using ENSEMBL (Equ Cab 2 version) in the Torrent server with proprietary ThermoFisher algorithms. Then, custom scripts were used to transform reads into transcript counts, and transcripts per kilobase million (TPM) scores for each gene were retrieved. A gene was considered expressed if the reads per kilobase or transcript model per million mapped reads was > 0.4. In order to evaluate gene expression differences between treatments (FRSH or CRYO embryos), we calculated two thresholds: first, we calculated the random TPM differences between FRSH and CRYO embryos by permutation of the TPM gene scores. Then we chose the genes whose expression difference between the two conditions was higher than in 95% ($P<0.05$) or in 99% ($P<0.01$) of the random cases. As a second score, we used a fold change ≥ 2 as a threshold in order to avoid expression biases in the selection of the candidate genes.

Gene ontology and pathway analysis

The annotations of the candidate genes selected after the RNA-seq analyses were explored to detect significant differences in molecular pathways between treatments. Specifically, the DAVID webserver [32] was used to retrieve the terms (gene ontology, up-expressed tissues, KEGG and reactome pathways, protein-protein interactions, etc.) with significant over-presence of the candidate genes, using a false discovery rate (FDR) < 0.05. We used the human genome as reference for the analysis because of its increased depth in terms of annotation.

Network analysis

STRING [33] was used to analyze the internal structure of the functional network obtained using the candidate genes. Data included co-expression, genetic fusion, co-occurrence or protein-protein interactions, among others. A high threshold (0.700) was selected for positive interaction between a pair of genes.

Results

A total of 12 conceptuses were analysed (2 FRSH and 2 CRYO at each day). An average of 29,196 transcripts per embryo were obtained.

Day-8 embryos

In Day-8 CRYO embryos, 100 transcripts showed increased abundance and 157 transcripts showed decreased abundance in respect to FRSH embryos of the same age from the same stallion and mare (Fig 1).

Of the 100 transcripts showing increased abundance in CRYO embryos, 23 could be aligned to the genome build (S1 Table). These included the progesterone receptor membrane component (8PGRMC1). Enriched biological processes (Fig 2A) included extracellular region genes, defensing beta 119, insulin like 3, prostaglandin D2 synthase and uteroglobin; genes associated with negative regulation of cysteine type endopeptidase activity involved in apoptotic processes including nuclear receptor subfamily 4 group A member and paired box 2; and genes involved in skeletal muscle cell differentiation including activating transcription factor 3 and nuclear receptor subfamily 4 group 4 A member. STRING analysis revealed no significant enrichments in functional networks for transcripts with increased abundance.

Transcripts showing decreased abundance in CRYO embryos provided more information, with 129 transcripts annotated in the equine database. The complete list of transcripts is presented in S2 Table. Due to the large number of genes retrieved, the threshold was reset at $P< 0.001$ and 62 transcripts were then retrieved (Table 1). Related gene ontology terms are shown in Fig 2B. Enriched terms in KEGG (Kyoto encyclopedia of gene and genomes) pathways included ribosome, Parkinson disease and oxidative phosphorylation (Fig 3). STRING analysis, performed using a threshold of 0.700, obtained a protein-protein interaction (PPI) enrichment P value of $< 1.0 \times 10^{-16}$ (Fig 4). The complete list of genes in this network with their clustering is presented in S3 Table. Enriched biological processes included cellular process, iron ion transport, cellular iron ion homeostasis, metabolic process, response to inorganic substance, biological regulation, single-organism process, cellular macromolecule metabolic process, single organism cellular process, cellular metabolic process, response to stimulus, cellular response to zinc ion, transport, regulation of biological process, oxidation-reduction process, cellular component disassembly, cellular nitrogen compound metabolic process, translation, single organism transport, gene expression, positive regulation of nitrogen compound

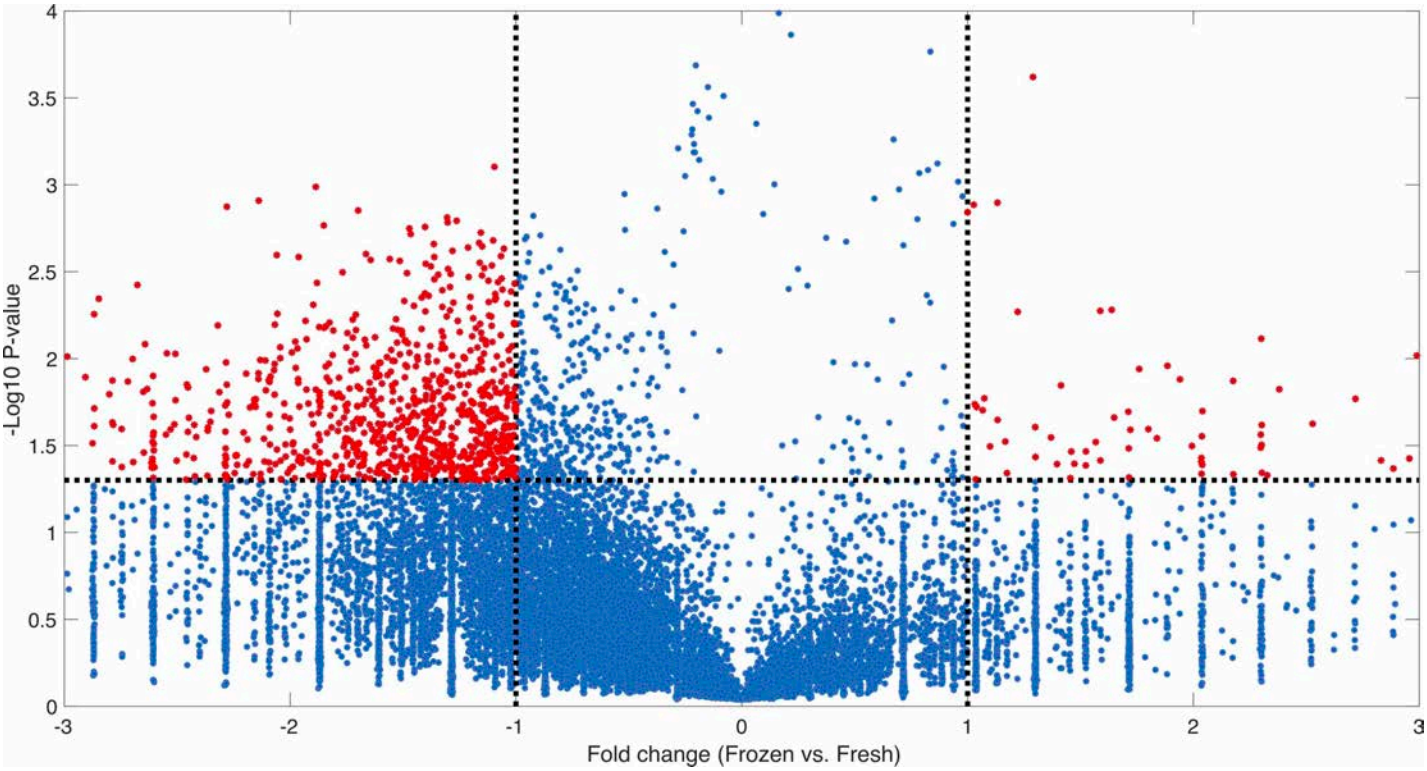


Fig 1. Volcano plot representing the RNA-seq results for Day-8 equine embryos conceived using fresh sperm (FRSH) or frozen-thawed sperm (CRYO). Each point represents a gene. On the X-axis, the fold change was calculated as the log2-ratio between the average gene expression in CRYO embryos and the average gene expression in FRSH embryos. Therefore, positive values indicate genes in which expression is higher in CRYO embryos, while negative values indicate genes whose expression is higher in FRSH embryos. On the Y-axis, the statistical significance for the difference in gene expression (see Methods) is represented as the (-log10) of the P-value. Dashed lines indicate the thresholds for significance on the two axes (-1 and +1 in the case of the X-axis for up-regulated and down-regulated genes, respectively, in CRYO embryos; and 1.30 (equal to a P-value = 0.05) in the case of the Y-axis). Red points mark differentially expressed genes.

https://doi.org/10.1371/journal.pone.0213420.g001

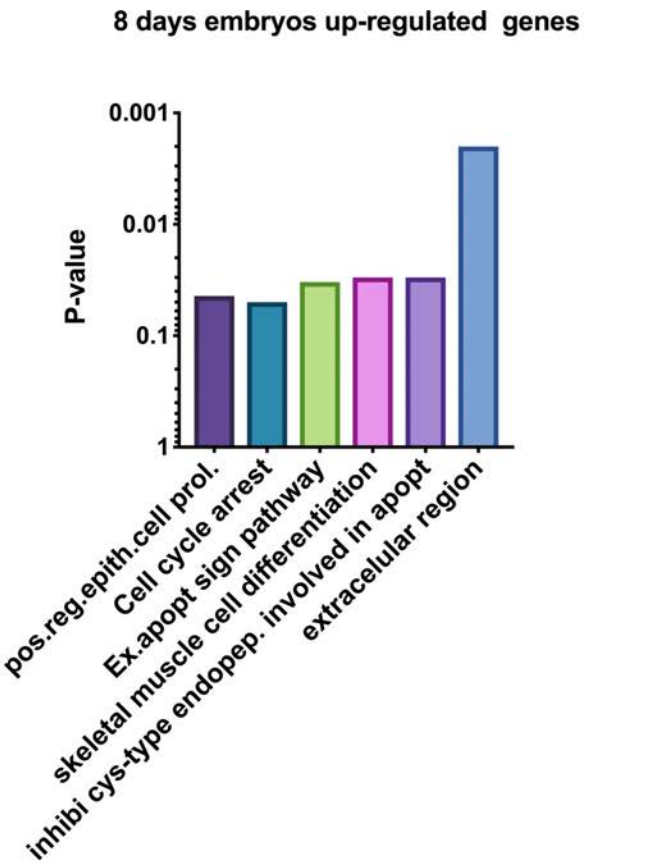
metabolic process, biological process, protein folding, cellular component organization, regulation of cell proliferation, and primary metabolic process.

Day-10 embryos

In Day-10 embryos 239 transcripts showed increased abundance ($P < 0.01$), and 206 showed decreased abundance, in CRYO embryos in comparison with FRSH embryos.

Of the 239 transcripts showing increased abundance in CRYO embryos, 53 aligned to the genome build (S4 Table). Functional annotation revealed these genes to be related to the GO terms and KEGG pathways nucleosome, systemic lupus erythematatosus, DNA replication-dependent nucleosome assembly, protein heterodemerization, alcoholism, nuclear chromosome, telomeric region, regulation of gene silencing, nucleosomal DNA binding, membrane, translation, poly (A) RNA binding, viral carcinogenesis, negative regulation of megakaryocyte differentiation, DNA replication independent nucleosome assembly, extracellular exosome, DNA-templated transcription, xenophagy, ribosome, positive regulation of defense to virus by host, DNA binding, mitochondrion, cytosolic large ribosomal subunit, extracellular space, transcriptional misregulation in cancer, innate immune response in mucosa, U1 snRNP, antibacterial humoral response, telomerase RNA binding and mitochondrial small ribosomal subunit (Fig 5A). STRING analysis revealed a PPI enrichment P value of $< 1.0 \times 10^{-16}$. Functional enrichment included the PFAM protein domain Core histone H2A/H2B/H3/HA and the

A


8 days embryos up-regulated genes
P-value
0.001
0.01
0.1
1
pos.reg.epith.cell prol.
Cell cycle arrest
Ex.apopt sign pathway
skeletal muscle cell differentiation
inhibi cys-type endopep. involved in apopt
extracelular region

B

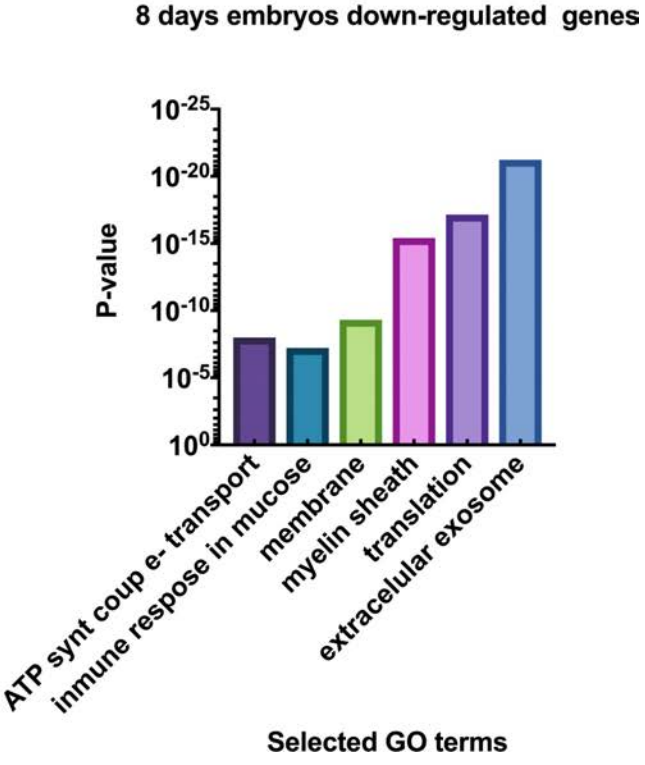

8 days embryos down-regulated genes
P-value
10-25
10-20
10-15
10-10
10-5
100
ATP synt coup e- transport
inmune respose in mucose
membrane
myelin sheath
translation
extracelular exosome
Selected GO terms

Fig 2. Selected enriched GO terms differentially regulated in Day-8 equine embryos obtained using fresh sperm (FRSH) and frozen-thawed sperm (CRYO), (A) transcripts down regulated in 8-Day CRYO embryos, (B) transcripts up regulated in 8-Day CRYO embryos.

https://doi.org/10.1371/journal.pone.0213420.g002

INTERPRO protein domains, including Histone fold, Histone H3/CNEP-A, Histone H2A/H2B/H3, Histone H4, Histone H4 conserved site, TATA box binding protein associated factor (TAF) and ribosomal protein L23/L15e core domain.

Of the 206 transcripts showing decreased abundance in CRYO embryos at Day 10, 115 were aligned. Enriched KEGG pathways that were also detected in Day-8 embryos (Table 2) included oxidative phosphorylation, Parkinson disease, Alzheimer disease, Hungtington disease, Metabolic pathways, Ribosome, cardiac muscle contraction, and non-alcoholic fatty liver disease. Three new KEGG enriched pathways, protein processing in endoplasmic reticulum, systemic lupus erythematosus and phagosome, were detected (Fig 6). More significantly represented GO terms were ATP synthesis coupled proton transport, translation, nucleosome assembly, cell redox homeostasis, extracellular exosome, myelin sheath, respiratory chain, mitochondrion, extracellular space, NADH dehydrogenase (ubiquinone) activity, structural constituent of ribosome, and proton transporting ATP synthase activity rotational mechanism (Fig 5B). A complete list of enriched GO terms retrieved are given in Table 3. STRING analysis revealed functional networks with a PPI enrichment P value of $< 1.0 \times 10^{-16}$ (Fig 7). Functional enrichment included the PAFM domains core histone H2A/H2B/H3/H4, thiorredoxin, NADH deshidrogenase, NADH-Ubiquinone and plastoquinone (Complex I), various chains.

Day-12 embryos

In Day-12 embryos, 149 transcripts showed increased abundance and 157 showed decreased abundance in CRYO embryos. Of the 149 transcripts with increased abundance, 61 were annotated (S5 Table). Enriched KEGG pathways included ribosome and Parkinson disease and the GOterms extracellular exosome, translation, structural constituent of ribosome, nuclear nucleosome, mitochondrial respiratory chain complex I, cytosolic large ribosomal subunit, nucleosome assembly, methylosome, and catalytic step 2 spliceosome (Fig 8A). On STRING analysis, the KEGG pathways Ribosome (Pathway ID 03010) and Parkinson disease (Pathway ID 05012) showed a PPI enrichment P value of $< 8 \times 10^{-10}$.

Of the 157 transcripts showing decreased abundance in Day-12 CRYO embryos, 60 transcripts aligned to the genome build (S6 Table). Enriched KEGG pathways, also detected in 8- and 10-day embryos, included oxidative phosphorylation, Parkinson disease, metabolic pathways, Alzheimer disease, Huntington disease, non-alcoholic fatty acid liver disease and cardiac muscle contraction. In addition a new pathway, folate biosynthesis, was enriched (Fig 9). GO terms enriched annotations (Fig 8B) were NADH dehydrogenase (ubiquinone) activity, mitochondrial respiratory chain complex I, nucleosome, DNA replication dependent nucleosome assembly, protein heterotetramerization, mitochondrion, respiratory chain, negative regulation of megakaryocyte differentiation, DNA template transcription initiation, ATP synthesis coupled electron transport, nuclear chromosome telomeric region, DNA binding, oxireductase activity, mitochondrial inner membrane, integral component of membrane, mitochondrial electron transport NADH to ubiquinone, and extracellular exosome. The complete list is given in Table 4

STRING analysis revealed functional networks with a PPI enrichment P value of $< 1.0 \times 10^{-16}$ (Fig 10). Functional enrichment included the PFAM protein domains core histone H2A/H2B/H3/H4, NADH dehydrogenase, and NADH-ubiquinone/plastoquinone (complex I various chains).

Table 1. Enriched biological processes from DEGs (downregulated) in 8 days embryos obtained after AI with frozen thawed sperm, as identified by DAVID functional annotation analysis.

Functional terms of overrepresented biological processes [a]	P value [b]
Chromosome (21, 35.78)	7.1×10^{-26}
Nucleosome core (19, 42.08)	9.2×10^{-25}
Extracellular exosome (62, 3.56)	5.7×10^{-22}
Histone fold (19, 30.02)	7.5×10^{-22}
Structural constituent of ribosome (24, 13.82)	5.8×10^{-20}
Ribosome (24, 12.89)	1.21×10^{-19}
Histone core (15, 36.91)	1.69×10^{-18}
Translation (21, 14.65)	7.15×10^{-18}
Mylein sheath (18, 16.63)	3.87×10^{-16}
Nucleosome (13, 31.72)	4.20×10^{-15}
Ribonucleoprotein (14, 24.48)	1.15×10^{-14}
Ribosomal protein (13, 29.28)	1.39×10^{-14}
Nucleosome assembly (13, 23.18)	2.14×10^{-13}
Poly (A) RNA binding (34, 4.29)	4.69×10^{-13}
Nuclear nucleosome (10, 40.67)	1.37×10^{-12}
Parkinson's disease (18, 9.52)	2.60×10^{-12}
Cytosolic small ribosomal subunit (10, 33.98)	8.65×10^{-12}
Cytosolic large ribosomal subunit (11, 24.85)	1.46×10^{-11}
Systemic lupus erythematosus (16, 10.14)	2.51×10^{-11}
Hungtinton's disease (19, 7.38)	4.19×10^{-11}
H2B (8, 52.06)	7.18×10^{-11}
Nucleus (26, 4.79)	8.79×10^{-11}
Oxidative phosphorylation (16, 9.01)	$1{,}43 \times 10^{-10}$
Histone H2B (8, 48.75)	1.48×10^{-10}
DNA binding (21, 6.06)	1.81×10^{-10}
Membrane (27, 4.21)	4.82×10^{-10}
Alzheimer disease (17, 7.38)	6.32×10^{-10}
Alcoholism (16, 7.17)	3.64×10^{-9}
ATP synthesis coupled proton transport (7, 41.39)	1.0×10^{-8}
Innate immune response in mucose (6, 51.80)	5.89×10^{-8}
Antibacterial humoral response (6, 48.15)	9.1×10^{-8}
Focal adhesion (15, 6.10)	1.38×10^{-7}
DNA binding (18, 4.18)	1.02×10^{-6}
DNA replication dependent nucleosome assembly (6, 30.64)	1.13×10^{-6}
Hydrogen ion transport (5, 55.38)	1.38×10^{-6}
Protein heterotetramerizacion (6, 29.31)	1.44×10^{-6}
Proton transporting ATP synthase activity, rotational mechanism (5, 51.26)	1.66×10^{-6}
Cytoplasm (11, 5.91)	1.60×10^{-5}
Cytoplasmatic translation (5, 29.56)	2.00×10^{-5}
H4 (4, 60.63)	2.86×10^{-5}
Viral carcinogenesis (13, 4.39)	3.09×10^{-5}
Cardiac muscle contraction (8, 8.53)	3.57×10^{-5}
Histone H4 (4, 56.81)	3.68×10^{-5}
Histone H4 conserved site (4, 56.81)	3.68×10^{-5}
TAF (4, 56.88)	5.56×10^{-5}
Defense response to gram positive bacterium (6, 49.69)	6.14×10^{-5}

(*Continued*)

Table 1. (Continued)

Functional terms of overrepresented biological processes [a]	P value [b]
Tata Box binding protein associated factor (TAF) (4, 14.04)	7.14×10^{-5}
Negative regulation of megakaryocite differentiation (4, 46.53)	1.04×10^{-4}
ATP hydrolysis coupled ion transport (5, 40.85)	1.14×10^{-4}
Acetylation (6, 19.37)	1.32×10^{-4}
H2A (4, 12.08)	3.67×10^{-4}
Mitochondrial electron transport, cytochrome c to oxygen (3, 27.33)	4.57×10^{-4}
Ribosomal large subunit assembly (4, 84.27)	4.94×10^{-4}
DNA replication independent nucleosome assembly (4, 24.96)	4.94×10^{-4}
Histone H2A (4, 24.37)	5.44×10^{-4}
V-ATPase proteolipid subunit C-like domain (3, 76.78)	5.86×10^{-4}
DNA templated transcription, initiation (4, 22.47)	6.81×10^{-4}
Nuclear chromosome, telomeric region (6, 8.22)	7.79×10^{-4}
Non alcoholic fatty liver disease (NAFLD) (9, 4.40)	8.75×10^{-4}
Lactate/malate dehydrogenase (3, 63.99)	8.74×10^{-4}
Lactate malate dehydrogenase, N–terminal (3, 63.99)	8.74×10^{-4}
Mitochondrial proton transporting ATP synthase complex (3, 61.00)	9.60×10^{-4}

[a] Values in parenthesis represent the number of genes involved in and the fold enrichment of the corresponding functional terms

[b] EASE score examine the significance of gene term enrichment with a modified Fisher's exact test

https://doi.org/10.1371/journal.pone.0213420.t001

Comparison of downregulated genes with the mouse genome database

In order to explore mechanisms that may relate to reduced viability in embryos obtained using cryopreserved semen, the Mouse Genome Database [34, 35] was queried to determine whether genes downregulated in CRYO equine embryos were orthologs to mouse genes with known associations with embryo lethality.

Day-8 embryos

In Day-8 CRYO embryos, transcripts of genes associated with the following terms were found to be of low abundance: failure of zygotic division, decreased embryo size, abnormal embryo size, embryonic growth arrest, embryonic growth retardation, embryonic lethality before implantation-complete penetrance, embryonic lethality between implantation and somite formation-complete penetrance, embryonic lethality between somite formation and embryo turning-complete penetrance, embryonic lethality prior to tooth bud stage, abnormal embryonic tissue morphology, abnormal extraembryonic tissue morphology, delayed allantois development, perinatal lethality incomplete penetrance, prenatal lethality-complete penetrance, preweaning lethality-complete penetrance, abnormal male germ cell apoptosis, abnormal spermatogenesis, azoospermia, male infertility, and female infertility.

