Award Winners (*Alphabetic order*)

A-1

Low polyvinylpyrrolidone concentration improved sperm apoptotic gene expression, cytokinetic of embryo, and intracytoplasmic sperm injection outcomes: An RCT

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Background: During the intracytoplasmic sperm injection (ICSI) procedure, 10% polyvinylpyrrolidone (PVP) is commonly used as a selection medium to decrease sperm motility.
Objective: The study aimed to determine the effect of different concentrations of PVP (5% and 10%) on sperm apoptotic transcripts, sperm DNA fragmentation, embryo cytokinetic and clinical characteristics in the ICSI program.
Materials and Methods: In this clinical trial study, 60 couples with male factor infertility underwent ICSI insemination using either 5% or 10% PVP concentration. Metaphase II oocytes were divided into 2 groups, with one group injected with spermin 5% PVP and the other in 10% PVP. After fertilization, the zygotes were cultured using a time-lapse microscope to assess morphokinetic parameters also, simultaneously with the ICSI procedure, sperm DNA fragmentation and apoptotic transcript levels were quantified using reverse transcription quantitative polymerase chain reaction.
Results: DNA fragmentation (20.27 ± 4.94% vs. 25.93 ± 5.73%) were lower in spermatozoa from the 5% PVP compared to 10% PVP groups ($p < 0.0001$). Also, DNA fragmentation was significant between 0% PVP group with 5% PVP and 10% PVP ($p = 0.00$). The expression levels of both *BCL2* and *HSP70* genes increased significantly with experimental when compared to control group. The transcript level of the *BAX* gene was significantly lower in experimental sample. There were higher rates of fertilization ($p = 0.049$) and high-quality embryo formation in group 5% PVP ($p = 0.03$). All cytokinetic variables were significantly different between groups ($p < 0.001$). Also, the median time to complete second synchronous divisions (S2) was significantly higher in 10% PVP embryos. In addition, the rates of embryo fragmentation, uneven blastomere, reverse cleavage and embryo arrest were significantly higher in 10% PVP group. The biochemical and clinical pregnancies, and implantation success rates were insignificantly increased in 5% PVP compared to the 10% PVP.
Conclusion: The study concluded that a low concentration of PVP (5%) is a suitable replacement for 10% PVP in ICSI cycles for male factor infertility.
Keywords: *Embryo cytokinetic, ICSI, Polyvinylpyrrolidone, Time-lapse.*
Registration ID in IRCT: IRCT20220725055550N1

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A-2

The effects of Wharton's jelly mesenchymal stem cells secretomes for restoring busulfan-induced reproductive toxicity in male mice

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Background: Numerous studies have explored the use of mesenchymal stem cells (MSCs) in the treatment of spermatogenic disorders.
Objective: Given the constraints associated with MSC applications, the current research aimed to compare the secretomes of Wharton's jelly MSCs, including conditioned medium at 10-fold concentration (CM10), 20-fold concentration (CM20), and extracellular vesicles (EVs), to mitigate busulfan-induced reproductive damage in male mice.
Materials and Methods: In this experimental study, 72 male BALB/c mice (8-9 wk, 29 ± 3 gr) were randomly divided into 9 groups (n = 8/group), including control, busulfan 1 month (1M), busulfan 2 months (2M), CM10, busulfan + CM10, CM20, busulfan + CM20, EVs, and busulfan + EVs. MSCs were isolated from Wharton's jelly in this experimental study. MSCs were characterized through morphology assessment using light microscopy, and flow cytometry was used to evaluate MSCs surface markers. Moreover, adipogenic differentiation was verified by Oil-Red O staining, and osteogenic differentiation was confirmed by Alizarin Red staining. The conditioned medium from passages 3-6 was used for EV isolation, and the remaining part was lyophilized at CM10 and CM20 concentrations for in vivo studies. EVs were characterized using transmission

electron microscopy and dynamic light scattering. The evaluation included sperm count, motility, morphology, plasma membrane integrity, DNA maturity, DNA fragmentation, and testicular gene expression.

Results: The analysis indicated that CM10 significantly enhanced sperm plasma membrane integrity ($p < 0.050$), sperm DNA maturity ($p \leq 0.001$), and decreased DNA fragmentation ($p < 0.050$) in the busulfan + CM10 group when compared to the Busulfan 2M group, while CM20 and EVs exhibited no significant improvements in these parameters. Furthermore, gene expression analysis revealed that busulfan treatment notably reduced the expression of androgen receptor, cAMP-responsive element-binding 1, and phospholipase C zeta genes, whereas CM10 significantly restored the expression of the cAMP-responsive element-binding 1 gene ($p < 0.050$).