Day 10 embryos

In Day-10 CRYO embryos, the following gene associations cited above for Day-8 embryos were found: decreased embryo size, abnormal embryo size, failure of zygotic cell division, embryonic lethality between implantation and somite formation, embryonic lethality between implantation and somite formation-complete penetrance, embryonic lethality prior to tooth bud stage, prenatal lethality-complete penetrance, perinatal lethality-incomplete penetrance,

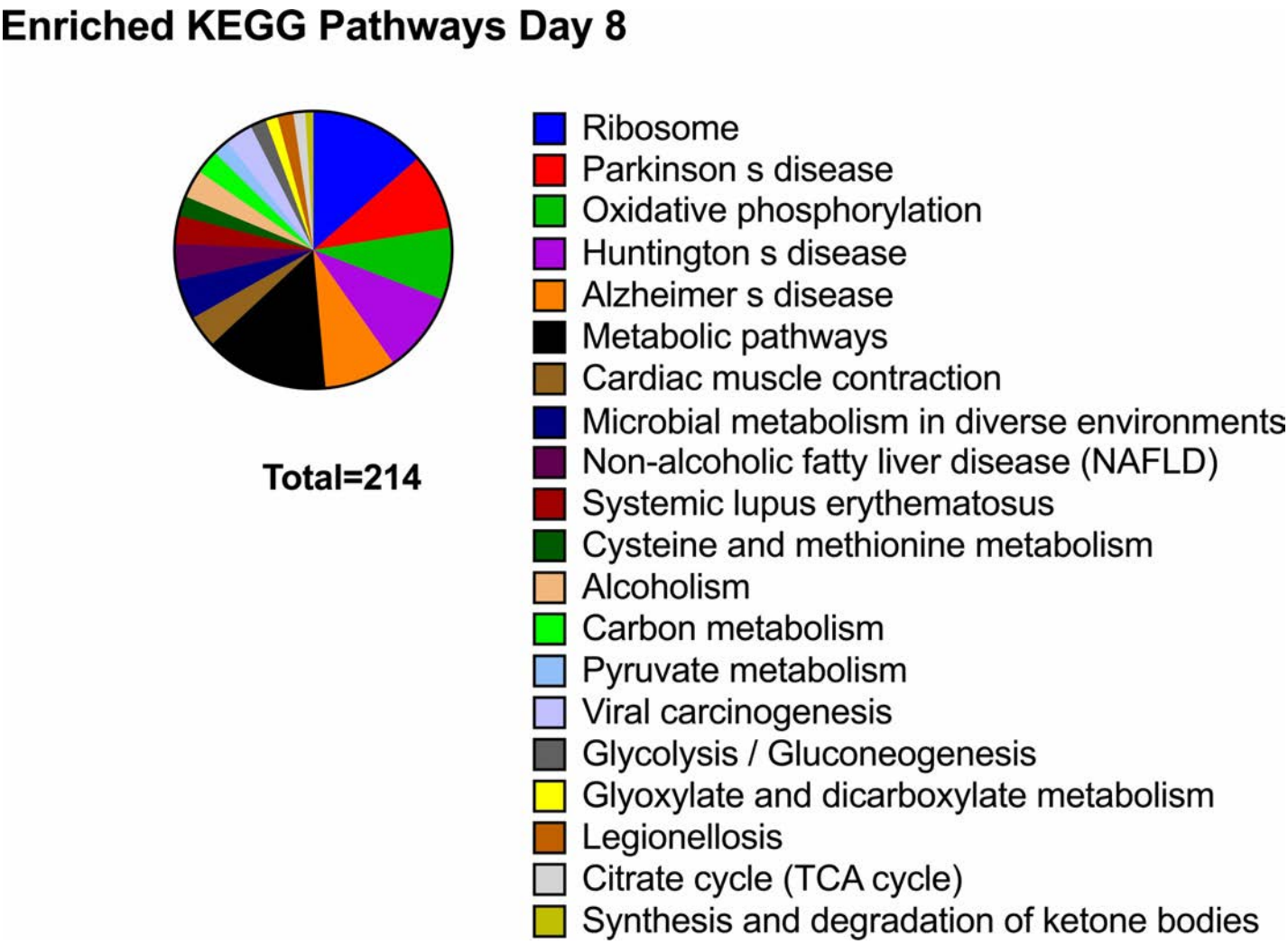


Fig 3. Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in transcripts downregulated in 8-Day embryos obtained with frozen-thawed spermatozoa.

https://doi.org/10.1371/journal.pone.0213420.g003

preweaning lethality, preweaning lethality-complete penetrance, and abnormal spermatogenesis.

In addition, the following associations were found: abnormal blastocyst morphology, absent blastocele, abnormal inner cell mass morphology, absent inner cell mass proliferation, empty decidua capsularis, embryonic growth retardation, failure of blastocyst to hatch from the zona pellucida, abnormal preimplantation embryo development, failure to gastrulate, embryonic lethality prior to embryogenesis, failure of embryo implantation, abnormal decidua basalis morphology, abnormal extraembryonic endoderm formation, prenatal lethality prior to heart atrial septation, decreased fetal size, preweaning lethality incomplete penetrance, abnormal gametogenesis, abnormal spermatid morphology, abnormal vas deferens morphology, decreased mature ovarian follicle number, reduced female fertility and small ovary.

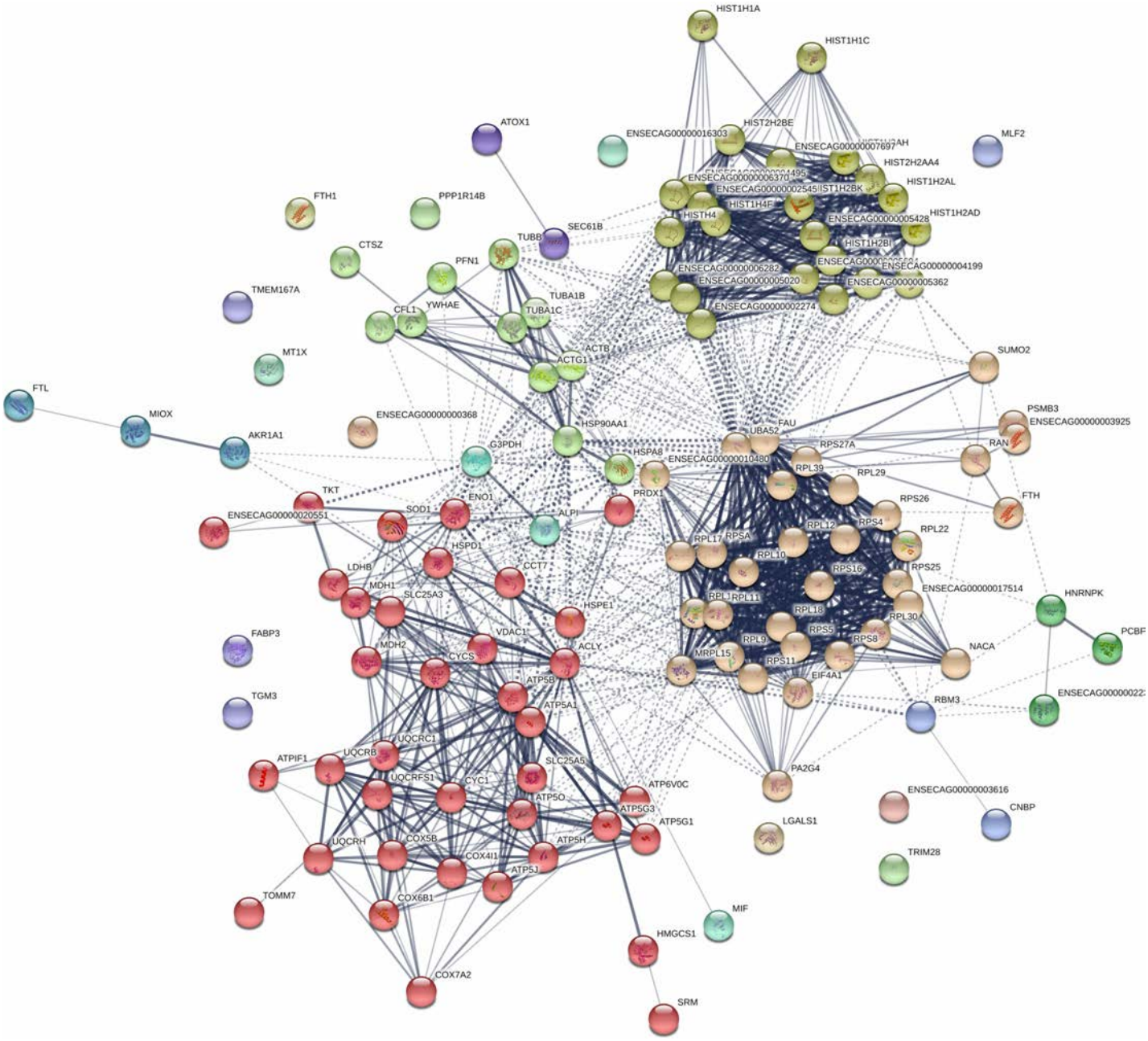


Fig 4. Functional networks (STRING) of transcripts downregulated in 8-Day equine embryos obtained with frozen-thawed sperm (CRYO embryos). Functional networks apply to histones and mitochondrial proteins. Controls are 8-Day embryos from the same mare, obtained with fresh semen from the same ejaculate that was frozen and used to produce the CRYO embryos. A list of the transcripts in each cluster obtained after STRING analysis are presented. Colors for each cluster are given in S3 Table.

https://doi.org/10.1371/journal.pone.0213420.g004

Day 12 embryos

In 12-day CRYO embryos the following gene associations cited above were found: abnormal embryo size, decreased embryo size, prenatal lethality prior to heart atrial septation, embryonic lethality prior to tooth bud stage, preweaning lethality-complete penetrance, male infertility, female infertility, and small ovary.

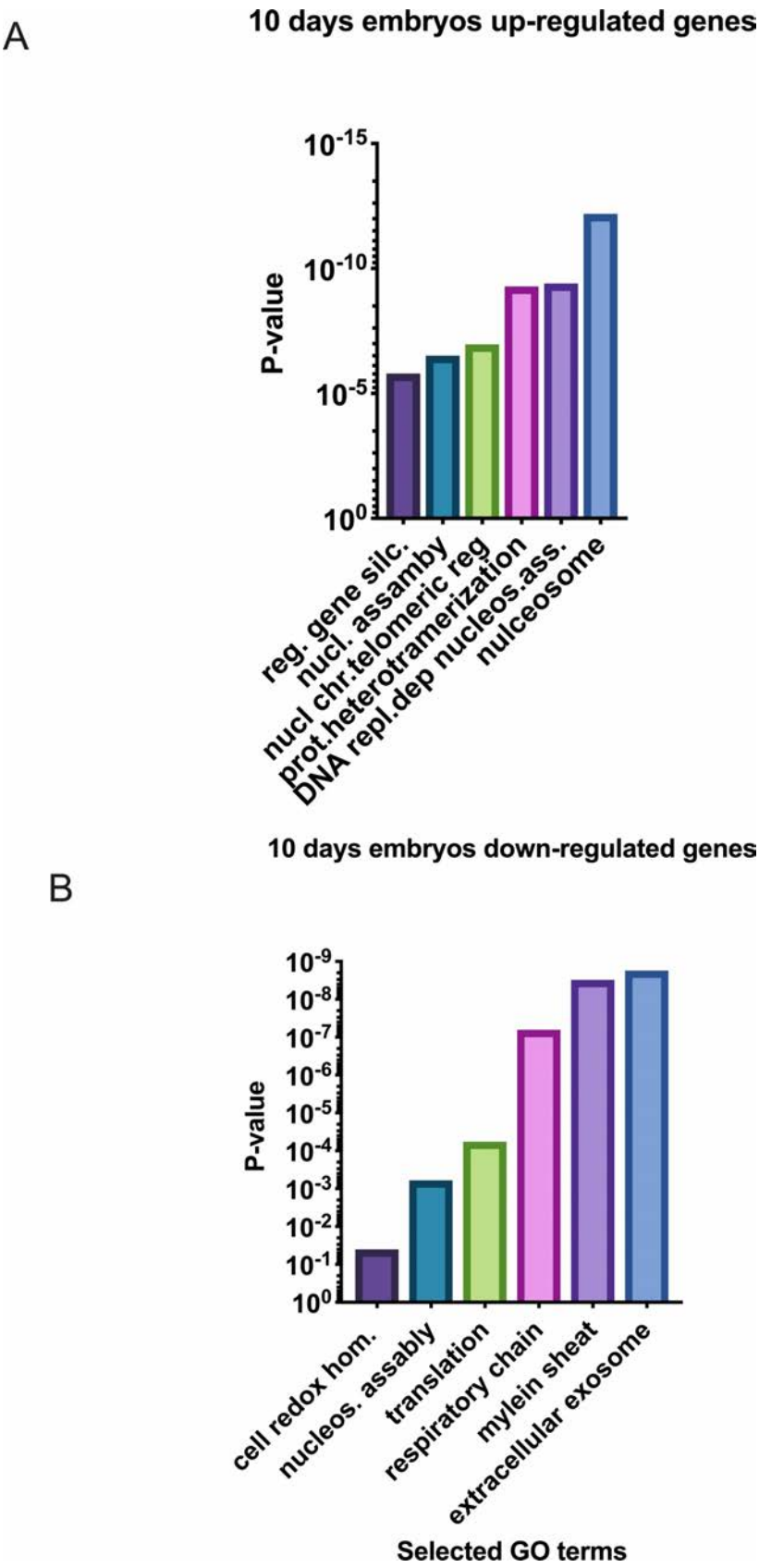

A
10 days embryos up-regulated genes
P-value
10-15
10-10
10-5
100
reg. gene silc.
nucl. assamby
nucl chr.telomeric reg
prot.heterotramerization
DNA repl.dep nucleos.ass.
nulceosome
B
10 days embryos down-regulated genes
P-value
10-9
10-8
10-7
10-6
10-5
10-4
10-3
10-2
10-1
100
cell redox hom.
nucleos. assably
translation
respiratory chain
mylein sheat
extracellular exosome
Selected GO terms

Fig 5. Selected enriched GO terms differentially regulated in 10-Day equine embryos obtained with fresh sperm (FRSH) and frozen-thawed sperm (CRYO), (A) transcripts down regulated transcripts 10-Day CRYO embryos, (B) transcripts up regulated in 10-Day CRYO embryos.

https://doi.org/10.1371/journal.pone.0213420.g005

In addition, the following associations were found: incomplete embryo turning, embryonic lethality prior to organogenesis, embryonic lethality during organogenesis-complete penetrance, decreased FSH level, small seminal vesicle, small seminiferous tubules, small testis, absent mature ovarian follicles, abnormal ovulation, abnormal corpus luteum morphology, uterus hypoplasia, and vaginal atresia.

Discussion

Here we report, for the first time, evidence that procedures performed during handling of sperm, such as freezing and thawing, have a significant impact on critical aspects of the early embryo transcriptome. The equine model used in our study has a number of advantages, including a long pre-attachment embryonic period in which the embryo remains spherical, which facilitates embryo collection, and the possibility of repeated embryo collections from the same animals over successive estrus cycles. Additionally, the stallion serves as an excellent model for the human male, as stallions are typically not selected for sperm quality nor the ability of semen to be cryopreserved, in contrast to males in production species, such as the bull. Moreover, since many stallions reach advanced age, the horse can be used as a model to study the impact of paternal age on embryo quality.

Our study, focused on three embryo ages (8, 10 and 12 days post ovulation), revealed a significant impact of sperm cryopreservation on the transcriptome of the resulting embryo. Importantly, transcripts with decreased abundance reflected genes related to DNA replication and assembly, and oxidative phosphorylation. Exploration of differentially-expressed genes at the molecular and cellular level revealed alterations in important functions including ATP synthesis, regulation of transcription, nucleosome assembly, chromatin silencing, protein synthesis, and redox regulation. Alterations in these genes help to explain the reduced fertility observed with cryopreserved sperm attributable to increased early embryo mortality [11, 12].

The pre-implantation period is a period of rapid embryo growth, requiring a ready supply of ATP. The equine embryo appears to have a significant capacity for glycolysis, but also uses oxidative phosphorylation [36]. The KEGG pathways analysis of downregulated genes revealed enriched annotations for oxidative phosphorylation, pyruvate metabolism, glycolysis, and the TCA cycle, suggesting compromised energy metabolism in CRYO embryos. A similar picture was observed in Day-10 and Day-12 embryos, with the pathways for oxidative

Table 2. Selected enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched in downregulated transcripts of in 10 days embryos obtained after AI with frozen thawed sperm.

KEGG pathway	Pathway description	observed gene count	false discovery rate
190	Oxidative phosphorylation	21	2,45E-23
5012	Parkinson s disease	20	4,64E-21
5010	Alzheimer s disease	15	1,05E-12
5016	Huntington s disease	15	3,42E-12
1100	Metabolic pathways	28	4,29E-09
3010	Ribosome	11	8,96E-09
4260	Cardiac muscle contraction	7	2,56E-06
4932	Non-alcoholic fatty liver disease (NAFLD)	9	3,96E-06
4141	Protein processing in endoplasmic reticulum	9	1,61E-05

https://doi.org/10.1371/journal.pone.0213420.t002

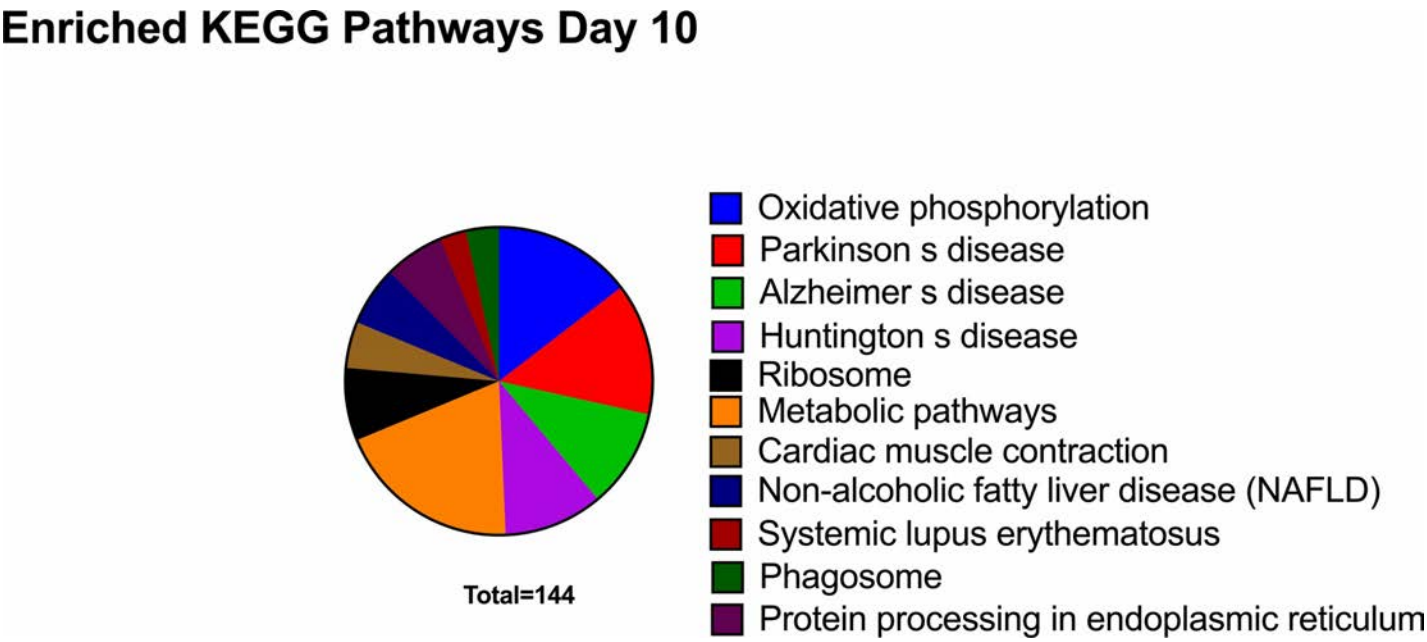


Fig 6. Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in transcripts downregulated in 10-Day embryos obtained with frozen-thawed spermatozoa.

https://doi.org/10.1371/journal.pone.0213420.g006

phosphorylation, metabolic pathways, and non alcoholic fatty liver disease significantly over-represented in transcripts with reduced abundance of all CRYO embryos obtained.

When we evaluated low-abundance equine transcripts for their mouse orthologs, we found that many of the genes downregulated in CRYO embryos have knockout database annotation terms related to reduced embryonic viability. This finding opens the possibility that not only genes related to the metabolism and thus growth of embryos, but also genes directly related to embryo organogenesis, embryo survival, and offspring health are affected by the use of cryopreserved sperm, and thus these genes warrant further investigation.

While the mechanisms behind the effects reported here are as yet unclear, a major factor may be the well-documented oxidative damage that the genome and epigenome experiences during cryopreservation and thawing [11–14]. Cryopreservation is a major cause of oxidative stress [37] and lipid peroxidation in stallion spermatozoa [10, 17, 38, 39]. Lipid peroxidation in spermatozoa surviving cryopreservation [37] is associated with increased levels of 4-hydroxinonenal (4-HNE) [17]. This compound is able to interact with DNA to form adducts that have been related directly to increased rates of mutation in important cell-cycle regulators [40, 41]. The production of 4-HNE during cryopreservation of stallion spermatozoa is well documented [10, 17, 39], and it is possible that significant amounts of 4-HNE and other toxic lipid aldehydes are incorporated to the oocyte, potentially causing alterations in embryo development. In addition to DNA damage, 4-HNE can alkylate the sperm centrioles, and in horses, as in humans, paternal centrioles are inherited by the embryos. Damaged centrioles may cause disrupted cytoskeletal protein organization during early cleavage [42].

Supporting this line of reasoning, recent reports have linked abnormal early cleavage events and changes in embryo transcript abundance to fertilization with spermatozoa showing oxidative stress. Macaque embryos obtained after fertilization with ROS-treated sperm showed significantly lower rates of development to the four- and eight-cell stages, and changes in transcript abundance for genes related to actin cytoskeleton organization, cell junction

Table 3. Gene ontology annotations enriched in downregulated transcripts of 10 days embryos obtained after AI with frozen thawed sperm.

Term	P Value
GO:0070062~extracellular exosome	1,72E-09
GO:0043209~myelin sheath	3,03E-09
GO:0070469~respiratory chain	6,30E-08
GO:0022625~cytosolic large ribosomal subunit	5,33E-07
GO:0008137~NADH dehydrogenase (ubiquinone) activity	8,07E-07
GO:0005747~mitochondrial respiratory chain complex I	3,12E-06
GO:0015986~ATP synthesis coupled proton transport	4,78E-06
GO:0003735~structural constituent of ribosome	1,66E-05
GO:0000788~nuclear nucleosome	2,25E-05
GO:0046933~proton-transporting ATP synthase activity, rotational mechanism	2,90E-05
GO:0005925~focal adhesion	4,41E-05
GO:0006412~translation	5,75E-05
GO:0046961~proton-transporting ATPase activity, rotational mechanism	1,59E-04
GO:0000786~nucleosome	1,73E-04
GO:0042773~ATP synthesis coupled electron transport	2,22E-04
GO:0006334~nucleosome assembly	5,99E-04
GO:0005743~mitochondrial inner membrane	0,001576512
GO:0004129~cytochrome-c oxidase activity	0,002995144
GO:0005739~mitochondrion	0,00444262
GO:0006336~DNA replication-independent nucleosome assembly	0,005364717
GO:0045261~proton-transporting ATP synthase complex, catalytic core F(1)	0,011191606
GO:0015991~ATP hydrolysis coupled proton transport	0,013628447
GO:0006457~protein folding	0,017943471
GO:0051603~proteolysis involved in cellular protein catabolic process	0,019505148
GO:0003677~DNA binding	0,022056606
GO:0006123~mitochondrial electron transport, cytochrome c to oxygen	0,024396132
GO:0044822~poly(A) RNA binding	0,032707627
GO:0005753~mitochondrial proton-transporting ATP synthase complex	0,0332053
GO:0005615~extracellular space	0,040768468
GO:0005687~U4 snRNP	0,044030068
GO:0045454~cell redox homeostasis	0,047994139
GO:0006122~mitochondrial electron transport, ubiquinol to cytochrome c	0,048204941
GO:1902166~negative regulation of intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator	0,054067016
GO:0006120~mitochondrial electron transport, NADH to ubiquinone	0,059893472
GO:0045653~negative regulation of megakaryocyte differentiation	0,065684522
GO:0030330~DNA damage response, signal transduction by p53 class mediator	0,065684522
GO:0016020~membrane	0,071097195
GO:0004185~serine-type carboxypeptidase activity	0,07400431
GO:0034719~SMN-Sm protein complex	0,075791838
GO:0005685~U1 snRNP	0,075791838
GO:0002227~innate immune response in mucosa	0,077161252
GO:0007569~cell aging	0,077161252
GO:0005682~U5 snRNP	0,080983251
GO:0071157~negative regulation of cell cycle arrest	0,082847353
GO:0019731~antibacterial humoral response	0,082847353
GO:0005975~carbohydrate metabolic process	0,083234703

(*Continued*)

Table 3. (Continued)

Term	P Value
GO:0005686~U2 snRNP	0,086145885
GO:0030970~retrograde protein transport, ER to cytosol	0,088498889
GO:0000784~nuclear chromosome, telomeric region	0,089088094
GO:0045787~positive regulation of cell cycle	0,094116067

https://doi.org/10.1371/journal.pone.0213420.t003

assembly and cell adhesion [43]. Although seen at a much later stage of development, in our study we also found that genes for cytoskeleton components tubulin alpha 1 a, tubulin beta 2

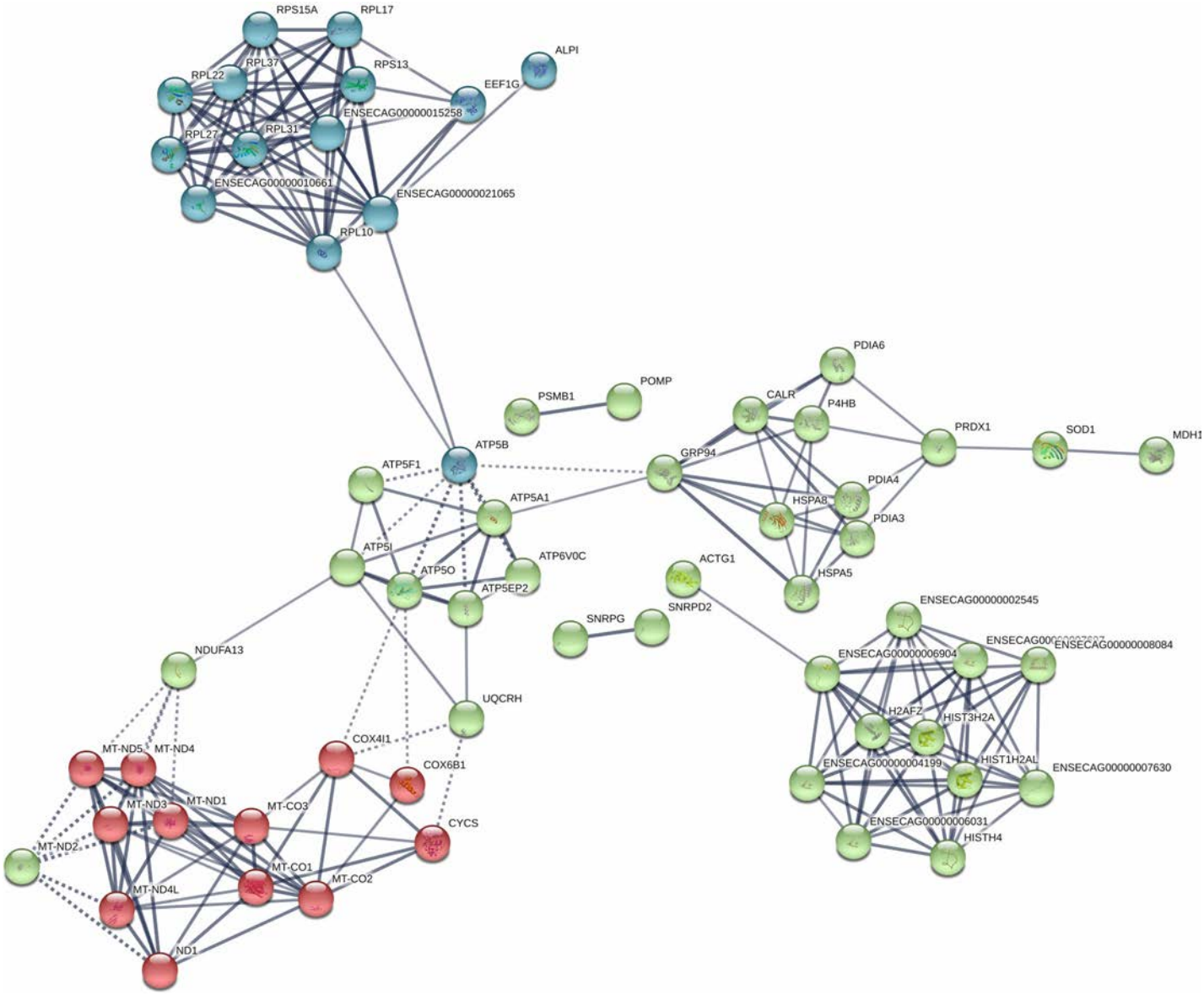


Fig 7. Functional networks (STRING) of transcripts downregulated in 10-Day equine embryos obtained with frozen-thawed sperm (CRYO embryos). Functional networks apply to histones and mitochondrial proteins. Controls are same-age embryos from the same mare, obtained with fresh semen from the same ejaculate that was frozen and used to produce the CRYO embryos.

https://doi.org/10.1371/journal.pone.0213420.g007

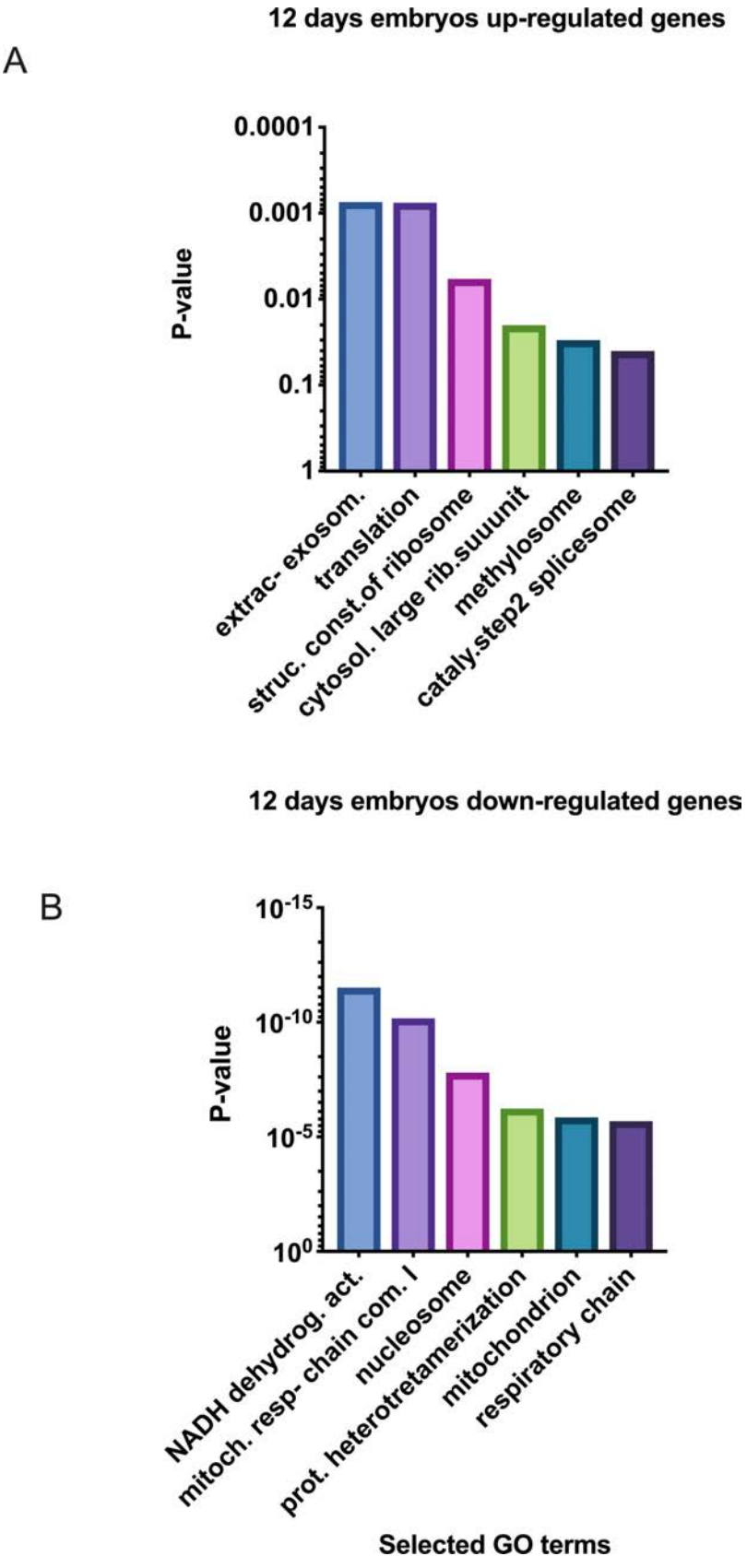

12 days embryos up-regulated genes
A
P-value
0.0001
0.001
0.01
0.1
1
extrac- exosom.
translation
struc. const.of ribosome
cytosol. large rib.suuunit
methylosome
cataly.step2 splicesome
12 days embryos down-regulated genes
B
P-value
10-15
10-10
10-5
100
NADH dehydrog. act.
mitoch. resp- chain com. I
nucleosome
prot. heterotretamerization
mitochondrion
respiratory chain
Selected GO terms

Fig 8. Selected enriched GO terms differentially regulated in 12-Day equine embryos obtained with fresh sperm (FRSH) and frozen-thawed sperm (CRYO), (A) transcripts down regulated in 12-Day CRYO embryos, (B) transcripts up regulated in 12-Day CRYO embryos.

https://doi.org/10.1371/journal.pone.0213420.g008

class II a and actin, cytoplasmic 1, N-terminally processed were downregulated in 8-day CRYO embryos.

Cryopreservation may also directly affect the epigenome of the paternal DNA; recent studies have shown that cryopreservation increases the level of DNA methylation in equine sperm [12] and the expression of genes important to intracellular regulation of epigenetic status [16]. Notably, we also found significant reduction in abundance of transcripts for histones in CRYO embryos.

The finding that many differentially regulated genes in CRYO embryos are orthologs of mouse genes that have knockout database annotation terms related to reduced embryonic viability provides further evidence linking cryopreserved sperm to reduced embryonic viability. These annotations consistently appeared on analysis of low-abundance transcripts in all CRYO embryos, and included genes related to embryonic growth retardation and embryo lethality. Interestingly, annotations related to male and female infertility were also present; this warrants further investigation on the effect of sperm origin on the fertility of resulting offspring.

In summary, the present study provides for the first time transcriptomic analysis of equine embryos in relation to the handling of semen used for their production, however we acknowledge the preliminary and descriptive nature of this report but our data provide strong evidence that cryopreservation of sperm exerts a profound impact on the transcriptome of early

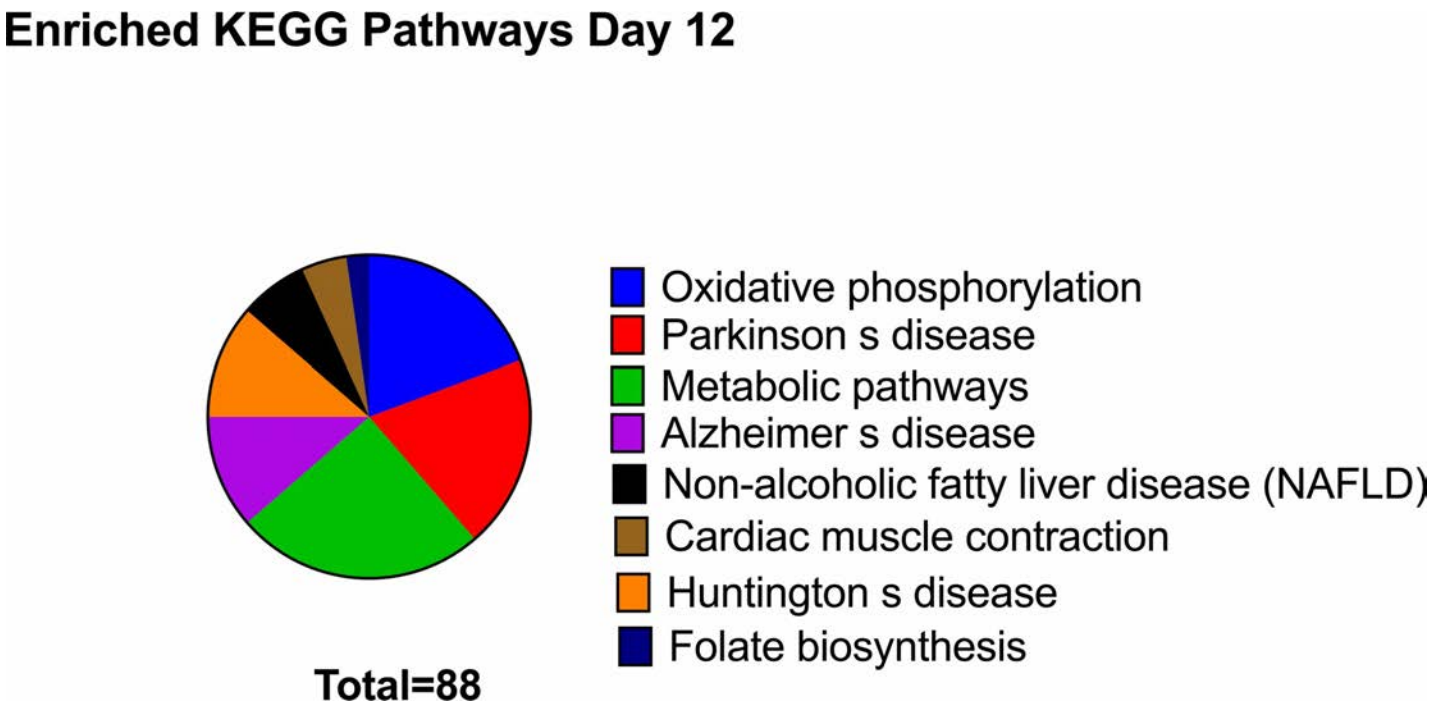


Fig 9. Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in transcripts downregulated in 12-Day embryos obtained with frozen thawed spermatozoa.

https://doi.org/10.1371/journal.pone.0213420.g009

Table 4. Functional annotation chart of differentially expressed genes (downregulated) in Day-12 equine embryos obtained after AI with frozen-thawed sperm.

Category	Term	Count	PValue
UP_KEYWORDS	Membrane	22	0,035165176
GOTERM_CC_DIRECT	GO:0016021~Integral component of membrane	19	0,023827798
KEGG_PATHWAY	ecb01100:Metabolic pathways	18	8,33E-05
GOTERM_CC_DIRECT	GO:0005739~Mitochondrion	14	1,40E-06
KEGG_PATHWAY	ecb00190:Oxidative phosphorylation	13	1,69E-12
KEGG_PATHWAY	ecb05012:Parkinson's disease	13	3,57E-12
GOTERM_CC_DIRECT	GO:0070062~Extracellular exosome	13	0,047249394
UP_KEYWORDS	Chromosome	10	4,30E-12
UP_KEYWORDS	Mitochondrion	10	1,63E-10
UP_KEYWORDS	DNA-binding	10	2,09E-05
UP_KEYWORDS	Transport	10	3,60E-05
UP_KEYWORDS	Nucleus	10	6,21E-04
UP_KEYWORDS	Respiratory chain	9	7,16E-16
UP_KEYWORDS	Electron transport	9	3,97E-14
UP_KEYWORDS	Nucleosome core	9	2,19E-11
INTERPRO	IPR009072:Histone-fold	9	2,07E-10
UP_KEYWORDS	Ubiquinone	8	3,96E-16
GOTERM_MF_DIRECT	GO:0008137~NADH dehydrogenase (ubiquinone) activity	8	3,09E-12
GOTERM_CC_DIRECT	GO:0005747~Mitochondrial respiratory chain complex I	8	6,67E-11
GOTERM_MF_DIRECT	GO:0003677~DNA binding	8	5,87E-04
UP_SEQ_FEATURE	Transmembrane region	8	9,18E-04
GOTERM_CC_DIRECT	GO:0000786~Nucleosome	7	1,55E-08
UP_KEYWORDS	NAD	7	5,92E-08
KEGG_PATHWAY	ecb05322:Systemic lupus erythematosus	7	3,03E-05
UP_KEYWORDS	Oxidoreductase	7	4,97E-05
KEGG_PATHWAY	ecb05034:Alcoholism	7	2,10E-04
GOTERM_CC_DIRECT	GO:0016020~Membrane	7	0,047593153
UP_KEYWORDS	Mitochondrion inner membrane	6	2,81E-07
KEGG_PATHWAY	ecb05010:Alzheimer's disease	6	0,001939005
KEGG_PATHWAY	ecb05016:Huntington's disease	6	0,003145256
GOTERM_BP_DIRECT	GO:0006335~DNA replication-dependent nucleosome assembly	5	4,77E-07
GOTERM_BP_DIRECT	GO:0051290~Protein heterotetramerization	5	5,76E-07
INTERPRO	IPR007125:Histone core	5	2,52E-05
GOTERM_CC_DIRECT	GO:0000784~Nuclear chromosome, telomeric region	5	2,30E-04
GOTERM_CC_DIRECT	GO:0005743~Mitochondrial inner membrane	5	0,001683893
SMART	SM00417:H4	4	6,25E-07
SMART	SM00803:TAF	4	1,22E-06
GOTERM_CC_DIRECT	GO:0070469~Respiratory chain	4	2,03E-06
INTERPRO	IPR019809:Histone H4, conserved site	4	2,80E-06
INTERPRO	IPR001951:Histone H4	4	2,80E-06
GOTERM_BP_DIRECT	GO:0045653~Negative regulation of megakaryocyte differentiation	4	4,00E-06
INTERPRO	IPR004823:TATA box binding protein associated factor (TAF)	4	5,47E-06
GOTERM_BP_DIRECT	GO:0006336~DNA replication-independent nucleosome assembly	4	1,95E-05
GOTERM_BP_DIRECT	GO:0006352~DNA-templated transcription, initiation	4	2,71E-05
INTERPRO	IPR020904:Short-chain dehydrogenase/reductase, conserved site	4	1,57E-04
INTERPRO	IPR002347:Glucose/ribitol dehydrogenase	4	6,29E-04
GOTERM_BP_DIRECT	GO:0006334~Nucleosome assembly	4	8,65E-04
GOTERM_MF_DIRECT	GO:0016491~Oxidoreductase activity	4	0,001585835

(*Continued*)

Table 4. (Continued)

Category	Term	Count	PValue
INTERPRO	IPR016040:NAD(P)-binding domain	4	0,016019866
KEGG_PATHWAY	ecb04932:Non-alcoholic fatty liver disease (NAFLD)	4	0,045179763
INTERPRO	IPR001750:NADH:ubiquinone/plastoquinone oxidoreductase	3	3,19E-05
GOTERM_BP_DIRECT	GO:0042773~ATP synthesis coupled electron transport	3	5,21E-05
GOTERM_CC_DIRECT	GO:0000788~Nuclear nucleosome	3	0,004620686
KEGG_PATHWAY	ecb04978:Mineral absorption	3	0,022822957
GOTERM_CC_DIRECT	GO:0000790~Nuclear chromatin	3	0,073903618

https://doi.org/10.1371/journal.pone.0213420.t004

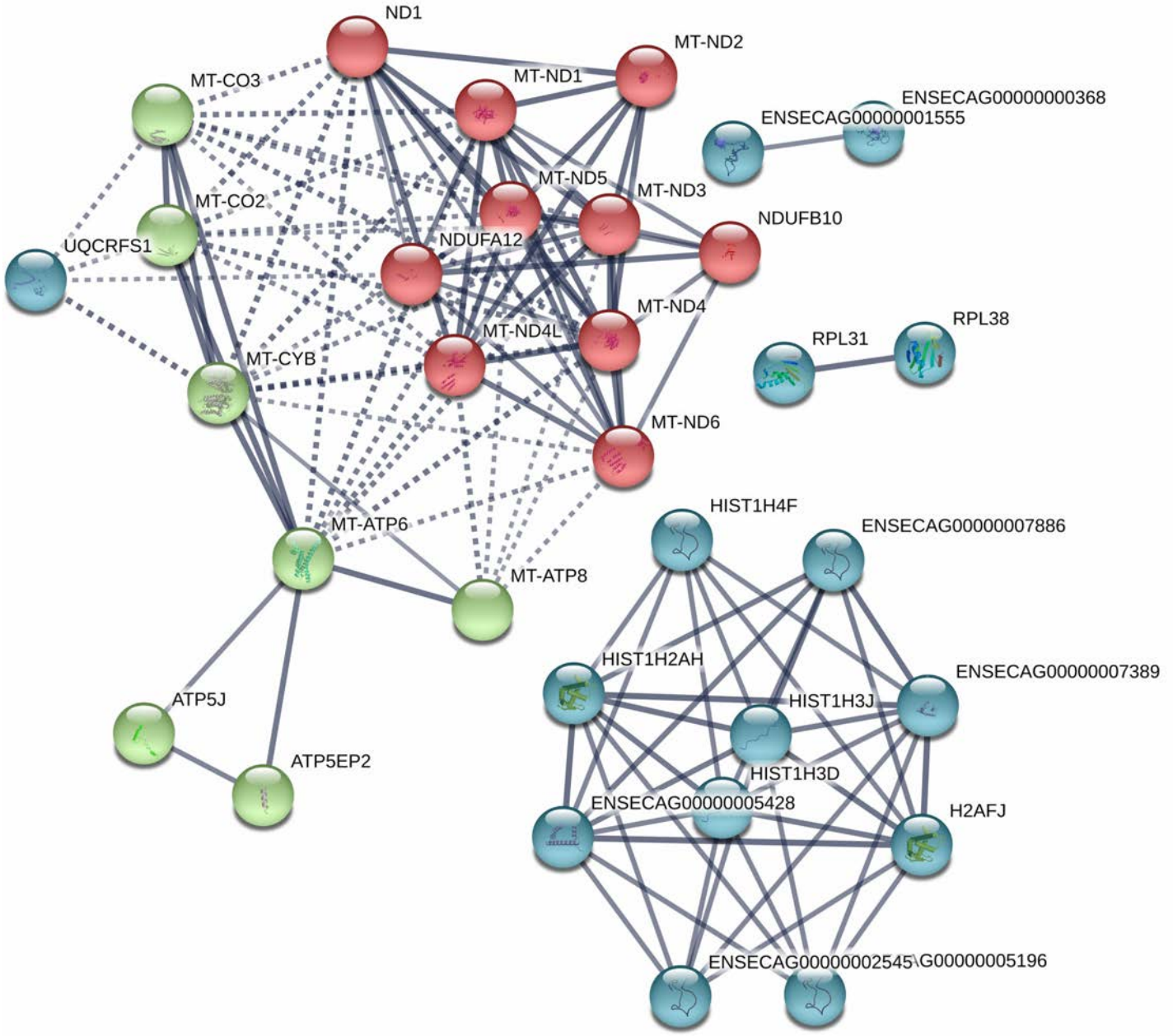


Fig 10. Functional networks (STRING) of transcripts downregulated in 12-Day equine embryos obtained with frozen thawed sperm (CRYO). Functional networks apply to histones and mitochondrial proteins. Controls are same-age embryos from the same mare obtained with fresh semen from the same ejaculate that was frozen and used to produce the CRYO embryos.

https://doi.org/10.1371/journal.pone.0213420.g010

embryos. Our findings may stimulate new lines of research to improve this biotechnology in humans and animals.

Supporting information

S1 Table. Transcripts upregulated in 8-Day embryos obtained with frozen-thawed spermatozoa with respect to controls obtained with fresh semen.
(XLSX)

S2 Table. Transcripts downregulated in 8-Day embryos obtained with frozen-thawed spermatozoa.
(XLSX)

S3 Table. Network analysis of transcripts downregulated in 8-Day embryos obtained with frozen-thawed spermatozoa. List of transcripts in each cluster obtained after STRING analysis; genes in each cluster are presented and colors for each cluster are given. Network is presented in Fig 4.
(XLSX)

S4 Table. Transcripts upregulated in 10-Day embryos obtained with frozen-thawed spermatozoa.
(XLSX)

S5 Table. Transcripts upregulated in 12-day embryos obtained with frozen-thawed spermatozoa.
(XLSX)

S6 Table. Transcripts downregulated in 12-Day embryos obtained with frozen-thawed spermatozoa.
(XLSX)

Author Contributions

Conceptualization: Heriberto Rodríguez-Martínez, Fernando J. Peña.

Data curation: Ángel Román.

Formal analysis: Alberto Álvarez-Barrientos, Ángel Román, Fernando J. Peña.

Funding acquisition: Fernando J. Peña.

Investigation: José M. Ortiz-Rodriguez, Cristina Ortega-Ferrusola, María C. Gil, Francisco E. Martín-Cano, Gemma Gaitskell-Phillips, Fernando J. Peña.

Methodology: Alberto Álvarez-Barrientos, Ángel Román.

Resources: Alberto Álvarez-Barrientos.

Software: Francisco E. Martín-Cano, Ángel Román.

Supervision: Katrin Hinrichs, Fernando J. Peña.

Validation: Ángel Román.

Visualization: Ángel Román.

Writing – original draft: Katrin Hinrichs, Fernando J. Peña.

Writing – review & editing: Heriberto Rodríguez-Martínez, Katrin Hinrichs, Fernando J. Peña.

References

1. Pena FJ, Garcia BM, Samper JC, Aparicio IM, Tapia JA, Ferrusola CO. Dissecting the molecular damage to stallion spermatozoa: the way to improve current cryopreservation protocols? Theriogenology. 2011; 76(7):1177–86. https://doi.org/10.1016/j.theriogenology.2011.06.023 PMID: 21835453.
2. Dearing CG, Jayasena CN, Lindsay KS. Human sperm cryopreservation in cancer patients: Links with deprivation and mortality. Cryobiology. 2017; 79:9–13. https://doi.org/10.1016/j.cryobiol.2017.10.003 PMID: 29031884.
3. Jiang XP, Zhou WM, Wang SQ, Wang W, Tang JY, Xu Z, et al. Multivariate model for predicting semen cryopreservation outcomes in a human sperm bank. Asian J Androl. 2017; 19(4):404–8. https://doi.org/10.4103/1008-682X.178488 PMID: 27080478; PubMed Central PMCID: PMC5507083.
4. Camillo F, Vannozzi I, Tesi M, Sabatini C, Rota A, Paciolla E, et al. Induction of ovulation with buserelin in jennies: in search of the minimum effective dose. Anim Reprod Sci. 2014; 151(1–2):56–60. Epub 2014/10/09. https://doi.org/10.1016/j.anireprosci.2014.09.011 PMID: 25293536.
5. Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang JA, Clark AM. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum Reprod. 2009; 24(9):2061–70. https://doi.org/10.1093/humrep/dep214 PMID: 19525298.
6. Panzani D, Rota A, Marmorini P, Vannozzi I, Camillo F. Retrospective study of factors affecting multiple ovulations, embryo recovery, quality, and diameter in a commercial equine embryo transfer program. Theriogenology. 2014; 82(6):807–14. Epub 2014/08/03. https://doi.org/10.1016/j.theriogenology.2014.06.020 PMID: 25085596.
7. Jodar M, Selvaraju S, Sendler E, Diamond MP, Krawetz SA, Reproductive Medicine N. The presence, role and clinical use of spermatozoal RNAs. Hum Reprod Update. 2013; 19(6):604–24. https://doi.org/10.1093/humupd/dmt031 PMID: 23856356; PubMed Central PMCID: PMC3796946.
8. Sendler E, Johnson GD, Mao S, Goodrich RJ, Diamond MP, Hauser R, et al. Stability, delivery and functions of human sperm RNAs at fertilization. Nucleic Acids Res. 2013; 41(7):4104–17. https://doi.org/10.1093/nar/gkt132 PMID: 23471003; PubMed Central PMCID: PMC3627604.
9. Davila MP, Munoz PM, Bolanos JM, Stout TA, Gadella BM, Tapia JA, et al. Mitochondrial ATP is required for the maintenance of membrane integrity in stallion spermatozoa, whereas motility requires both glycolysis and oxidative phosphorylation. Reproduction. 2016; 152(6):683–94. https://doi.org/10.1530/REP-16-0409 PMID: 27798283.
10. Pena FJ, Plaza Davila M, Ball BA, Squires EL, Martin Munoz P, Ortega Ferrusola C, et al. The Impact of Reproductive Technologies on Stallion Mitochondrial Function. Reprod Domest Anim. 2015; 50(4):529–37. https://doi.org/10.1111/rda.12551 PMID: 26031351.
11. Kopeika J, Thornhill A, Khalaf Y. The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence. Hum Reprod Update. 2015; 21(2):209–27. https://doi.org/10.1093/humupd/dmu063 PMID: 25519143.
12. Aurich C, Schreiner B, Ille N, Alvarenga M, Scarlet D. Cytosine methylation of sperm DNA in horse semen after cryopreservation. Theriogenology. 2016; 86(5):1347–52. https://doi.org/10.1016/j.theriogenology.2016.04.077 PMID: 27242182.
13. Valcarce DG, Carton-Garcia F, Riesco MF, Herraez MP, Robles V. Analysis of DNA damage after human sperm cryopreservation in genes crucial for fertilization and early embryo development. Andrology. 2013; 1(5):723–30. https://doi.org/10.1111/j.2047-2927.2013.00116.x PMID: 23970451.
14. Valcarce DG, Carton-Garcia F, Herraez MP, Robles V. Effect of cryopreservation on human sperm messenger RNAs crucial for fertilization and early embryo development. Cryobiology. 2013; 67(1):84–90. https://doi.org/10.1016/j.cryobiol.2013.05.007 PMID: 23727067.
15. Teperek M, Simeone A, Gaggioli V, Miyamoto K, Allen GE, Erkek S, et al. Sperm is epigenetically programmed to regulate gene transcription in embryos. Genome Res. 2016; 26(8):1034–46. https://doi.org/10.1101/gr.201541.115 PMID: 27034506; PubMed Central PMCID: PMC4971762.
16. Zeng C, Peng W, Ding L, He L, Zhang Y, Fang D, et al. A preliminary study on epigenetic changes during boar spermatozoa cryopreservation. Cryobiology. 2014; 69(1):119–27. https://doi.org/10.1016/j.cryobiol.2014.06.003 PMID: 24974820.
17. Martin Munoz P, Ortega Ferrusola C, Vizuete G, Plaza Davila M, Rodriguez Martinez H, Pena FJ. Depletion of Intracellular Thiols and Increased Production of 4-Hydroxynonenal that Occur During Cryopreservation of Stallion Spermatozoa Lead to Caspase Activation, Loss of Motility, and Cell Death. Biol Reprod. 2015; 93(6):143. https://doi.org/10.1095/biolreprod.115.132878 PMID: 26536905.
18. Plaza Davila M, Martin Munoz P, Tapia JA, Ortega Ferrusola C, Balao da Silva CC, Pena FJ. Inhibition of Mitochondrial Complex I Leads to Decreased Motility and Membrane Integrity Related to Increased

Hydrogen Peroxide and Reduced ATP Production, while the Inhibition of Glycolysis Has Less Impact on Sperm Motility. PLoS One. 2015; 10(9):e0138777. https://doi.org/10.1371/journal.pone.0138777 PMID: 26407142; PubMed Central PMCID: PMC4583303.

19. Timme-Laragy AR, Hahn ME, Hansen JM, Rastogi A, Roy MA. Redox stress and signaling during vertebrate embryonic development: Regulation and responses. Semin Cell Dev Biol. 2017. https://doi.org/10.1016/j.semcdb.2017.09.019 PMID: 28927759; PubMed Central PMCID: PMC5650060.

20. Fernandez-Gonzalez R, Moreira PN, Perez-Crespo M, Sanchez-Martin M, Ramirez MA, Pericuesta E, et al. Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. Biol Reprod. 2008; 78(4):761–72. https://doi.org/10.1095/biolreprod.107.065623 PMID: 18199884.

21. Bouckenheimer J, Assou S, Riquier S, Hou C, Philippe N, Sansac C, et al. Long non-coding RNAs in human early embryonic development and their potential in ART. Hum Reprod Update. 2016; 23(1):19–40. https://doi.org/10.1093/humupd/dmw035 PMID: 27655590.

22. Chen Q, Yan W, Duan E. Epigenetic inheritance of acquired traits through sperm RNAs and sperm RNA modifications. Nat Rev Genet. 2016; 17(12):733–43. https://doi.org/10.1038/nrg.2016.106 PMID: 27694809; PubMed Central PMCID: PMC5441558.

23. Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, et al. PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. Development. 2002; 129 (15):3533–44. PMID: 12117804.

24. Fujimoto S, Yoshida N, Fukui T, Amanai M, Isobe T, Itagaki C, et al. Mammalian phospholipase Czeta induces oocyte activation from the sperm perinuclear matrix. Dev Biol. 2004; 274(2):370–83. https://doi.org/10.1016/j.ydbio.2004.07.025 PMID: 15385165.

25. Banrezes B, Sainte-Beuve T, Canon E, Schultz RM, Cancela J, Ozil JP. Adult body weight is programmed by a redox-regulated and energy-dependent process during the pronuclear stage in mouse. PLoS One. 2011; 6(12):e29388. https://doi.org/10.1371/journal.pone.0029388 PMID: 22216268; PubMed Central PMCID: PMC3247262.

26. Ozil JP, Huneau D. Activation of rabbit oocytes: the impact of the Ca2+ signal regime on development. Development. 2001; 128(6):917–28. Epub 2001/02/27. PMID: 11222146.

27. Iqbal K, Chitwood JL, Meyers-Brown GA, Roser JF, Ross PJ. RNA-seq transcriptome profiling of equine inner cell mass and trophectoderm. Biol Reprod. 2014; 90(3):61. https://doi.org/10.1095/biolreprod.113.113928 PMID: 24478389; PubMed Central PMCID: PMC4435230.

28. Graf A, Krebs S, Zakhartchenko V, Schwalb B, Blum H, Wolf E. Fine mapping of genome activation in bovine embryos by RNA sequencing. Proc Natl Acad Sci U S A. 2014; 111(11):4139–44. Epub 2014/03/05. https://doi.org/10.1073/pnas.1321569111 PMID: 24591639; PubMed Central PMCID: PMC3964062.

29. Ortega Ferrusola C, Anel-Lopez L, Ortiz-Rodriguez JM, Martin Munoz P, Alvarez M, de Paz P, et al. Stallion spermatozoa surviving freezing and thawing experience membrane depolarization and increased intracellular Na(). Andrology. 2017; 5(6):1174–82. https://doi.org/10.1111/andr.12419 PMID: 28973824.

30. Rodriguez AM, Ferrusola CO, Garcia BM, Morrell JM, Martinez HR, Tapia JA, et al. Freezing stallion semen with the new Caceres extender improves post thaw sperm quality and diminishes stallion-to-stallion variability. Anim Reprod Sci. 2011; 127(1–2):78–83. https://doi.org/10.1016/j.anireprosci.2011.07.009 PMID: 21821371.

31. Cuervo-Arango J, Aguilar J, Newcombe JR. Effect of type of semen, time of insemination relative to ovulation and embryo transfer on early equine embryonic vesicle growth as determined by ultrasound. Theriogenology. 2009; 71(8):1267–75. https://doi.org/10.1016/j.theriogenology.2008.12.020 PMID: 19246082.

32. Jiao X, Sherman BT, Huang da W, Stephens R, Baseler MW, Lane HC, et al. DAVID-WS: a stateful web service to facilitate gene/protein list analysis. Bioinformatics. 2012; 28(13):1805–6. https://doi.org/10.1093/bioinformatics/bts251 PMID: 22543366; PubMed Central PMCID: PMC3381967.

33. Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, et al. STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic Acids Res. 2015; 43(Database issue):D447–52. https://doi.org/10.1093/nar/gku1003 PMID: 25352553; PubMed Central PMCID: PMC4383874.

34. Blake JA, Bult CJ, Eppig JT, Kadin JA, Richardson JE, Mouse Genome Database G. The Mouse Genome Database genotypes::phenotypes. Nucleic Acids Res. 2009; 37(Database issue):D712–9. https://doi.org/10.1093/nar/gkn886 PMID: 18981050; PubMed Central PMCID: PMC2686566.

35. Bult CJ, Eppig JT, Kadin JA, Richardson JE, Blake JA, Mouse Genome Database G. The Mouse Genome Database (MGD): mouse biology and model systems. Nucleic Acids Res. 2008; 36(Database

issue):D724–8. https://doi.org/10.1093/nar/gkm961 PMID: 18158299; PubMed Central PMCID: PMC2238849.

36. Lane M, O'Donovan MK, Squires EL, Seidel GE Jr., Gardner DK. Assessment of metabolism of equine morulae and blastocysts. Mol Reprod Dev. 2001; 59(1):33–7. https://doi.org/10.1002/mrd.1004 PMID: 11335944.

37. Ortega Ferrusola C, Gonzalez Fernandez L, Morrell JM, Salazar Sandoval C, Macias Garcia B, Rodriguez-Martinez H, et al. Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes. Reproduction. 2009; 138(1):55–63. https://doi.org/10.1530/REP-08-0484 PMID: 19380427.

38. Ortega-Ferrusola C, Anel-Lopez L, Martin-Munoz P, Ortiz-Rodriguez JM, Gil MC, Alvarez M, et al. Computational flow cytometry reveals that cryopreservation induces spermptosis but subpopulations of spermatozoa may experience capacitation-like changes. Reproduction. 2017; 153(3):293–304. https://doi.org/10.1530/REP-16-0539 PMID: 27965398.

39. Munoz PM, Ferrusola CO, Lopez LA, Del Petre C, Garcia MA, de Paz Cabello P, et al. Caspase 3 Activity and Lipoperoxidative Status in Raw Semen Predict the Outcome of Cryopreservation of Stallion Spermatozoa. Biol Reprod. 2016; 95(3):53. https://doi.org/10.1095/biolreprod.116.139444 PMID: 27417910.

40. Feng Z, Hu W, Amin S, Tang MS. Mutational spectrum and genotoxicity of the major lipid peroxidation product, trans-4-hydroxy-2-nonenal, induced DNA adducts in nucleotide excision repair-proficient and -deficient human cells. Biochemistry. 2003; 42(25):7848–54. https://doi.org/10.1021/bi034431g PMID: 12820894.

41. Wei X, Yin H. Covalent modification of DNA by alpha, beta-unsaturated aldehydes derived from lipid peroxidation: Recent progress and challenges. Free Radic Res. 2015; 49(7):905–17. https://doi.org/10.3109/10715762.2015.1040009 PMID: 25968945.

42. Lu Y, Lin M, Aitken RJ. Exposure of spermatozoa to dibutyl phthalate induces abnormal embryonic development in a marine invertebrate Galeolaria caespitosa (Polychaeta: Serpulidae). Aquat Toxicol. 2017; 191:189–200. https://doi.org/10.1016/j.aquatox.2017.08.008 PMID: 28843738.

43. Burruel V, Klooster KL, Chitwood J, Ross PJ, Meyers SA. Oxidative damage to rhesus macaque spermatozoa results in mitotic arrest and transcript abundance changes in early embryos. Biol Reprod. 2013; 89(3):72. https://doi.org/10.1095/biolreprod.113.110981 PMID: 23904511; PubMed Central PMCID: PMC4094196.

ANDROLOGY

ISSN: 2047-2919

ANDROLOGY

ORIGINAL ARTICLE

Correspondence:
Stephanie T. Page, Box 356426, 1959 NE Pacific Street, Seattle WA 98195.
E-mail: page@u.washington.edu

*These authors contributed equally to this work.Clinical Trial Number: NCT01382069

Keywords:
androgen, dimethandrolone, male contraception, pharmacokinetics, suppression of gonadotropins

Received: 17-Jun-2016
Revised: 20-Sep-2016
Accepted: 20-Oct-2016

doi: 10.1111/andr.12303

Comparison of the single dose pharmacokinetics, pharmacodynamics, and safety of two novel oral formulations of dimethandrolone undecanoate (DMAU): a potential oral, male contraceptive

[1,*]R. Ayoub, [2,*]S. T. Page, [1]R. S. Swerdloff, [1]P. Y. Liu, [2]J. K. Amory, [1]A. Leung, [1]L. Hull, [3]D. Blithe, [3]A. Christy, [2]J. H. Chao, [2]W. J. Bremner and [1]C. Wang

[1]Department of Medicine, Division of Endocrinology, Harbor-UCLA Medical Center and Los Angeles Biomedical Research Institute, Torrance, CA, USA, [2]Department of Medicine, University of Washington, Seattle, WA, USA, and [3]Contraception Research Branch, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA

SUMMARY

Dimethandrolone (DMA, 7α,11β-dimethyl-19-nortestosterone) has both androgenic and progestational activities, ideal properties for a male hormonal contraceptive. In vivo, dimethandrolone undecanoate (DMAU) is hydrolyzed to DMA. We showed previously that single oral doses of DMAU powder in capsule taken with food are well tolerated and effective at suppressing both LH and testosterone (T), but absorption was low. We compared the pharmacokinetics and pharmacodynamics of two new formulations of DMAU, in castor oil and in self-emulsifying drug delivery systems (SEDDS), with the previously tested powder formulation. DMAU was dosed orally in healthy adult male volunteers at two academic medical centers. For each formulation tested in this double-blind, placebo-controlled study, 10 men received single, escalating, oral doses of DMAU (100, 200, and 400 mg) and two subjects received placebo. All doses were evaluated for both fasting and with a high fat meal. All three formulations were well tolerated without clinically significant changes in vital signs, blood counts, or serum chemistries. For all formulations, DMA and DMAU showed higher maximum ($p < 0.007$) and average concentrations ($p < 0.002$) at the 400 mg dose, compared with the 200 mg dose. The powder formulation resulted in a lower conversion of DMAU to DMA ($p = 0.027$) compared with both castor oil and SEDDS formulations. DMAU in SEDDS given fasting resulted in higher serum DMA and DMAU concentrations compared to the other two formulations. Serum LH and sex hormone concentrations were suppressed by all formulations of 200 and 400 mg DMAU when administered with food, but only the SEDDS formulation was effectively suppressed serum T when given fasting. We conclude that while all three formulations of oral DMAU are effective and well tolerated when administered with food, DMAU in oil and SEDDS increased conversion to DMA, and SEDDS may have some effectiveness when given fasting. These properties might be advantageous for the application of DMAU as a male contraceptive.

INTRODUCTION

Current methods of male contraception include condoms and vasectomy, both of which have drawbacks. While condoms are reversible and widely available, they have a high user failure rate. Vasectomies are efficacious, but invasive and not readily reversible. Therefore, efforts are underway to develop alternative male contraceptives with agents of known mechanisms of action. Male hormonal contraception uses exogenous sex steroids to suppress gonadotropin secretion and spermatogenesis, is reversible, and might provide additional health benefits for men

if optimally designed (Liu *et al.*, 2006; Page *et al.*, 2008; Nieschlag, 2010; Piotrowska *et al.*, 2016; Wang *et al.*, 2016). Contraceptive efficacy studies in men with weekly intramuscular (IM) injections of testosterone enanthate or monthly injections of testosterone undecanoate have been encouraging, with high efficacy rates and few side effects (World Health Organization Task Force on Methods for the Regulation of Male Fertility, 1990; World Health Organization Task Force on the Regulation of Male Fertility, 1996; Gu *et al.*, 2009). However, suppression of spermatogenesis with exogenous testosterone alone is not uniform across all ethnic groups and requires supraphysiologic dosing. By combining testosterone with a progestin, suppression of spermatogenesis is enhanced (Meriggiola & Bremner, 1997; Liu *et al.*, 2008; Page *et al.*, 2008; Wang & Swerdloff, 2010). For example, the combination of testosterone implants and long-acting injections of the progestin depo-medroxyprogesterone acetate has excellent contraceptive efficacy (Turner *et al.*, 2003). However, injections and implants may be less desirable for some men than an oral medication such as DMAU. Oral contraceptives that are user-controlled, easy to administer, and have shorter 'on and off' rates, might be desirable for many couples (Liu *et al.*, 2006; Glasier, 2010).

Dimethandrolone (DMA) is a novel derivative of 19-nortestosterone that binds to both the androgen and the progesterone receptors, making it an attractive candidate as a single-agent male contraceptive (Attardi *et al.*, 2006). DMA undecanoate (DMAU), includes a long carbon chain ester at the C-17 position (Cook & Kepler, 2005). DMAU has been shown to be an effective, reversible contraceptive when dosed orally in pre-clinical studies. DMAU is hydrolyzed to DMA in vivo where it effectively decreases fertility in rabbits without appreciable toxicity, and is similarly non-toxic in rats and monkeys (Hild *et al.*, 2010; Attardi *et al.*, 2011a,b). We previously reported the pharmacokinetics, safety, and tolerability of DMAU when given as a single oral dose as a powder in capsule to healthy male volunteers. However, we noted that this formulation resulted in low serum concentrations of DMA, likely because of the poor conversion of DMAU to DMA (about 3%) and that concomitant administration with food was required for both appreciable absorption and for conversion to DMA (Surampudi *et al.*, 2014). In an effort to increase the bioavailability of DMA, we reformulated oral DMAU into capsules in either castor oil or self-emulsifying drug delivery systems (SEDDS) and assessed their pharmacokinetics and pharmacodynamics when administered orally to healthy men. We hypothesized that utilization of these lipophilic drug delivery entities would enhance absorption and hydrolysis of DMAU, might negate the need for concomitant administration with food, and would optimize DMA serum concentrations for future evaluations of oral DMAU as a male hormonal contraceptive.

RESEARCH PARTICIPANTS AND METHODS

Research participants

Healthy men, age 18–50 years, with no significant medical history or illnesses, and normal physical examination, blood count, clinical chemistries, hepatitis panel, liver function tests, prostate-specific antigen (PSA) levels, electrocardiogram, and BMI <33 kg/m^2, were included in the study. Men were excluded if they had participated in a clinical trial involving an investigational drug within 30 days, had used hormonal therapy within the last 3 months, had a disorder of the hypothalamus/pituitary/testis, desired fertility within a year, or had a pregnant partner, or had clinically significant abnormal physical or laboratory findings, or an elevated PSA. Participants were recruited and enrolled at the Harbor-UCLA Medical Center/Los Angeles Biomedical Research Institute in Torrance, California and the University of Washington in Seattle, Washington. The study protocol was approved by the institutional review boards for both participating institutions. All participants provided written informed consent prior to any study procedures. The medical monitor and the investigators reviewed adverse events and safety data weekly with the provision that an external independent data safety monitoring board be notified if and when a grade 3 adverse event occurred.

Study medications

DMAU is manufactured by Evestra Inc (San Antonio, TX, USA). For the powder in capsule formulation, DMAU was micronized by Micron Technologies, Inc. (Malvern, PA, USA) and encapsulated as 25 or 200 mg DMAU powder and placebo in capsules (Pharmtek Laboratories, Inc., San Diego, CA, USA) in a cGMP environment. SRI International (Menlo Park, CA, USA) manufactured 100 mg DMAU in castor oil/benzyl benzoate (70 : 30 volume : volume) and 50 mg DMAU in SEDDS capsules and corresponding placebo under Good Manufacturing Practice standards.

Study design

All participants were assessed by a study physician to ensure that all inclusion and exclusion criteria were met. For all three formulations, at each dose level evaluated, 10 men received active drug and two received placebo in a double-blind fashion. For the castor oil and SEDDS formulations, 10 participants received 100, 200, and 400 mg DMAU (for) both fasting and after a high fat meal, and two men received identical placebo capsules. One subject randomized to the DMAU in SEDDS group had no measurable DMA levels on two pharmacokinetics sampling days and his data were not included in the study (despite approval for additional procedures by the UCLA-IRB, he declined re-dosing). For the DMAU powder in capsules, participants were administered 100, 200, and 400 mg DMAU or identical placebo with a high fat meal as previously described (Surampudi *et al.*, 2014).

All participants were admitted to the clinical research unit within the Clinical and Translational Science Institute at each site and were observed for 24 h with hourly vital signs monitoring following dosing. An electrocardiogram was performed 4–6 and 24 h after drug administration. Safety laboratory tests (clinical chemistry panel, liver function tests) were measured at baseline and 24 h after each DMAU dose. Fasting lipids and complete blood counts were quantified at baseline, and approximately, 7 days following each dose. Serum hormones [T, free T, estradiol, dihydrotestosterone (DHT), LH, FSH, and sex hormone-binding globulin (SHBG)], were measured before drug administration (time zero) and either every 4 (castor oil and SEDDS formulations) or 12 (powder formulation) h post-administration. In all cases, DMA and DMAU concentrations were quantified in the blood drawn -0.5 before, 0, and 0.5, 1, 2, 4, 6, 8, 12, 18, and 24 h after oral administration of DMAU. The participants returned to the clinic at least 7 days after the dose of

DMAU for safety laboratory tests, adverse event reporting, vital signs, and hormone evaluation. Halting parameters for predefined safety criteria and adverse events were included in the protocol, but these parameters were never reached.

Analytical methods

Safety laboratory tests and lipid panels were performed at each institution's respective clinical laboratory. All hormones were quantified by the licensed Endocrine and Metabolic Research Laboratory at Harbor-UCLA/LA Biomed using validated methods. Serum T, DHT, estradiol, DMAU, and DMA were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Shiraishi *et al.*, 2008; Rothman *et al.*, 2011; Surampudi *et al.*, 2014) and free T was calculated using a standard formula (Vermeulen *et al.*, 1999). Serum LH, FSH, and SHBG were measured using sensitive fluoroimmunometric assays as previously described (Swerdloff *et al.*, 2000).

Statistical analyses

The primary endpoints of the trial were safety and tolerability of the three formulations of DMAU. Secondary endpoints included the 24-h PK of DMAU and DMA after oral dosing fasting and with food, as well as suppression of gonadotropins and testosterone production.

The number of participants for this Phase 1 clinical study was powered to provide at least a 0.80 probability to exclude at least 20% of participants developing Grade 3 adverse events assuming a two-sided 95% confidence interval with each dose and formulation given. The PK parameters for each full sampling day for DMAU were determined by non-compartmental methods and were primarily assessed using the area under the curve from 0–24 h (AUC_{0-24}) of serum DMAU/DMA levels generated by the 10 blood sampling times over 24 h for each dose of DMAU and computed using the trapezoid method. Other PK parameters assessed included C_{avg} (average concentration over 24 h), C_{max} (maximum concentration over 24 h), C_{min} (minimum concentration over 24 h), and T_{max} (time to reach C_{max}). The elimination half-life, $T_{1/2}$, was calculated assuming exponential decay when there were at least three measurable concentrations after C_{max}.

As a dose effect is not anticipated for zero dose, the zero dose was removed from the analysis. Also, as we anticipated a marked effect of food, we planned separate analyses under the fed and fasting conditions a priori, assuming this was verified. Mixed models incorporated repeated measurements within subjects using a compound symmetrical covariance structure were constructed to examine the effect of dose (0, 200, and 400 mg), formulation (powder, SEDDS, castor oil), and the interaction on AUC, C_{avg}, C_{max}, T_{max}, and $T_{1/2}$ for serum DMA, serum DMAU and the ratio of DMA to DMAU. Post hoc Bonferroni-adjusted testing was performed only when a significant main effect was detected. All analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA), with two-tailed $p < 0.05$ construing statistical significance. Data are presented as mean ± SEM.

The effect of oral DMAU treatment on serum T, DHT, estradiol (E2), LH, FSH, and calculated free T was analyzed by mixed model analogously for C_{avg} and C_{min}. Models here were constructed separately using three levels of formulation (powder, SEDDS, castor oil) as well as two levels of formulation (SEDDS and castor oil), as serum concentrations for hormones were measured less frequently when the powder formulation was administered than when SEDDS and castor oil formulations were dosed. Analyses under both models yielded congruent findings, and hence, we present analyses that include all three formulations, unless otherwise indicated.

RESULTS

Research participants' demographics, disposition, and safety

There were a total of 44 participants between the two study sites, 19 for powder in capsule, 12 for castor oil, and 13 for SEDDS. A total of eight men discontinued during the dosing periods and were replaced to ensure that for all doses, 12 participants were evaluated for both fasting and fed; seven discontinued dosing during the evaluation of powder in capsule and one from SEDDS dosing. In all cases, discontinuation was because of scheduling or personal reasons and not because of adverse effects. Demographics for the participants in each phase of the study are shown in Table 1. Across all three groups, men had an average age of 33 years and BMI of 25.

All three formulations were well tolerated and there were no serious adverse events. One participant had an AST >twofold the upper limit of normal thought to be related to binge alcohol intake; this elevation resolved without treatment. There were no significant changes in chemistry or lipid panels and hematocrit was not significantly different between baseline and the end of the study. EKGs and QTc intervals in all participants were not significantly different from baseline and there were no clinically significant changes in vital signs in any of the participants (data not shown).

Food effects

There were marked food effects on DMAU and DMA pharmacokinetics in all three formulations. When DMAU was administered with a high fat meal (50% calories as fat), DMAU absorption and DMA serum concentrations were increased leading to a significantly higher AUC, C_{avg}, and C_{max} for all formulations compared to administration fasting (Fig. 1, $p < 0.001$ in all cases). Hence, the data were analyzed separately for the fasting and fed conditions.

Pharmacokinetics of DMA and DMAU when DMAU is given with food

DMA and DMAU showed higher AUC, C_{avg}, and C_{max} ($p \leq 0.001$ for each) and DMA/DMAU AUC ratio ($p = 0.044$) at the 400 mg dose, compared with the 200 mg dose (2). This dose effect was true for all three formulations. Comparing the three formulations, the powder in capsule resulted in higher AUC and C_{max} for DMAU than dosing in castor oil ($p = 0.007$ and $p = 0.029$, respectively). Despite these higher serum concentrations of DMAU, administration in powder did not increase serum DMA concentrations compared to the other two formulations. In fact, powder in capsule resulted in the lowest proportion of DMAU conversion to DMA (DMA/DMAU AUC ratio) compared with either of the other two formulations ($p < 0.02$ for powder in capsule compared to both castor oil and SEDDS, Table 2).

There was no dose effect on the time to maximum concentration, $T_{max,,}$ for either DMAU or DMA. However, T_{max} for both

Table 1 Characteristics of the participants (±standard error of the mean)

	Total (44)	Powder (19)	Castor oil (12)	SEDDS (13)
Race				
American Indian or Alaska Native	2 (4.5%)	2 (10.5%)	0	0
Asian	1 (2.3%)	0	0	1 (7.7%)
Native Hawaiian or other Pacific Islander	1 (2.3%)	1 (5.3%)	0	0
Black or African American	5 (11.4%)	2 (10.5%)	3 (25%)	0
White	33 (75%)	12 (63.2%)	9 (75%)	12 (92.3%)
Other	2 (4.5%)	2 (10.5%)	0	0
BMI (kg/m^2)	25.4 ± 0.5	25.9 ± 0.71	25.2 ± 1.1	24.9 ± 1.1
Age (years)	33.2 ± 1.4	31.7 ± 2.3	36.4 ± 2.6	32.4 ± 2.4
Screening T (ng/dL)	546 ± 29	546 ± 43	481 ± 63	610 ± 45

Figure 1 Serum DMA (upper panel) and DMAU (lower panel) concentrations after oral administration of DMAU in three formulations as a single dose 0, 100, 200, or 400 mg after fasting overnight (left panels) or a high fat meal (50% fat) (right panels). Note, *y*-axis is log scale. (Conversion DMA 1 ng/mL = 3.29 nmol/L and DMAU 1 ng/mL = 2.12 nmol/L).

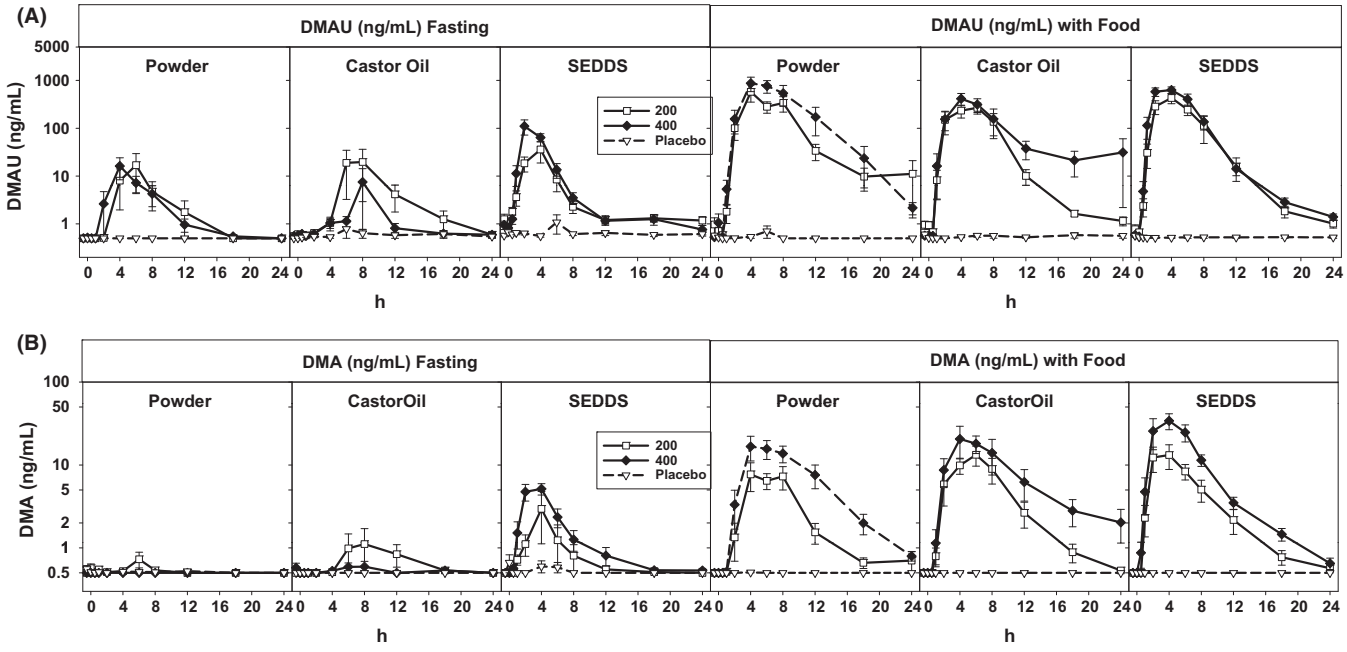


Table 2 Comparison of PK parameters after single oral dose of DMAU in three formulations (±standard error of the mean).

Fed DMAU	200 mg SEDDS (n = 10)	Castor oil (n = 10)	Powder in capsule (n = 10)	400 mg SEDDS (n = 9)	Castor oil (n = 10)	Powder in capsule (n = 10)
AUC (ng/mL/24 h)	2233 ± 340	1706 ± 247	3149 ± 470	3534 ± 289	2583 ± 400	6153 ± 1143
C_{avg} (ng/mL)	93.0 ± 14.1	71.1 ± 10.3	131.2 ± 19.6	147.3 ± 12.1	107.6 ± 16.7	256.4 ± 47.6
C_{max} (ng/mL)	599 ± 90	485 ± 59	854 ± 194	889 ± 50	665 ± 109	1410 ± 283
T_{max} (h)	3.8 ± 0.6	4.6 ± 0.7	5.8 ± 0.6	3.3 ± 0.6	6.4 ± 2.0	6.2 ± 1.1
HalfLife(h)	2.0 ± 0.63	1.94 ± 0.87	1.72 ± 0.57	2.13 ± 0.71	2.30 ± 0.76	1.83 ± 0.61
DMA						
	(n = 10)	(n = 10)	(n = 10)	(n = 9)	(n = 10)	(n = 10)
AUC (ng/mL/24 h)	96.0 ± 16.3	103.1 ± 16.3	66.7 ± 12.0	222.5 ± 28.1	187.6 ± 35.3	163.9 ± 23.2
C_{avg} (ng/mL)	4.0 ± 0.7	4.3 ± 0.7	2.8 ± 0.5	9.3 ± 1.2	7.8 ± 1.5	6.8 ± 1.0
C_{max} (ng/mL)	19.2 ± 4.7	19.2 ± 3.3	12.5 ± 2.8	47.9 ± 8.1	36.1 ± 8.2	27.7 ± 4.3
T_{max} (h)	3.8 ± 0.8	4.8 ± 0.5	5.8 ± 0.5	3.8 ± 0.5	5.6 ± 0.9	6.4 ± 1.0
HalfLife(h)	2.84 ± 1.0	2.68 ± 0.85	2.44 ± 0.92	3.23 ± 1.07	3.10 ± 1.03	3.31 ± 1.10
AUC ratio						
DMA/DMAU AUC	0.050 ± 0.009	0.063 ± 0.007	0.028 ± 0.006	0.112 ± 0.051	0.075 ± 0.008	0.031 ± 0.004

serum concentrations of DMA and DMAU were significantly delayed with powder in capsule compared with SEDDS ($p < 0.05$ for both DMAU and DMA). In contrast, there was an effect of dose on the elimination half-lives of DMA and DMAU ($p < 0.05$ for both, Table 2); importantly, however, the elimination half-lives for both DMA and DMAU were not significantly affected by formulation.

Pharmacodynamic effects of DMAU given with food

Significant dose effects were detected in C_{avg} and C_{min} for LH, T, free T, DHT, and E2 ($p < 0.01$ in all cases). Significant post hoc Bonferrroni-adjusted differences are illustrated in Fig. 2A–E, and consistently show that 400 mg was more suppressive than placebo, both in overall suppression (C_{avg}) and the minimum concentration achieved (C_{min}) (Table 2). A total quantity of 400 mg achieved a greater maximal suppression than 200 mg for steroids T, DHT, and E2: see Fig. 2C–E which shows the C_{min} for each dose and formulation. There were no significant effects of the formulation on LH or any of the sex steroids examined. In contrast, there were no significant dose effects on FSH C_{avg}, but there was a significant dose by drug interaction for FSH C_{min} ($p < 0.05$, Fig. 2B). Post hoc testing indicated that the 400 mg of SEDDS DMAU significantly suppressed FSH compared with SEDDS placebo ($p = 0.004$), but no other significant differences were detected.

Figure 2 Serum average (C_{avg}) and minimum (C_{min}) concentrations over 24 h of LH (A), FSH (B), T (C), free T (D), estradiol (E), and DHT (F) after administration of a single dose 0 (placebo), 200, 400, mg of DMAU with a high fat meal. For simplicity, the statistical differences between doses were marked by brackets only in the combined responses of gonadotropins or sex steroids of all three formulations (ALL).

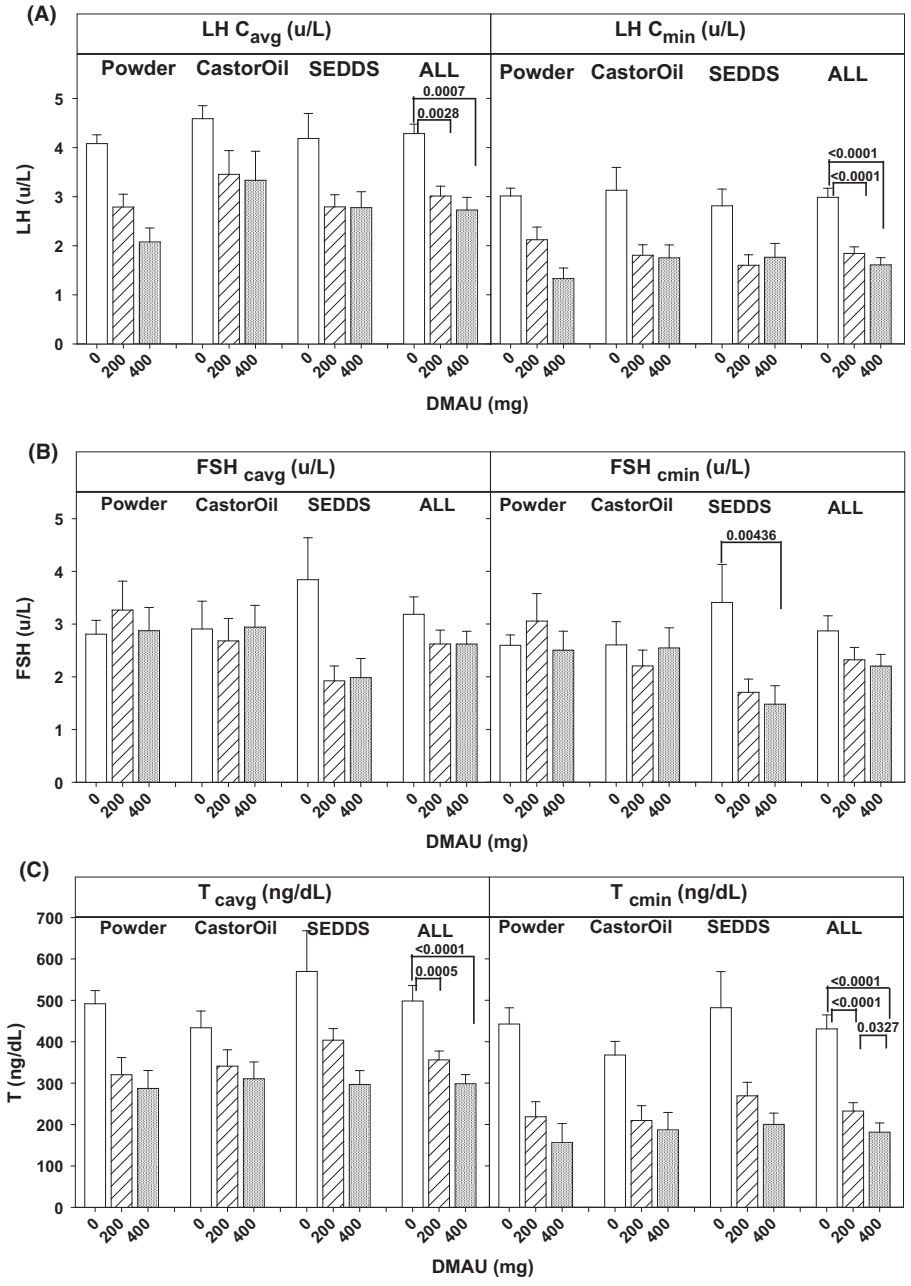
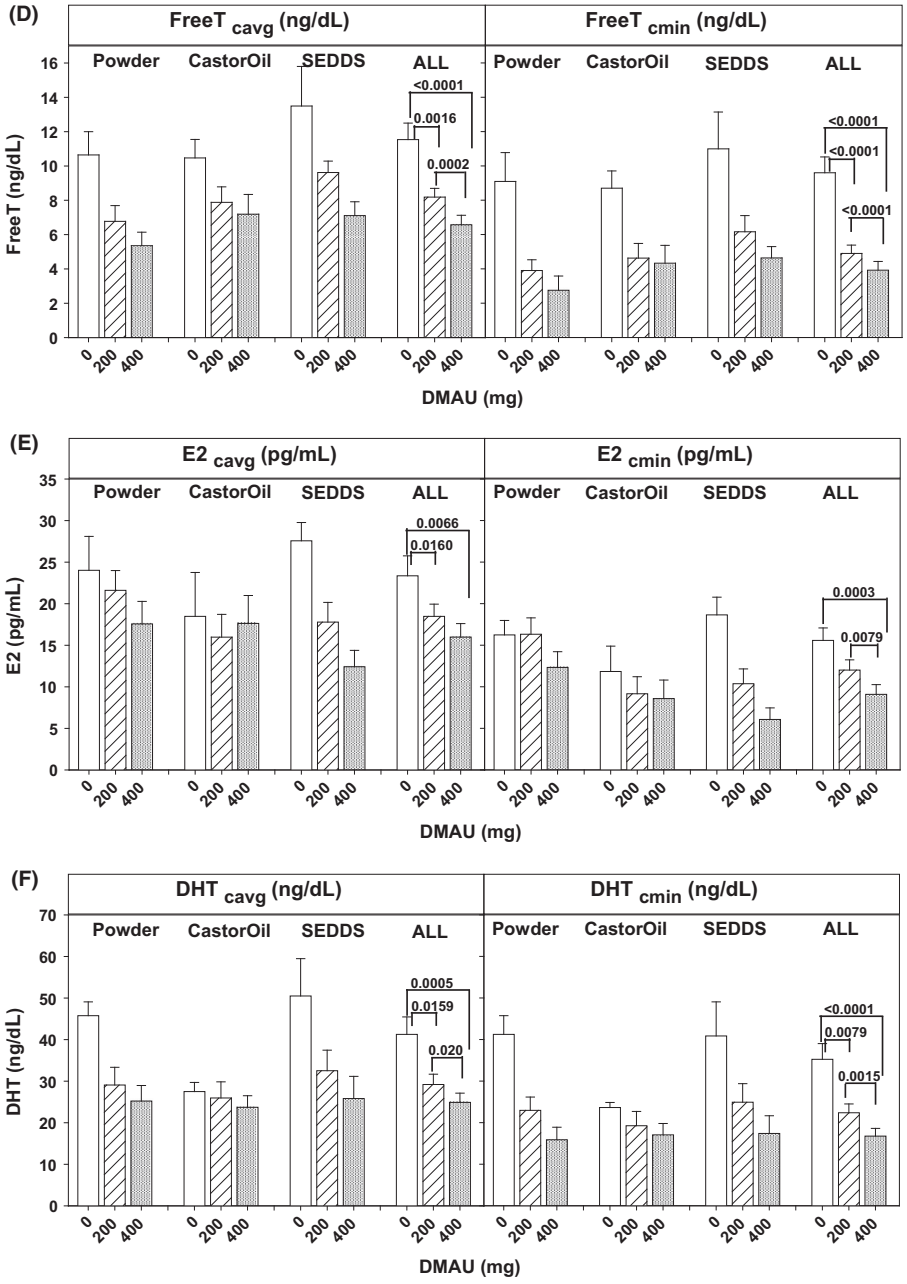


Figure 2 Continued



Pharmacokinetics and pharmacodynamics of DMAU administered when fasting

When administered fasting, oral DMAU in the SEDDS formulation resulted in higher blood concentrations of DMAU and DMAU than the other two formulations (Fig. 1). When the 400 mg dose was compared, these differences were significant, with SEDDS DMAU resulting in higher C_{avg} for both DMA and DMAU than the castor oil and powder in capsule formulations ($p < 0.005$ for each comparison for formulation by dose interaction, Bonferroni-adjusted post hoc $p < 0.03$ for each comparison).

As only negligible concentrations of DMA were achieved when DMAU was administered in powder or castor oil, their pharmacodynamics effects were not further evaluated. We investigated whether DMAU given fasting in SEDDS decreased serum LH, FSH, or T in a dose-dependent manner. When given fasting, DMAU in SEDDS resulted in a dose-dependent decrease in T C_{avg} ($p = 0.0005$) and C_{min} ($p = 0.015$), and FSH C_{min} ($p < 0.0001$) without significantly impacting LH (data not shown).

DISCUSSION

In this study, we compared the pharmacokinetics of DMAU and DMA after oral administration of single, escalating doses of three formulations of DMAU. These studies are consistent with our earlier observation that administration of DMAU with food, even when formulated in oil, markedly increases absorption (Surampudi *et al.*, 2014), even when formulated in oil, and result

in dose incremental increases in DMAU and DMA levels. Although administering DMAU in oil did not negate the significant enhancing effect of co-administration of food on DMAU absorption, oil-based formulations improved the conversion of DMAU to DMA compared to powder in capsule. Moreover, these newer formulations of DMAU dynamically suppress LH, T, and the downstream metabolites of T, DHT, and E2, when given with food. In contrast to the powder and castor oil formulations, under fasting conditions, administration of DMAU in SEDDS provided sufficient DMA to suppress T and FSH, even after a single dose, despite resulting in markedly lower DMAU and DMA concentrations than when given with food.

This study builds upon our previous work, further demonstrating that a single oral dose of DMAU of 200 or 400 mg with food significantly suppressed serum LH, T, free T, and its metabolites E_2 and DHT compared with placebo. The suppressive effect of all three formulations on sex steroids was dose dependent. The suppression of serum FSH was only evident when DMAU was given at the highest dose, 400 mg, with the SEDDS preparation, which also resulted in the highest DMA AUC (Fig 2B and Table 2). This is consistent with our prior observation that a single dose of DMAU as powder in capsule is a very effective suppressor of production of T, and suggests that oral DMAU may, when given repeatedly over time, be a potent suppressor of spermatogenesis (Surampudi *et al.*, 2014). The very rapid suppression of LH and endogenous T in response to oral DMAU may be because of the dual action of DMA on both the androgen and progesterone receptors (Attardi *et al.*, 2006). Combinations of androgens and progestins are more effective suppressors of spermatogenesis than T alone in male contraceptive clinical trials (Liu *et al.*, 2008).

We were somewhat surprised that DMAU had greater oral bioavailability when given as a powder than the other two formulations when given in the fed state (Fig. 1 and Table 2), achieving greater serum DMAU C_{avg} than the castor oil formulation. Testosterone and its short chain esters undergo rapid first-pass hepatic metabolism, limiting oral bioavailability (Tauber *et al.*, 1986), whereas testosterone undecanoate (TU) in castor oil has enhanced lipophilicity with absorption occurring via the intestinal lymphatics when TU is given with food (Shackleford *et al.*, 2003). Presumably, DMAU is also lymphatically absorbed; thus, we expected that oil emulsions would enhance absorption via this route, but this was not evident in this study. However, there was no effect of formulation on the AUC for DMA, the active metabolite of DMAU, because of the enhanced conversion of DMAU to DMA in vivo when given in either emulsified/oil formulation compared to powder (Table 2). How administration of DMAU in oil enhances de-esterification is not clear; however, given that only 5–10% of the DMAU is metabolized to DMA systemically, the improved DMA/DMAU ratio achieved when DMAU is given in oil is likely to be a significant advantage in multiple dose studies, allowing for markedly lower amounts of DMAU to be administered to achieve equivalent pharmacodynamic effects when DMAU is given in oil/SEDDS vs. powder. This hypothesis remains to be tested in repeat dose studies as we did not observe an effect of formulation in this single dose study on the degree or extent of LH or endogenous steroid production.

Although the effects were modest, we did observe a significant effect of formulation on DMA, LH, and endogenous steroid production when DMAU was administered fasting. In particular, the SEDDS formulation was superior to both powder in capsule and the castor oil formulation in achieving significant DMA and DMAU concentrations. SEDDS has also been shown to enhance the absorption of TU (Yin *et al.*, 2012). While the AUC for DMAU and DMA when DMAU is administered in SEDDS is still vastly lower than when given with food, by roughly an order of magnitude (Fig. 1), these low levels of serum DMA achieved when DMAU is given in SEDDS may be important in longer term, real use studies. As a potential contraceptive, the levels of DMA achieved with DMAU-SEDDS occasionally dosed without concomitant food, may be sufficient for maintaining gonadotropin suppression, and perhaps inhibition of spermatogenesis, in long-term daily users.

There were very few adverse events that were ascribed to DMAU. Importantly, as the studies presented here include only single doses, and involve multiple blood draws, androgenic effects such as stimulation of erythropoiesis, suppression of sex hormone-binding globulin, and potential reductions in high-density lipoprotein cholesterol concentrations could not be adequately assessed in this study. Longer term, repeat dose studies are required to further evaluate the safety of DMAU in men. To assess the effects of DMAU on spermatogenesis as well non-gonadal, androgen-sensitive organs including the prostate, bone, and muscle, longer term DMAU administration studies will be necessary. While DMAU has both androgenic and progestational activity in vitro and in pre-clinical rodent models, the long-term impact of LH and testosterone suppression on these hormonally sensitive tissues remains to be assessed and will be vital in developing DMAU as a male hormonal contraceptive.

In summary, single escalating doses of DMAU in three formulations: powder in capsule, castor oil, and SEDDS, up to 400 mg, were well tolerated in healthy male volunteers. When a single oral dose of DMAU was administered with a high fat meal, serum DMAU and DMA showed dose incremental increases sufficient to reversibly suppress LH and endogenous sex hormone production with all three formulations. Administration of DMAU in castor oil or SEDDS resulted in enhanced conversion of DMAU to DMA in vivo, which might be an advantage further development of these formulations over the powder in capsule. Furthermore, DMAU given in SEDDS was superior to the other two formulations when given in the fasting state, resulting in higher serum DMA concentrations sufficient for suppression of T and FSH. Further development of oral DMAU is ongoing with the goal of assessing its safety and efficacy in suppressing endogenous gonadotropins, sex steroids, and, in the long run, sperm production. These studies demonstrate that the formulation of DMAU in oil may have some advantages over powder in capsule; whether these observations hold true with multiple, repeat dosing remains to be evaluated. DMAU holds promise as a potential single agent, reversible, male hormonal contraceptive.

ACKNOWLEDGEMENTS

The Los Angeles Center was supported by the Eunice Kennedy Shriver National Institute of Child Health and Development (NICHD) Contraceptive Clinical Trial Network Contract HHSN27520130024I Task Orders HHSN 27500001, 27500002, 27500006; the Endocrinology, Metabolism and Nutrition training Grant (T32 DK007571), and the UCLA Clinical and Translational Science Institute (UL1TR000124) at Harbor-UCLA/LA BioMed.

The Seattle Center was supported by NICHD Contraceptive Clinical Trial Network Contract HHSN275201100075U Task Orders HHSN 27500001, and 27500002, the National Center For Advancing Translational Sciences of the National Institutes of Health under Award Number UL1TR000423, the Center for Research in Reproduction and Contraception U54 HD 04245 (NICHD), and the National Institute of Diabetes and Digestive and Kidney Diseases training grant 5T32 DK007247-35. We thank Peter Christenson, PhD, formerly at LA Biomed for his advice in study design and data analysis plans. We thank our research coordinators Xiao-Dan Han, Elizabeth Ruiz, Kathryn Torrez-Duncan for their assistance with the study and the technical assistance of Feng Bai and Andrew Leung for DMA and DMAU assay development and validation. Finally, we thank our research volunteers and the staff of the Endocrine and Metabolic Research Laboratory at Harbor-UCLA/LA Biomed and the University of Washington Center for Research in Reproduction and Contraception.

DISCLOSURES

CW received research funding from Clarus Therapeutics, Lipocine, and Besins Healthcare. She is a temporary consultant to TesoRx and Lipocine. JKA has received research funding from Clarus Therapeutics. RSS received research funding from Clarus Therapeutics, Lipocine, Antares, and Aeterna Zentaris, Inc. He has served as a temporary consultant for Clarus Therapeutics, Novartis, TesoRex, Antares, and Aeterna Zentaris, Inc.

AUTHORS' CONTRIBUTIONS

STP, RSS, JKA, DB, AC, WJB, and CW designed the research study, analyzed the data, and wrote the paper. RA, JHC, AL, LH conducted the research study and analyzed the samples and reviewed and revised the paper.

REFERENCES

Attardi BJ, Hild SA & Reel JR. (2006) Dimethandrolone undecanoate: a new potent orally active androgen with progestational activity. *Endocrinology* 147, 3016–3026.

Attardi BJ, Engbring JA, Gropp D & Hild SA. (2011a) Development of dimethandrolone 17beta-undecanoate (DMAU) as an oral male hormonal contraceptive: induction of infertility and recovery of fertility in adult male rabbits. *J Androl* 32, 530–540.

Attardi BJ, Marck BT, Matsumoto AM, Koduri S & Hild SA. (2011b) Long-term effects of dimethandrolone 17beta-undecanoate and 11beta-methyl-19-nortestosterone 17beta-dodecylcarbonate on body composition, bone mineral density, serum gonadotropins, and androgenic/anabolic activity in castrated male rats. *J Androl* 32, 183–192.

Cook CE & Kepler JA. (2005) 7alpha,11beta-dimethyl-19-nortestosterone: a potent and selective androgen response modulator with prostate-sparing properties. *Bioorg Med Chem Lett* 15, 1213–1216.

Glasier A. (2010) Acceptability of contraception for men: a review. *Contraception* 82, 453–456.

Gu Y, Liang X, Wu W, Liu M, Song S, Cheng L, Bo L, Xiong C, Wang X, Liu X, Peng L & Yao K. (2009) Multicenter contraceptive efficacy trial of injectable testosterone undecanoate in Chinese men. *J Clin Endocrinol Metab* 94, 1910–1915.

Hild SA, Attardi BJ, Koduri S, Till BA & Reel JR. (2010) Effects of synthetic androgens on liver function using the rabbit as a model. *J Androl* 31, 472–481.

Liu PY, Swerdloff RS, Christenson PD, Handelsman DJ, Wang C & Hormonal Male Contraception Summit, G. (2006) Rate, extent, and modifiers of spermatogenic recovery after hormonal male contraception: an integrated analysis. *Lancet* 367, 1412–1420.

Liu PY, Swerdloff RS, Anawalt BD, Anderson RA, Bremner WJ, Elliesen J, Gu YQ, Kersemaekers WM, McLachlan RI, Meriggiola MC, Nieschlag E, Sitruk-Ware R, Vogelsong K, Wang XH, Wu FC, Zitzmann M, Handelsman DJ & Wang C. (2008) Determinants of the rate and extent of spermatogenic suppression during hormonal male contraception: an integrated analysis. *J Clin Endocrinol Metab* 93, 1774–1783.

Meriggiola MC & Bremner WJ. (1997) Progestin-androgen combination regimens for male contraception. *J Androl* 18, 240–244.

Nieschlag E. (2010) Clinical trials in male hormonal contraception. *Contraception* 82, 457–470.

Page ST, Amory JK & Bremner WJ. (2008) Advances in male contraception. *Endocr Rev* 29, 465–493.

Piotrowska K, Wang C, Swerdloff RS & Liu PY (2016) Male hormonal contraception: hope and promise. Lancet Diabetes Endocrinol 16, 34–36. *Epub* February 22, 2016.

Rothman MS, Carlson NE, Xu M, Wang C, Swerdloff R, Lee P, Goh VH, Ridgway EC & Wierman ME. (2011) Reexamination of testosterone, dihydrotestosterone, estradiol and estrone levels across the menstrual cycle and in postmenopausal women measured by liquid chromatography-tandem mass spectrometry. *Steroids* 76, 177–182.

Shackleford DM, Faassen WA, Houwing N, Lass H, Edwards GA, Porter CJ & Charman WN. (2003) Contribution of lymphatically transported testosterone undecanoate to the systemic exposure of testosterone after oral administration of two andriol formulations in conscious lymph duct-cannulated dogs. *J Pharmacol Exp Ther* 306, 925–933.

Shiraishi S, Lee PW, Leung A, Goh VH, Swerdloff RS & Wang C. (2008) Simultaneous measurement of serum testosterone and dihydrotestosterone by liquid chromatography-tandem mass spectrometry. *Clin Chem* 54, 1855–1863.

Surampudi P, Page ST, Swerdloff RS, Nya-Ngatchou JJ, Liu PY, Amory JK, Leung A, Hull L, Blithe DL, Woo J, Bremner WJ & Wang C. (2014) Single, escalating dose pharmacokinetics, safety and food effects of a new oral androgen dimethandrolone undecanoate in man: a prototype oral male hormonal contraceptive. *Andrology* 2, 579–587.

Swerdloff RS, Wang C, Cunningham G, Dobs A, Iranmanesh A, Matsumoto AM, Snyder PJ, Weber T, Longstreth J & Berman N. (2000) Long-term pharmacokinetics of transdermal testosterone gel in hypogonadal men. *J Clin Endocrinol Metab* 85, 4500–4510.

Tauber U, Schroder K, Dusterberg B & Matthes H. (1986) Absolute bioavailability of testosterone after oral administration of testosterone-undecanoate and testosterone. *Eur J Drug Metab Pharmacokinet* 11, 145.

Turner L, Conway AJ, Jimenez M, Liu PY, Forbes E, McLachlan RI & Handelsman DJ. (2003) Contraceptive efficacy of a depot progestin and androgen combination in men. *J Clin Endocrinol Metab* 88, 4659–4667.

Vermeulen A, Verdonck L & Kaufman JM. (1999) A critical evaluation of simple methods for the estimation of free testosterone in serum. *J Clin Endocrinol Metab* 84, 3666–3672.

Wang C & Swerdloff RS. (2010) Hormonal approaches to male contraception. *Curr Opin Urol* 20, 520–524.

Wang C, Festin MP & Swerdloff RS. (2016) Male hormonal contraception: where are we now? *Curr Obstet Gynecol Rep* 5, 38–47.

World Health Organization Task Force on Methods for the Regulation of Male Fertility. (1990) Contraceptive efficacy of testosterone-induced azoospermia in normal men. World health organization task force on methods for the regulation of male fertility. *Lancet* 336, 955–959.

World Health Organization Task Force on the Regulation of Male Fertility. (1996) Contraceptive efficacy of testosterone-induced azoospermia and oligozoospermia in normal men. *Fertil Steril* 65, 821–829.

Yin AY, Htun M, Swerdloff RS, Diaz-Arjonilla M, Dudley RE, Faulkner S, Bross R, Leung A, Baravarian S, Hull L, Longstreth JA, Kulback S, Flippo G & Wang C. (2012) Reexamination of pharmacokinetics of oral testosterone undecanoate in hypogonadal men with a new self-emulsifying formulation. *J Androl* 33, 190–201.

Khilwani et al. Basic and Clinical Andrology (2020) 30:2
https://doi.org/10.1186/s12610-020-0099-1

Basic and Clinical Andrology

REVIEW ARTICLE Open Access

RISUG® as a male contraceptive: journey from bench to bedside

Barkha Khilwani, Ayesha Badar, Abdul S. Ansari and Nirmal K. Lohiya*

Abstract

Even after decades of research men still lack reliable and reversible contraceptive methods comparable to female methods of contraception. Traditional methods of male contraception present a high failure rate and also involve high risk both when used for contraception and for protection against sexually transmitted diseases. Various chemical, hormonal, immunological, vas based and herbal methods of contraception have been examined by scientists world over during the past four decades. Among the possible lead approaches, exogenous hormonal contraception, either alone or in combination with progesterone or antiandrogen, is being viewed at low profile because of their insufficiency in inducing uniform suppression of spermatogenesis and steroid related long term complications. As an alternative to vasectomy, among various intravasal devices being examined, RISUG® (Reversible Inhibition of Sperm Under Guidance), a co-polymer of styrene and maleic anhydride offers long term contraception with safety, efficacy and it can be delivered by no-scalpel injection. Thus it is the only male contraceptive procedure currently under Phase- III Clinical Trial. The non-invasive reversal technique, successfully demonstrated in langur monkeys and functional reversal achieved with dimethyl sulphoxide (DMSO) and sodium bicarbonate ($NaHCO_3$) in rats and rabbits with safety at F_1 generation (first filial generation) have projected RISUG® as a better alternative to vasectomy. In this narrative review we revisit the long journey of RISUG® beginning with formulation on a bench towards reaching the market as a safe and effective contraceptive method, discussing various milestones and roadblocks of this expedition awaiting the mandatory regulatory clearance from the Government of India. Successful completion of ongoing phase III clinical trials with demonstration of reversal in human volunteers will give an indigenously developed male contraceptive to the world.

Keywords: Male contraception, RISUG®, Clinical trials, Azoospermia, Reversibility

Résumé

Malgré plusieurs décennies de recherche, il manque toujours pour les hommes des méthodes de contraception fiables et réversibles qui soient comparables aux méthodes de contraception féminine. Les méthodes de contraception masculine traditionnelles ont un taux d'échec élevé ; elles sont aussi à risque lors d'utilisations à la fois comme contraceptif et comme protection contre les infections sexuellement transmissibles. Au cours des 40 dernières années, le monde scientifique a évalué différentes méthodes de contraception masculine basées sur des approches chimiques, hormonales, immunologiques, déférentielles ou à base de plante.
Parmi les pistes possibles d'approche, la contraception par apport d'hormone exogène, soit seule soit associée à un progestatif ou à un anti androgène, est actuellement perçue comme ayant un faible profil en raison de son incapacité à induire une suppression uniforme de la spermatogenèse et des complications à long termes des
(Continued on next page)

* Correspondence: lohiyank@gmail.com
Centre for Advanced Studies, Department of Zoology, University of Rajasthan, Jaipur 302004, India

(Continued from previous page)
stéroïdes.
Parmi les alternatives à la vasectomie, plusieurs dispositifs intra déférentiels ont été évalués dont le RISUG® (Reversible Inhibition of Sperm Under Guidance). Ce dernier est un copolymère de styrène et d'anhydride maléique qui offre une contraception de longue durée avec innocuité et efficacité, pouvant être mise en place par injection sans scalpel. C'est ainsi actuellement la seule procédure contraceptive masculine pour laquelle un essai clinique de Phase-III est en cours. La technique de réversibilité sans scalpel, démontrée avec succès chez le singe *Langur*, ainsi que la réversibilité fonctionnelle par le sulfoxyde de diméthyle (DMSO) et le bicarbonate de sodium ($NaHCO_3$) confirmée chez le rat et le lapin, sans atteintes sur la génération F1 (première génération de petits), ont fait apparaître le RISUG® comme une meilleure alternative à la vasectomie.
Dans cette revue narrative, nous réévaluons le long chemin du RISUG® depuis une formulation de paillasse de laboratoire jusqu'à sa mise sur le marché comme méthode de contraception sans risque et efficace, en discutant les différents jalons et obstacles rencontrés au cours de cette expédition dans l'attente de l'autorisation réglementaire obligatoire du Gouvernement Indien. Le succès des essais de Phase-III en cours par la preuve d'une réversibilité chez des hommes volontaires apportera au Monde une méthode de contraception développée localement.

Mots-clés: Contraception masculine, RISUG®, Essais cliniques, Azoospermie, Réversibilité

Family planning: the way forward

Family planning is crucial for the achievement of sustainable development goals and subsequent efforts need to be made to improve access and strengthen quality of family planning services. Research shows that adequate attention to family planning in countries with high birth rates can not only reduce poverty and hunger but can also avert maternal and childhood deaths [1].

In 2012, the global community at the London Summit on Family Planning formulated a global partnership 'Family Planning 2020' (FP2020), with an aim to add 120 million women and girls to the category having access to effective and safe family planning methods and services by the year 2020. Towards achieving the FP2020 goal national governments, civil societies and the private sectors joined hands to address the barriers that affect access and use of contraceptives. According to FP2020, modern methods of contraception can prevent a large number of unintended pregnancies, unsafe abortions and maternal deaths [2]. In the FP2020 focus countries modern contraceptive prevalence rate was estimated to be 45.7% in 2017 with 21.6% unmet need for modern contraceptive methods. Usage of modern contraceptive methods by women globally, increased by 28.8 million between 2012 and 2017. Modern contraceptives are the most relied method in Western Europe with 95.5% population using condoms, hormonal contraceptives or sterilization and only 4.5% users rely on traditional methods. In Eastern and Central Europe users of modern methods of contraception is 77.5% with rest of the population (22.5%) still following traditional methods of contraception [3]. India's contraception prevalence rate among all women was 39.2 in the year 2012, 39.57 in 2017 and is predicted to rise to 40.87 by the year 2020 [2]. About three-fourths of these were using female sterilization, which is by far the most prevalent birth-control method in India. However, role of male partner in family planning has been highly limited, specifically in developing nations like India.

Traditional methods of male contraception have long included periodic abstinence, non-vaginal ejaculation, condoms and vasectomy [4]. The lack of modern methods of contraception for men does not, however, explain the low prevalence of male sterilization, given that vasectomy is more effective, less expensive to perform and has fewer complications than female sterilization [1, 5]. However, for men to share more equally the burdens as well as the benefits of family planning, more effective reversible male contraceptive methods need to be available. The resultant diminished male role may have inadvertently undermined the many societal efforts at birth control. Many men, young and old, still perceive contraception as primarily a woman's responsibility, for after all, she suffers most directly from contraceptive failure; this attitude is unfortunate [6]. Since decisions about pregnancy affect both partners, both should share the contraceptive burden equitably. Limited choices and access to methods, attitudes of men towards family planning, perceived fear of side-effects, poor quality of available services, cultural or religious oppositions and gender-based barriers are some of the reasons for lesser participation of men in family planning.

Methods of contraception for male with limitations

In 1950s and 1960s males were overlooked by family planners even after being an integral part of the family unit [7]. Drug companies were reluctant to invest in developing contraceptives for male consumers. There were various misconceptions and misbelieves regarding side-

effects like loss of libido and so called "manhood". Further, there were unproven assumptions regarding male attitudes in sharing responsibility of family planning [8]. Development of male contraceptive thus lagged behind due to both societal and technological stereotypes. Considering the dismal past of male contraceptive research, in 1960 R. J. Ericsson, an early pioneer in male reproductive research, quoted it as "almost an illegitimate specialty within reproductive biology" [9]. Present available contraceptive methods for male have been listed below with their advantages and disadvantages (Table 1).

- **Male condoms**: Condoms are made from very thin latex (rubber), polyisoprene or polyurethane. Condoms are being practiced by people worldwide for contraception and prevention of sexually transmitted diseases as they are cheap and easily available [11]. They are associated with infidelity, reduce the spontaneity and sensitivity of sexuality, present problem of storage and disposal and have high failure rate (3–15%). Condom failure due to condom breakage, slippage, incorrect use and latex allergies also occur [12, 26].
- **Coitus interruptus (Withdrawal):** Coitus interruptus is the practice of ending sexual intercourse before ejaculation. The main risk for coitus interruptus is related to perform correctly or in a timely manner. Disadvantages of this method include the fact that it requires high motivation and is highly frustrating to some couples. Another disadvantage is that any sperm deposited before withdrawal, or left on the vulva wall during withdrawal, could reach the cervix. These factors account for the high failure rate of coitus interruptus [10].
- **Hormonal approaches:** The hormonal approach is based on the reversible suppression of gonadotropins leading to reversible suppression of the spermatogenetic process. Over the last decades studies have been performed to evaluate the level of acceptability of possible hormonal methods for male contraception. Medroxyprogesterone acetate is a hormonal medication of the progestin type that is shown to prevent spermatogenesis in combination with the topical application of testosterone gel [13]. Testosterone enanthate in clinical trials showed good efficacy with few drawbacks [14]. Most of the hormonal approaches have reached to clinical trials, but none of them has been approved for acceptability in public use. Major drawbacks in use of male hormonal contraceptive regimens are side effects like proatherogenic or antiatherogenic action,

Table 1 Methods of male contraception

Method	Advantages	Disadvantages	References
Abstinence	No side effects. No cost.	Difficult to abstain for long duration.	[4]
Withdrawal	No Cost.	High risk of pregnancy if not withdrawn at time. Pregnancy may occur by pre-ejaculate.	[10]
Male condoms	Easy availability. Helps in prevention of STIs.	Decrease spontaneity. May break during use. High failure rate.	[11, 12]
Hormonal approaches	Non-surgical procedure.	Lack of uniform efficacy, Complex formulations, Impractical systemic delivery system, Poor availability, High cost	[13–16]
Immuno-contraceptives	Target specific effect. Long-term efficacy. No surgical interventions.	Still under research phase.	[17, 18]
Non-injectable Plugs	No-scalpel method. Size available according to vas, thus avoids vas rupture.	Lower efficacy. Delayed azoospermia Reversal – less assured	[19, 20]
Vasectomy	Safe and effective. Risk involved in surgical intervention.	Microsurgical skills required. Antisperm antibody development. Reversal is expensive and partially successful.	[5, 21]
Non-Scalpel Vasectomy	No surgical procedure. Easy technique. High efficiency.	Reversal is expensive and partially successful.	[22, 23]
RISUG®	Easy approach. Single intervention. Early contraception Minimal systemic interference. No undue side effects. Better scope for reversal.	No protection against Sexually Transmitted Diseases (STDs).	[24, 25]

association with insulin resistance, hematopoetic action, etc. World Health Organization (WHO) conducted trials with men who receive twice weekly injections of testosterone. As noted earlier, this has the effect of suppressing sperm production by decreasing levels of gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Good results were obtained, but they found that the frequent injections posed a commercial and psychological barrier. More troubling, high levels of circulating testosterone led to increased irritability, acne and reduced levels of good cholesterol in many test subjects [15]. A recent review on male contraception highlighted that delay in development of male hormonal contraception is multifactorial. However, willingness to use a new male method is driving new clinical trials closer than ever to bringing viable products to market [16]. Thus, no hormonal regimes have yet been approved for contraceptive use and focus has been shifted from hormonal to non-hormonal strategies.

- **Immunocontraceptives:** Immunocontraception involves the administration of vaccine that induces an adaptive immune response which causes an animal to become infertile. The method promises for high target specificity, long term action but not permanent, relatively inexpensive, lack of endocrine or metabolic side effects, easy to use without surgical intervention. The immune system is employed as a contraceptive by targeting sperm- or egg-specific proteins, or even gonadotropins because antisperm antibodies can play a role in infertility. Few examples of target proteins for immunocontraception are SPAM1, MDC, SP-10, FA-1, SP-17, NZ-1, NZ-2, LDH-C, SAGA-1, hESP, rSMP-B, SAMP-32, 80 kDa HSA, BS-17, EP-20, DE Protein, SFP2, AKAP, TSA-1, YLP-12 and Izumo [17, 18]. The development of immunocontraceptives is still at the research stage. The future of contraceptive vaccines holds great promise in terms of comfort, price, efficacy, complications, and possibly non-selective action in animal populations as well as in humans.
- **Non-injectable plugs:** Silicone plugs, called the Shug is composed of two silicone plugs with nylon tails to help anchor the plugs to the vas. Double plugs could be more reliable than the single one. The Shug can be inserted into the vas by the no-scalpel method and removed by minor surgery. In monkeys, fertility was returned after seven months of Shug use [19]. Clinical trials reported in men with 97% reduction in sperm motility. The Shug has several advantages: the size of the plug could be controlled according to the size of the vas deferens, thus avoiding the possible rupture of the vas; the anchoring mechanism can prevent the migration of the plug along the length of the vas. The preformed plug also avoided the possibility of entry of toxic substances during the hardening processes as in the case of injectable silicone rubber [20].
- **Vasectomy/Male sterilization:** Vasectomy is a safe and effective mode of permanent male contraception used by 42–60 million men worldwide. Vasectomy is safe with no mortality, effective, simple, convenient, requires only 10–15 min and inexpensive compared with female sterilization, which relatively costly and risky. Minor side effects are bruising, scrotal swelling, acute pain, hemorrhage, haematoma and surgical infection. But there are many reasons for its low acceptance. There is a possibility of prostate cancer after 20 years of vasectomy, due to enhanced dihydrotestosterone levels [27]. Issue of reversal on desire which requires skilled microsurgery and is less assured due to sperm antibodies development. It provides no protection against STD, and reversal is expensive with only partial success [22, 28].

Conventional vasectomy

Involves bilateral scrotal incisions through which the vas deferens is mobilized and transacted. This approach is effective but difficult to be reversed [29]. **No-scalpel vasectomy (NSV)** uses a unique puncture technique that reduces trauma to the scrotum and vas deferens (Fig. 1). The urologist uses a special clamp to puncture the scrotal skin, retrieve the vas and separate it from the surrounding structures in the scrotal sac without cutting the nerves or blood vessels near the scrotum. NSV is associated with no incision, no stitches, faster procedure, faster recovery, less chance of bleeding, less discomfort and high efficiency, which have helped the technique to increase the acceptability of male sterilization in many parts of the world [22, 23].

Vasovasostomy is a form of microsurgery first performed by the Australian Surgeon, Dr. Earl Owen in 1971 [5]. Pregnancy occurs in approximately 50% of couples after vasovasostomy and 30% of couples after vasoepididymostomy within 1 year of vasectomy reversal. Approximately 50 - 80% of vasectomies men develop antisperm antibodies [30]. A scar tissue develops at the site of reversal causing a blockage in 5–10% of vasovasostomies [31]. The vasectomy reversal will probably fail if an epididymal blowout has occurred at the time of vasectomy reversal surgery. The epididymis is adversely affected by elevated pressure due to long time vas deferens blockage that results in poor sperm motility [32]. NSV procedure requires surgical skills, handling of special instruments and manual skills. There are also

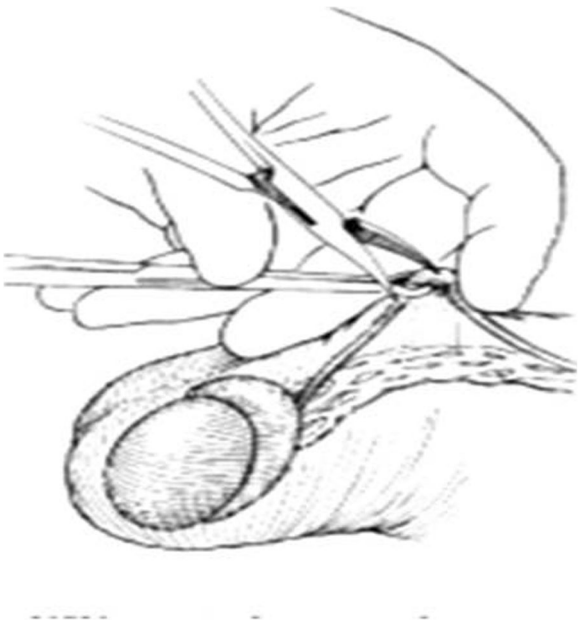


Fig. 1 Non-scalpel vasectomy approach. The No Scalpel Ring Clamp isolates and secures the vas deferens without penetrating the skin. The No Scalpel Dissecting Forceps pierces the scrotal sac to expose the vas deferens. The vas deferens is lifted out of the scrotum with the No Scalpel Dissecting Forceps and occluded

physiological effects of vasectomy on male reproductive system that makes vasectomy a potentially permanent method of contraception [21].

Currently, there are several promising traditional, herbal, hormonal, non-hormonal, immune based and vas based contraceptives at various stages of research and development. There is clearly a desire and need for more contraceptive options [33]. Couples desire more choices for fertility control, and unplanned pregnancies continue to occur at alarming rates. Through further research, advocacy and support, male contraceptives are likely to become a valuable addition to the current choices of family planning. The shortcomings of currently few available male contraceptive methods are a major barrier to the involvement of men in family planning. Current research into male contraceptives will potentially increase the equitability of family planning between males and females.

Birth of RISUG® (reversible inhibition of sperm under guidance)

The limitations of available male methods in controlling birth rate raise the idea to develop a new method which overcome the constraints and provide an ideal method to use. Prof. Sujoy K. Guha, School of Medical Science and Technology, Indian Institute of Technology, Kharagpur, India known for his innovative techniques worked on developing a method for stimulating the flow of blood through the human body. Later, his idea was utilized by ship manufacturers to design a pump known today as magneto-hydrodynamic propulsion units. In the 1970s while investigating some cost effective techniques to purify rural water systems, he discovered that when pipes were coated with a common polymer called styrene maleic anhydride (SMA), it could kill bacteria present in the water supply. In concert with Government of India, Prof. Guha worried regarding rapidly growing population of the country and suggested use of SMA to be developed as a male contraceptive [34, 35].

The proposed design was modified to work safely inside male genitalia and then considering, vas deferens similar to a water pipe and sperm travelling through the narrow tubes analogous to microbes, reproductive tract of male rats were injected with SMA. Positive results, indicated by sterility in rats, were observed and published in 1979 [34]. Later on, the procedure was further refined and also tested in rhesus and langur monkeys.

Journey begins as male contraceptive

In 1979, Prof. Guha proposed a radically new technique of male contraception, styrene maleic anhydride (SMA), a co-polymer dissolved in dimethyl sulphoxide (DMSO) was injected into vas deferens in rats. RISUG® (Reversible Inhibition of Sperm Under Guidance), a co-polymer of SMA dissolved in DMSO, was developed as a new perspective in non-hormonal male contraception methods [24]. RISUG® was formulated as an occlusive polymer which was claimed to sterilize subjects by single injection and reversed at any time following vas occlusion. Within 72 h of injection, RISUG® forms electrically charged precipitates in the lumen and further layers the lumen wall and inner walls of vas deferens. Precipitates are dominated with positive charge creating an acidic environment. Passing through the RISUG® injected vas deferens, sperms suffer ionic and pH stress, causing acrosomal damage, rendering them unable to fertilize oocytes. Afterwards it was demonstrated that SMA polymer injected into vas deferens of rhesus monkey could occlude the vas deferens lumen and also inhibits the fertilizing ability of spermatozoa by virtue of the pH lowering effect. Preclinical toxicity evaluated in rodents (Charles Foster rats) showed safety of the compound [36]. The polymer was proposed to be injected into the vas deferens through the non scalpel procedure thus avoiding surgery in the initial sterilization procedure. After being introduced in 1980 successful pre-clinical efficacy and safety studies on various species of animals including primates, RISUG® has also been tested successfully in number of human volunteers during Phase-I, Phase-II and Phase-III clinical trials. Presently the drug is under extended Phase-III clinical trials at various centers in India.

Composition

RISUG® is synthesized by dissolving 60 mg SMA in 120 μL DMSO. The DMSO is strongly alkaline and hygroscopic, minimal concentrations reported with no cytotoxicity. DMSO was chosen as solvent vehicle as it helps the penetration of polymer into the folds of inner wall of the vas deferens and its retention [24]. A part of SMA is converted into styrene maleic acid, which neutralizes the alkaline pH of DMSO. This action reduces the reactivity of DMSO. However, because of the sulphur moiety, DMSO is highly reactive. When SMA is mixed with this particular form of DMSO, the sulphur moiety of DMSO interacts with the etheric oxygen (–O-) of maleic anhydride moiety of SMA thereby leading to the formation of an intermediate unstable complex of SMA and DMSO. The carbonyl oxygen of SMA being resonance stabilized is not affected.

Mode of action

The complex of SMA and DMSO was suggested to act through vas occlusion, pH lowering and charge disturbance effect [25]. When RISUG® is injected into the vas deferens, it comes under the influence of the proteins in the spermatic fluid of the vas deferens. The polar amino acids react with the SMA - DMSO complex. Due to the formation of SMA - DMSO complex, there is a chemical instability which enables the polar amino acids to detach DMSO from SMA, while retaining the broken bond and polyelectrolyte nature of SMA. Thus, the COOH of maleic anhydride exists as COO^- and H^+. The reactions with the proteins take 48 h to complete. During this period, the DMSO helps in the entry of SMA into the folds of the vas deferens inner wall which promotes anchorage and retention of the contraceptive (Fig. 2). When the reaction is complete all the DMSO is detached and gets absorbed into the surrounding tissue and the blood stream for ultimate secretion [24]. The place of DMSO is then taken over by the proteins of the spermatic fluid with the polar amino acids of the proteins linked to the SMA and sustaining the polyelectrolyte nature induced into the SMA. The negative charge of COO^- ions and the positive charge of H^+ ions are maintained in a bound state. The proteins form a layer around SMA. An electrical charge double layer formation occurs with the proteins covering the SMA. The amino acids of proteins are zwitter ions having both positive and negative charged groups. In SMA, COO^- ions are structurally larger than the H^+ ions, but are less active. The more active H^+ ions tied up with the negative charged groups of amino acids, thus rendering them less effective in giving an external charge. Hence, the positive charges of the amino acids are left to give an external influence.

The protein - SMA complex has a positive charged surface which can influence the sperms. Also the protein layer over the SMA gives a protection to SMA from dissolution. This phenomenon gives the long-term contraception in the vas deferens. The hydrolyzed RISUG® in the vas deferens is claimed to have a pH of 4.0–4.5 which is likely to lower the motility, but would it completely immotile the sperms is an important question. The sperm damages due to RISUG® are very much similar to that of the damages caused by oxidative stress. The generation of excess of intracellular or extracellular reactive oxygen species (ROS) such as, O_2^-, H_2O_2, $ROO^•$, $OH^•$ are associated with many cell damages, including morphological defects, DNA fragmentation, lipid peroxidation, decrease in acrosome reaction and

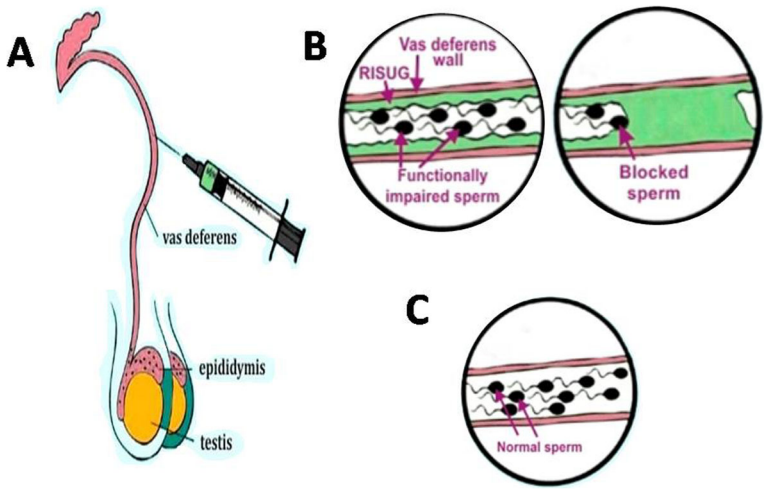


Fig. 2 RISUG: mode of action. (**A**) Vas is exposed from inguinal region and RISUG is injected in both vas deferens towards distal region by a micro-syringe. (**B**) RISUG coats the wall of the vas deferens blocking sperm movement. (**C**) Complete reversal obtained after DMSO/$NAHCO_3$ is injected bilaterally, flushing component of RISUG [37]

fusiogenic ability and impaired fertilization [38–41]. The concept of implantation of the SMA in the rat vas deferens have been confirmed by transmission electron microscopic (TEM) examination and fluorescence microscopy of vas fluid and prostate tissue [42]. In extended Phase III clinical trial, 60 mg styrene maleic anhydride dissolved in 120 μL of DMSO (1: 2) induced azoospermia in 84% of the subjects with presence of occasional abnormal sperm along with low neutral α-glucosidase activity was indicating 'partial' and not 'complete' vas occlusion. However, no further study was published to support this statement [43].

Preclinical trials

Various animal models have been used for attaining contraception with RISUG®, through vas occlusion, before initiation of clinical trials, for the safety evaluation with contraceptive effects.

- **Rat:** In an early study, co-polymer of styrene and maleic anhydride was dissolved in DMSO and injected into the vas deferens of rats. Histological observations indicated that the polymer was retained in the vas deferens and the morphological changes detected were confined to the mucosa. When the polymer was removed by flushing DMSO, the mucosal structure became normal within 2 weeks [44]. Later, further studies were carried out in rats related to its reversibility aspects.
- **Rabbit:** For the first time, SMA was evaluated in male rabbits as a contraceptive by Sethi et al. and the results showed no teratogenic potential at the doses of 1.25 mg, 2.5 mg and 5.0 mg used in the experiment [45].
- **Rhesus monkey:** SMA, was injected in to the vas deferens of male rhesus monkeys for safety evaluation at the dose of 100 mg (contraceptive dose, CD), 250 mg (CD × 2.5) and 500 mg (CD × 5.0). The observed behavioural, haematological, biochemical and histopathological parameters in treated monkeys were comparable to controls. The results suggested the polymer SMA to be safe up to 5 times CD in monkeys [46]. Similarly, another study in rhesus monkey showed that the polymer has the dual feature that it can occlude the vas deferens lumen and also can inhibit the fertilising ability of spermatozoa by virtue of the pH lowering effect. Matings with females were carried out when the lumen was completely occluded giving azoospermia as well as with partial block and spermatozoa present in the semen. All matings were infertile. Data up to 1 year were presented and indicated that the contraceptive effects last for a considerably long period [38]. Later, Guha et al. presented alterations in sperm plasma membrane, mitochondria as well as in the sperm structural components through histological data of monkey, providing a means of causing changes in the sperm that inhibit the fertilizing ability. Therefore, achieving non-obstructive vas-based contraception, without genotoxic or teratogenic effects caused by infertile sperm passing into the semen, is feasible [47].
- **Langur monkey:** The findings were also presented in langur monkeys with the changes in the physical characteristics of semen and ultrastructure of the spermatozoa after vas occlusion with SMA. Scanning electron microscopy (SEM) revealed severe coiling of tail, rupture of acrosomal envelope, and bent midpiece associated with damaged mitochondrial sheath. Observations by transmission electron microscopy (TEM) revealed vacuolization in the nucleus, membrane damage in the acrosome, loss of segmented columns, and numeric aberrations in the centriole of the neck, as well as degeneration of mitochondrial sheath and axoneme in the midpiece, and absence of outer plasma membrane in the midpiece and tail. The results indicated that the necrospermic status of the spermatozoa during initial ejaculations may offer instant sterility after vas occlusion with SMA [48]. After that, routine hematology, clinical chemistry, the serum testosterone and sperm antibody titers were studied that remained unchanged from their pretreatment values until 540 days of vas occlusion. Histology of testes revealed continued spermatogenesis throughout the study period. The results suggested focal degeneration of seminiferous epithelium in the central portion of the testis following long-term vas occlusion with SMA [49].

Clinical journey

After fertility control investigations, toxicological studies and further successful safety evaluation on albino rats and rhesus monkeys the Indian Council of Medical Research (ICMR) and Drugs Controller General of India (DCGI) permitted to conduct Clinical Trials in 1989 (Table 2). Study was planned to assess the contraceptive effectiveness and safety of the intra-vas deferens injections of complex comprised of SMA in a solvent vehicle of DMSO.

- **Phase-I of the clinical journey**: Phase-I clinical trial was initiated at a few centers with 38 healthy adult male volunteers with normal reproductive system [50]. Female partners of all the volunteers enrolled in the Phase-I clinical trials had already undergone tubal occlusion, thus the efficacy was

Table 2 Clinical journey of RISUG®

	No. of Subjects	Dose regimen	Sperm count (million/ml)	Remarks	References
Phase I	38	5 mg to 140 mg	For 60–140 mg dose azoospermia was reported during 20–389 days post injection	Phase I clinical trial showed that the injection of DMSO and DMSO-SMA mixture into the lumen of the vas deferens is a safe procedure with no long-term adverse effects.	[50]
Phase II	12	60 mg	All subjects were azoospermic within 5–243 days	The results of Phase II clinical trials reconfirm the safety and show that for a period of at least one year, the treatment leads to azoospermia in the male and gives pregnancy protection.	[51]
Phase III	315	60 mg	After 2.5 months 92.6% subjects and after 6 months 96.7% subjects showed azoospermia post RISUG® injection.	Contraceptive efficacy was found to be 99.02% with 0.3% method failure and 0.98% overall failure in the drug efficacy.	[52, 53]

obtained as indirect evidence in terms of semenological studies. The Phase I clinical trial was focused at confirming the safety and side effects of the drug preparation evaluated on the basis of clinical parameters, as the drug was being used for the first time in medical studies. After complete medical examination, with local anesthesia, a small incision of about 7 mm length in the scrotal skin to the left of the midline and at a level 15–20 mm above the upper pole of testis was made. In the distal direction while maintaining proximal compression, 5 mg to 140 mg doses of the polymeric drug of SMA was injected into the vas deferens using a 23-gauge needle. After injection clinical assessment and semenology was periodically performed for more than 2 years. Drug with 60–140 mg SMA was found to be effective showing azoospermia during 20–389 days post injection. Most effective outcomes were observed with 70 mg SMA dose which showed azoospermia in nearly 3 weeks and the subjects stayed azoospermic for 292 days. The Phase-I clinical trial of RISUG® with more than 2 years of follow-up study demonstrated that the procedure does not lead to any clinical complications in the urogenital system and other parts of the body.

- **Phase-II of the clinical journey**: Phase II clinical trials with RISUG® injection were initiated to assess efficacy of intravasal injection based on azoospermia and no pregnancy in the female partner that have a normal reproductive profile, had not undergone sterilization and have not being using any other conventional contraceptive. Under the Phase-II study, 12 healthy adult male volunteers were injected with 60 mg of SMA. All the subjects underwent pre- and post-injection clinical examination that included sperm count, motility and morphology assessment. Results of the Phase-II studies showed injection with 60 mg of SMA can induce azoospermia immediately and was observed for more than 12 months. Azoospermia was observed in all the subjects with no side effects [51, 54]. Female partners of all the subjects retained good health throughout the study and no pregnancies were reported during the period of study [51].
- **A parallel journey:** In Phase-II, 60 mg of SMA resulted in an immediate contraceptive effect, parallel a 2 year clinical efficacy trial was performed with variable doses of RISUG® [54]. The study included 20 subjects who were injected with 40, 50, 60, 65 and 70 mg of SMA and were monitored for the maximum of 1407 days. Results of the study suggested dosages ranging from 40 to 70 mg of SMA were effective in giving more than 2 years of contraception regardless of azoospermic or non-azoospermic stage of the subjects. One subject amongst the 20 subjects under study had a normal child after 145 days of injection, due to slippage during injection. All subjects maintained good health during the course of vas occlusion with RISUG®, indicating efficacy and safety of the drug.
- **Phase-III of the clinical journey**: Long term follow up of human volunteers with RISUG® during Phase-II clinical trials showed the method to be effective and safe. However the study involved only a limited number of volunteers, thus Phase-III clinical trials were designed to evaluate safety and effectiveness of intravasal RISUG® injections in a larger sample.

In 2003, a brief study was performed wherein 25 healthy adult male volunteers were injected 60 mg SMA dissolved in 120 μL of DMSO [43]. Based on assessment of levels of neutral α-glucosidase, the biochemical marker for epididymis, acid phosphatase activity and fructose levels in the seminal plasma, RISUG® was shown to be effective as a partially occluding agent in the vas deferens. Semen and biochemical analyses were done for a period of 6 months post-injection and the results showed predominantly showing immotile and abnormal spermatozoa in all subjects after injection of RISUG®.

Phase-III clinical trials with RISUG® were initiated by ICMR at four different centers in the country with 64 healthy adult male volunteers [55]. All the subjects were injected with 60 mg SMA dissolved in 120 μL of DMSO and were followed up for efficacy and safety. Post injection, all the male volunteers and their female partners underwent clinical evaluation after 3, 7 and 21 days. Further, clinical and laboratory examinations that included monitoring for infections, pyrexia, pain and/or swelling in scrotum, liver and kidney function tests, blood and urine examinations, ultra-sonography of scrotum/lower abdomen and vital organs, etc. were performed and noted for all the subjects at 1.5 months, 2.5 months, 4 months, 5 months, 6 months and then after every 6 month interval till 5 years post RISUG® injection. ICMR reported no side effects of the drug with all subjects maintaining high clinical efficacy. 92.6% of subjects were reported to achieve azoospermia at 2.5 months post injection and in 96.7% of the subjects azoospermia was reported at 6 months after RISUG® injection [52]. In 2018, a total of 315 subjects enrolled at 5 different centers in the country were reported to show no adverse side-effects of the drug with overall contraceptive efficacy of 99.02%. Few subjects were reported to be lost in follow up due to personal reasons, 0.3% method failure and 0.98% overall failure in efficacy of the drug has been observed [53]. A multi-centric limited Phase III clinical trial of RISUG® reported no pregnancy among the subjects that received complete dose of RISUG® and indicated it is an effective and safe male contraceptive with majority of the individuals under study achieving either oligozoospermia or azoospermia within 2 months after injection [56].

Advantages of RISUG® over other methods of male contraception

RISUG® creates a physical and chemical barrier preventing sperm from reaching the oocyte. The polymer is injected into the vas deferens through the non scalpel technique, thus avoiding surgery in the initial sterilization procedure. There are few major advantages of RISUG® as mentioned below, that has made it a potential male contraceptive.

- **Early azoospermia:** Contraception is an all or none game, a single sperm is sufficient for fertilization resulting in an unplanned pregnancy. Three most commonly used methods of male contraception that have been in use for hundreds of years present a very high first-year failure rates (periodic abstinence - 20%, withdrawal - 19%, condoms - 3-14%). Vasectomy is a surgical method of male sterilization considered to be highly effective and permanent form of contraception. However, absence of sperms in ejaculates is mostly observed at-least 12 weeks after the procedure. Clinical trials with RISUG® demonstrate promising results showing azoospermia in subjects as early as 4 weeks after the injection that is sustained over years. A few sperms that are observed in ejaculates after RISUG® were found to be functionally inactive [25].
- **Reversibility**: In any contraceptive method a great concern is the reestablishment of fertility when required. RISUG® presents an advantage over other male contraceptive methods like vasectomy with its effective and easy reversibility, as observed in different animal models. Removal of SMA co-polymer (RISUG®) can be induced by injecting DMSO or $NaHCO_3$ that acts as partial solvent. After preclinical trials in various animal models based on blockage of vas deferens without any toxicity, the studies have been moved toward its reversibility aspect without affecting cellular integrity. Despite the promising results of reversibility in animal models, the reversibility studies have not yet been carried out in humans.

i. **Rat:** The SMA polymer was removed by flushing dimethyl sulphoxide in vas occluded rats and observed that the mucosal structure of vas deferens became normal [44, 57]. Subsequently, the functional success and safety of vas occlusion reversal by DMSO was reported in rat model along with teratogenicity studies [58]. After that, sodium bicarbonate (10%), pH 8.9, was used to flush the polymeric material from the vas deferens lumen in rats. Histological observations of vas deferens indicated potential role of $NaHCO_3$ in reversal of vas deferens blockage [59]. The reversal with $NaHCO_3$ in rats resulted into an early resumption of fertility when compared with DMSO and the procedure found to be successful, feasible and safe up to F_1 generation [60]. It is also concluded that vas occlusion with RISUG® at the contraceptive dose regimen is not associated with genotoxicity in leukocytes or the testis of pre- and post-reversal rats [61]. The study using both DMSO and $NaHCO_3$ for reversal of RISUG®-induced contraception was successful without any toxicity at the cellular levels [62].

ii. **Rabbit:** The results of reversal studies in rabbit suggested that DMSO and $NaHCO_3$ were feasible, with normal progeny, following short- and long-term contraception. The safety evaluation following vas occlusion with RISUG® and its reversal using genotoxicity tests and apoptotic marker assays

concluded that it has not been correlated with any toxicity [63, 64].

iii. **Langur monkey:** Non-invasive reversal approaches (palpation, percutaneous electrical stimulation of the vas deferens, forced vibratory movement, suprapubic percussion and per rectal digital massage of the vas deferens) have been applied in monkeys. The results suggested that non-invasive reversal was feasible even after long-term vas occlusion with SMA and is safe without adverse side effects [65–67]. Ultrastructural changes in the vas deferens of langur monkeys after 150 days of vas occlusion with styrene maleic anhydride (SMA) and after 150 days of non-invasive reversal were also reported. The results suggested that the exfoliation of the epithelium due to vas occlusion by SMA regains normalcy after 150 days of non invasive reversal [68, 69]. Degeneration of seminiferous epithelium was evident in some of the tubules and following 420 days of vas occlusion, the central portion of the testis showed regressed seminiferous tubules depicting various shapes and devoid of germ cells, which continued until 540 days of vas occlusion [69].

iv. **Other added advantages**: In the field of contraception, RISUG® has several advantages such as effectiveness, no interruption before the sexual act, cost factor, outpatient procedures means patients can leave the hospital immediately after an injection and resume their normal sex lives within a week, duration of effect that for at least 10 years no side effects with greater reversibility. Apart from male contraception, RISUG® shows antibacterial effect on *Escherichia coli* illustrated through a SEM, TEM and AFM based study. RISUG® based on its composition i.e. SMA, was hypothesised to demonstrate antimicrobial activity against various microorganisms like *Candida albicans, Pseudomonas auroginosa, Staphylococcus aureus, Escherichia coli,* etc. [70]. It has been also suggested that viruses could be more sensitive towards antimicrobial action of RISUG® than bacteria and based on this assumption RISUG® has been presented as a potential candidate for developing antiretroviral drug/ male vas deferens implant for HIV free semen [70, 71]. RISUG® was thus taken as a potential antiretroviral drug, still study needs further confirmation and mechanism needs to be elucidated.

Female contraception with RISUG®

A recent study was initiated to examine the tissue specific reaction and the histo-architecture of the female tract that receive the polymer implant. The above finding indicates that the drug is compatible within the fallopian tube and therefore needs to be explored further for its contraceptive potential in females [72]. The contraceptive efficacy of intratubular injection of RISUG® and its reversal assessed in female rats was found to be safe without any untoward side-effects [73].

Why the drug is still not in market after 3–4 decades of research?

Towards answering this one must understand that regulatory measures take time and these requirements help protect people from potentially harmful products. Looking for an alternative, effective and reversible male contraceptive, hormonal methods of male contraception were developed, but none could reach the markets due to undue side effects, lack of uniformity in results and also need for long-term administration. Preclinical and clinical journey of RISUG® demonstrates high efficacy and safety of the drug. With regard to reversibility, safety and efficacy trials have been performed only on animal models. Before putting RISUG® into market, its reversibility needs to be clinically verified. Another major concern inhibiting the progress of RISUG® is lack of interest from pharmaceutical industries. In 2000, a survey found 83% of men from various countries are willing to accept male contraceptive. Despite, pharmaceutical companies are reluctant to pursue the idea to avoid losing the thriving global markets for female contraceptives and condom that value to billions each year. Initially, RISUG® attracted some interest from pharmaceutical companies. However, considering it as an inexpensive one time procedure manufactures retracted. Taking in to account the ever increasing population of countries like India, there is a demand for family planning, thus RISUG® caught attention of the Government. Apart from scientific and monetary matters, major hindrance that stands in the way of this revolutionary male contraceptive is men itself. In the male dominating society it has always being tough for men accepting the responsibility of family planning. Today the world communities are evolving and there is increased focus on involvement of men from supporting and understanding female partner's reproductive health to engaging men as contraceptive users. Various studies are coming up engaging men as potential clients of family planning and surveys indicate about young adults being more willing to use male contraceptive methods [74, 75]. The perspective also varies by country and demographical backgrounds; a wider acceptability has been reported amongst men with advanced educational background and stable employment [76]. Nevertheless, scientists are pushing ahead and the momentum and buzz in the field is reflecting fresh optimism.

Conclusion- present scenario and future perspectives

The RISUG® has surely created a new concept of contraception with great feasibility and long lasting sterility. After being introduced in 1980 successful pre-clinical efficacy and safety studies on various species of animals including primates, RISUG® has also been tested successfully in number of human volunteers during Phase-I, Phase-II and Phase-III clinical trials. Presently the drug is under extended Phase-III clinical trials at various centers in India waiting for approval from DCGI for mass production. Although, many leads have been taken towards making of an effective male contraceptive, many of these failed, many of these succeeded at first and then failed, many are still struggling for recognition, RISUG® on the other hand, provides a hope which has a slow pace and drawbacks, but it is in a right direction.

Abbreviations

AFM: Atomic Force Microscopy; CD: Contraceptive Dose; DCGI: Drugs Controller General of India; DMSO: Dimethyl Sulphoxide; FAMS: Fellow of National Academy of Medical Sciences (India); FEMSI: Fellow of Electron Microscopy Society of India; FIAES: Fellow of Indian Academy of Environmental Sciences; FNASc: Fellow of National Academy of Sciences, India; FP2020: Family Planning 2020; FSH: Follicle-Stimulating Hormone; GnRH: Gonadotropin-Releasing Hormone; HIV: Human Immunodeficiency Virus; ICMR: Indian Council of Medical Research; LH: Luteinizing Hormone; NASI: National Academy of Sciences, India; NSV: No Scalpel Vasectomy; RISUG®: Reversible Inhibition of Sperm Under Guidance; SEM: Scanning Electron Microscope; SMA: Styrene Maleic Anhydride; STDs: Sexually Transmitted Diseases; STIs: Sexually Transmitted Infections; TEM: Transmission Electron Microscopy; WHO: World Health Organization

Acknowledgements

The authors are thankful to Prof. Sujoy K Guha, School of Medical Science and Technology, Indian Institute of Technology, Kharagpur, India and Dr. R. S. Sharma, Division of Reproductive Biology, Maternal and Child Health, Indian Council of Medical Research, New Delhi, India for their keen interest and valuable inputs. National Academy of Sciences, India is thankfully acknowledged for the award of NASI Senior Scientist's assignment to NKL and Research Associate fellowship to BK.

Authors' contributions

All authors made substantial contributions to conception and design, acquisition, analysis and interpretation of data; BK and AB participated in drafting the manuscript. ASA and NKL provided important intellectual contents. Each author participated sufficiently in this work and takes public responsibility for contents reported in the manuscript. All authors read and approved the final manuscript.

Funding

The studies carried out on RISUG® were supported by the Ministry of Health and Family Welfare, Government of India; Indian Council of Medical Research, New Delhi and National Academy of Sciences, India.

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 31 October 2019 Accepted: 13 January 2020
Published online: 13 February 2020

References

1. Muttreja P, Singh S. Family planning in India: the way forward. Indian J Med Res. 2018;148:S1–9.
2. Family Planning 2020. www.familyplanning2020.org .
3. Dereuddre R, Van de Velde S, Bracke P. Gender inequality and the 'east-West'divide in contraception: an analysis at the individual, the couple, and the country level. Soc Sci Med. 2016;161:1–2.
4. Handelsman D, Waites G. Traditional methods. In: Schill WB, Comhaire FH, Hargreave TB, editors. Andrology for the clinician. Berlin, Heidelberg: Springer; 2006. p. 122–124.
5. Shih G, Turok DK, Parker J. Vasectomy: the other (better) form of sterilization. Contraception. 2011;83:310–5.
6. Brown S. They think it's all up to the girls: gender, risk and responsibility for contraception. Cult Hlth Sexual. 2015;17:312–25.
7. Kogan P, Wald M. Male contraception: history and development. Urolog Clin. 2014;41:145–61.
8. Eberhardt J, van Wersch A, Meikle N. Attitudes towards the male contraceptive pill in men and women in casual and stable sexual relationships. BMJ Sexual Reprod Hlth. 2009;35:161–5.
9. Hartmann B. Reproductive rights and wrongs: the global politics of population control. Chicago, Illinois: Haymarket Books, South End Press; 1995.
10. Rogow D, Horowitz S. Withdrawal: a review of the literature and an agenda for research. Stud Fam Plan. 1995;26:140–53.
11. Jones RE. Contraception. In: Jones RE and Lopez KH, editors. Human reproductive biology. California, USA: Academic Press; 1997; p. 271–297.
12. Grady WR, Klepinger DH, Billy JO, Tanfer K. Condom characteristics: the perceptions and preferences of men in the United States. Fam Plan Perspect. 1993;25:67–73.
13. Finn R. Male contraceptive methods are in the pipeline. Ob Gyn News. 2007;42:28.
14. Amory JK, Page ST, Bremner WJ. Recent advances in male hormonal contraception. Nat Clin Pract Endocrinol Metab. 2006;2:32–41.
15. Gava G, Meriggiola MC. Update on male hormonal contraception. Ther Adv Endocrinol Metab. 2019;10:1–9.
16. Reynolds-Wright JJ, Anderson RA. Male contraception: where are we going and where have we been? BMJ Sexual Reprod Hlth. 2019;45:236–42.
17. Kaur K, Vijay P. Immunocontraceptives: new approaches to fertility control. Biomed Res Int. 2014;868196. https://doi.org/10.1155/2014/868196.
18. Naz RK. Vaccine for human contraception targeting sperm Izumo protein and YLP_{12} dodecamer peptide. Protein Sci. 2014;23:857–68.
19. Zaneveld LJD, Depel W, Burns JW, Shapiro S, Beyler S. Development of a potentially reversible vas deferens occlusion device and evaluation in primates. Fertil Steril. 1998;49:527–33.
20. Zaneveld LJD, De Castro MP, Faria G, Derrick F, Ferraro R. The soft, hollow plug (Shug): A potentially reversible vas deferens occlusive device. In: Rajalakshmi M, Griffin PD, editors. Male contraception: Present and Future. New Delhi, India: New Age Int (P) Ltd; 1999. p. 293–307.
21. Wespes E. Vasectomy in male contraception and its reversal. Europ Urol Suppl. 2014;13:68–72.
22. Li SQ, Goldstein M, Zhu J, Huber D. The no-scalpel vasectomy. J Urol. 1991; 145:341–4.
23. Monoski MA, Li PS, Baum N, Goldstein M. No-scalpel, no-needle vasectomy. Urology. 2006;68:9–14.
24. Guha SK. Contraceptive for use by a male. 1996. USA Patent No. 5,488,075.
25. Lohiya NK, Alam I, Hussain M, Khan S, Ansari AS. RISUG: An intravasal injectable male contraceptive. Indian J Med Res. 2014;140:S63–72.
26. Levy DA, Khouader S, Leynadier F. Allergy to latex condoms. Allergy. 1998; 53:1107–8.
27. Mo ZN, Huong X, Zhang SC, Yang JR. Early and long term effects of vasectomy on serum testosterone, dehydrotestosterone, luteinizing hormone and follicle stimulating hormone levels. J Urol. 1995;154:2065–9.
28. Schwingl PJ, Guess HA. Safety and effectiveness of vasectomy. Fertil Steril. 2000;73:923–36.
29. Aradhya KW, Best K, Sokal DC. Recent developments in vasectomy. Br Med J. 2005;330:296–9.
30. Ansbacher R. Humoral sperm antibodies: a 10-year follow-up of vas-ligated men. Fertil Steril. 1981;36:222–4.

31. Eisenberg ML, Walsh TJ, Turek PJ. Use of viscoelastic solution to improve visualization during urologic microsurgery: evaluation of patency after vasovasostomy. Urology. 2009;73:134–6.
32. Schwarzer JU, Steinfatt H. Current status of vasectomy reversal. Nat Rev Urol. 2013;10:195–205.
33. Kuang C. Beyond the male pill. Popular Science May. 2008;20. http://www.popsci.com/scitech/article/2008-05/beyond-male-pill .
34. Misro MM, Guha SK, Singh H, Mahajan S, Ray AR, Vasudevan P. Injectable non-occlusive chemical contraception in the male-I. Contraception. 1979;20:467–73.
35. Gifford B. The revolutionary new birth control method for men. Wired April. 2011;26 http://www.wired.com/magazine/2011/04/ff_vasectomy/all/1.
36. Misro MM, Kaur H, Mahajan S, Guha SK. An intravasal non-occlusive contraceptive device in rats. J Reprod Fert. 1982;65:9–13.
37. Lohiya NK, Alam I, Ansari AS. Male Contraception. In: Kumar A, Sharma M, editors. Basics of human Andrology. Singapore: Springer; 2017. p. 493–508.
38. Guha S, Ansari S, Anand S, Farooq A, Misro M, Sharma D. Contraception in male monkeys by intra-vas deferens injection of a pH lowering polymer. Contraception. 1985;32:109–18.
39. Fraczek M, Kurpisz M. The redox system in human semen and peroxidative damage of spermatozoa. Postepy Hig Med Dosw. 2005;59:523–34.
40. Lemkecher T, Dartigues S, Vaysse J, et al. Leucocytospermia, oxidative stress and male fertility: facts and hypotheses. Gynecol Obstet Fertil. 2005;33:2–10.
41. Aitken RJ, Baker MA. Oxidative stress, sperm survival and fertility control. Mol Cell Endocrinol. 2006;250:66–9.
42. Guha SK, inventor. Styrene maleic anhydride based formulation for male contraception and prostate cancer. United States patent application US 12/736,112. 2011 Jan 6.
43. Chaki S, Das H, Misro MM. A short-term evaluation of semen and accessory sex gland function in phase III trial subjects receiving intravasal contraceptive RISUG. Contraception. 2003;67:73–8.
44. Verma K, Misro MM, Singh H, Mahajan S, Ray AR, Guha SK. Histology of the rat vas deferens after injection of a non-occlusive chemical contraceptive. J Reprod Fert. 1981;63:539–42.
45. Sethi N, Srivastava RK, Singh RK. Male mediated teratogenic potential evaluation of new antifertility compound SMA in rabbit (*Oryctolagus cuniculus*). Contraception. 1990;42:215–23.
46. Sethi N, Srivastava RK, Singh RK, Bhatia GS, Sinha N. Chronic toxicity of styrene maleic anhydride, a male contraceptive, in rhesus monkeys (*Macaca mulatta*). Contraception. 1990;42:337–47.
47. Guha SK. Biophysical mechanism-mediated time-dependent effect on sperm of human and monkey vas implanted polyelectrolyte contraceptive. Asian J Androl. 2007;9:221–7.
48. Lohiya NK, Manivannan B, Mishra PK. Ultrastructural changes in the spermatozoa of langur monkeys *Presbytis entellus entellus* after vas occlusion with styrene maleic anhydride. Contraception. 1998;57:125–32.
49. Mishra PK, Manivannan B, Pathak N, et al. Status of spermatogenesis and sperm parameters in langur monkeys following long-term vas occlusion with styrene maleic anhydride. J Androl. 2003;24:501–9.
50. Guha SK, Singh G, Anand S, Ansari S, Kumar S, Koul V. Phase I clinical trial of an injectable contraceptive for the male. Contraception. 1993;48:367–75.
51. Guha SK, Singh G, Ansari S, et al. Phase II clinical trial of a vas deferens injectable contraceptive for the male. Contraception. 1997;56:245–50.
52. Indian Council of Medical Research (ICMR), New Delhi, Annual Report, 2016–2017.; p. 89. (https://www.icmr.nic.in/sites/default/files/annual_repoorts/ICMR_AR_English_2016_17.pdf). Accessed 30 Dec 2019.
53. Indian Council of Medical Research (ICMR), New Delhi, Annual Report, 2017–2018. p. 70 (https://www.icmr.nic.in/sites/default/files/annual_repoorts/ICMR_AR_English_2017-18_final.pdf). Accessed 30 Dec 2019.
54. Guha SK, Singh G, Srivastava A, et al. Two-year clinical efficacy trial with dose variations of a vas deferens injectable contraceptive for the male. Contraception. 1998;58:165–74.
55. Indian Council of Medical Research (ICMR), New Delhi, Annual Report, 2011–2012; p. 112. (https://www.icmr.nic.in/sites/default/files/annual_repoorts/Annual-Report-2011-2012-English.pdf). Accessed 30 Dec 2019.
56. Sharma RS, Mathur AK, Singh R, et al. Safety and efficacy of an intravasal, one-time injectable and non-hormonal male contraceptive (RISUG): a clinical experience. Indian J Med Res. 2019;150:81–6.
57. Sethi N, Srivastava RK, Singh RK. Histological changes in the vas deferens of rats after injection of a new male antifertility agent "SMA" and its reversibility. Contraception. 1990;41:333–9.
58. Lohiya NK, Suthar R, Khandelwal A, Goyal S, Ansari AS, Manivannan B. Sperm characteristics and teratology in rats following vas deferens occlusion with RISUG and its reversal. Int J Androl. 2010;33:e198–206.
59. Koul V, Srivastav A, Guha SK. Reversibility with sodium bicarbonate of styrene maleic anhydride, an intravasal injectable contraceptive, in male rats. Contraception. 1998;58:227–31.
60. Ansari AS, Hussain M, Khan SR, Lohiya NK. Relative suitability of DMSO and $NaHCO_3$ for reversal of RISUG® induced long-term contraception. Andrology. 2016;4:306–13.
61. Ansari AS, Alam I, Hussain M, Khan SR, Lohiya NK. Evaluation of genotoxicity in leukocytes and testis following intra-vasal contraception with RISUG and its reversal by DMSO and $NaHCO_3$ in Wistar albino rats. Reprod Toxicol. 2013;36:53–9.
62. Ansari AS, Hussain M, Khan SR, Badar A, Lohiya NK. Toxicity and mutagenicity evaluation following RISUG® contraception reversal in rats. Int J Toxicol. 2018;20:1–9.
63. Ansari AS, Badar A, Balasubramanian K, Lohiya NK. Contraception with RISUG® and functional reversal through DMSO and $NaHCO_3$ in male rabbits. Asian J Androl. 2017;19(3):89–95.
64. Ansari AS, Badar A, Lohiya NK. Safety evaluation through genotoxicity and apoptotic markers following RISUG® induced contraception and its reversal in male rabbits. Reprod Toxicol. 2018;81:84–92.
65. Lohiya NK, Manivannan B, Mishra PK, Pathak N, Balasubramanian SPA. Intravasal contraception with styrene maleic anhydride and its noninvasive reversal in langur monkeys (*Presbytis entellus entellus*). Contraception. 1998;58:119–28.
66. Lohiya NK, Manivannan B, Mishra PK. Repeated vas occlusion and non-invasive reversal with styrene maleic anhydride for male contraception in langur monkeys. Int J Androl. 2000;23:36–42.
67. Lohiya NK, Manivannan B, Bhande SS, Panneerdoss S, Garg S. Perspectives of contraceptive choices for men. Indian J Exp Bio. 2005;43:1042–7.
68. Manivannan B, Mishra PK, Lohiya NK. Ultrastructural changes in the vas deferens of langur monkeys *Presbytis entellus entellus* after vas occlusion with styrene maleic anhydride and after its reversal. Contraception. 1999;59:137–44.
69. Manivannan B, Bhande SS, Panneerdoss S, Sriram S, Lohiya NK. Safety evaluation of long-term vas occlusion with styrene maleic anhydride and its non-invasive reversal on accessory reproductive organs in langurs. Asian J Androl. 2005;7:195–204.
70. Guha SK. RISUG™ (reversible inhibition of sperm under guidance)–an antimicrobial as male vas deferens implant for HIV free semen. Med Hypotheses. 2005;65:61–4.
71. Banerjee S, Guha SK. RISUG®: a potential candidate for the entry inhibitor group of antiretroviral drugs. Med Hypotheses. 2009;73(2):150–2.
72. Pal M, Bhatoa PK, Bhardwaj JC. Histo-architecture of the rabbit fallopian tube following short-term RISUG implant. Int J of Pharma Sci Res. 2014;5:903–7.
73. Ansari AS, Sevliya K, Badar A, Lohiya NK. Studies on RISUG® induced intratubular contraceptive efficacy and its reversal with DMSO and $NaHCO_3$ in female albino rats. Int J Biomed Res. 2019; (In Press).
74. Dismore L, Van Wersch A, Swainston K. Social constructions of the male contraception pill: when are we going to break the vicious circle? J Health Psychol. 2016;21:788–97.
75. Peterson LM, Campbell MA, Laky ZE. The next frontier for men's contraceptive choice: college men's willingness to pursue male hormonal contraception. Psychol Men Mascul. 2019;20:226–37.
76. Sax M, Hurley E, Rossi R, Thakore S, Hasija A, Sroga-Rios J. Young adult males perspectives of male hormonal contraception. Obstet Gynecol. 2019;133:204S. https://doi.org/10.1097/01.AOG.0000559144. 82213.99.

Publisher's Note