Conclusion: This study shows that CM10 is more effective than CM20 or EVs in alleviating busulfan-induced reproductive toxicity.

Keywords: *Toxicity, Spermatozoa, Mesenchymal stem cells, Extracellular vesicles, Conditioned medium, DNA fragmentation.*

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A-3
Effect of advanced glycation end products on sperm parameters and function in C57Bl/6 mice

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Background: Advanced glycation end products (AGEs) are frequently observed in metabolic disorders such as diabetes, obesity, and conditions associated with infertility. These AGEs are strongly correlated with adverse effects on cell and tissue health.

Objective: To better understand how AGEs affect both sperm structure and function, our research used mouse models exposed to tailored diets that promote AGE accumulation.

Materials and Methods: In this experimental study, we used 2 groups of 5-wk-old C57BL/6 mice: one group received a control diet, while the other was fed a diet enriched in AGEs daily. After 13 wk, we measured several parameters, including fasting blood glucose, body weight, and food intake. In addition, we evaluated sperm quality by assessing sperm concentration, motility, morphology, histone retention, protamine deficiency, DNA damage, lipid peroxidation, cytoplasmic reactive oxygen species, and sperm malondialdehyde levels. In addition, we assessed testicular superoxide dismutase levels, malondialdehyde content, total antioxidant capacity, Johnson scores, and receptor of AGE and carboxymethyl lysine protein contents.

Results: After 13 wk, we observed significant differences between AGE and control groups. The AGE group showed increased body weight and fasting blood sugar levels compared with the control group ($p < 0.001$). However, there were no significant differences in mean testicular volume and testicular weight between groups ($p > 0.05$), except for adipose tissue epididymal white adipose tissue, which showed a significant difference ($p = 0.003$) between the groups but only unilaterally. With regard to sperm parameters, the AGE group showed lower mean values and a higher percentage of sperm abnormalities, including nuclear histone retention, chromatin deficiencies, DNA fragmentation, and increased membrane lipid peroxidation, compared to the control group ($p < 0.005$). In addition, the AGE group showed a significant reduction in total testicular antioxidant capacity and a lower Johnson score compared to the control group ($p = 0.04$). Mean levels of testicular superoxide dismutase did not differ significantly between groups. However, the AGE group had the highest mean level of testicular malondialdehyde content, as well as a higher accumulation of receptor of AGE and carboxymethyl lysine proteins compared to the control group ($p = 0.006$, $p = 0.002$ respectively).

Conclusion: AGEs have negative effects on male reproductive health, causing metabolic problems, sperm abnormalities and oxidative stress, highlighting the role these compounds can play in male infertility, particularly in the case of metabolic disorders.

Keywords: *Advanced glycosylation end products, Carboxymethyl lysine, Receptor of advanced glycation end products, Diabetes mellitus, Oxidative stress.*

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A-4
Identifying causative genes and variants of nonobstructive azoospermia through whole-genome sequencing

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The knowledge of the causes of spermatogenetic failure in non-obstructive azoospermia (NOA) is very limited. The goal is to uncover the causative genes, variants and biological processes implicated in spermatogenic failure. Whole blood samples were collected from tow NOA cases who were referred to Yazd Reproductive Sciences Institute, Yazd, Iran between 2023 and 2024. Cases had normal karyotype and no microdeletions of the Y chromosome. Whole genome sequencing was performed as a collaborative project with Institute of Human Genetics Polish Academy of Sciences, Gdansk, Poland using Illumina HiSeq X. The quality of raw data was evaluated using the FastQC. Reads were aligned to the reference human genome GRCh38 using bwa aligner. Variants were called using freeBayes v0.9.21and annotated using Ensembl Variant Effect Predictor.30.Variants filtered with allele frequency less than 0.01 in gnomAD, high and moderate variant impact, recessive disease model, expression in testis, and potential function and relation with fertility/spermatogenesis. We identified a homozygous variant (c.899C>T; p.Ser300Phe) in SRY-box transcription factor 30 (*SOX30*) in a NOA case. The variant was not previously reported in a homozygous state in any allele frequency databases. According to the PolyPhen-2 and SIFT result, this mutation is predicted to be probably damaging and not tolerated substitution, respectively. *SOX30* is a key regulator of mouse spermiogenesis. We also identified two variants for the other NOA case. The first was a hemizygous novel variant (c.2354A>G; p. Gln785Arg) in the X chromosome-linked A-kinase anchor protein 4 (*AKAP4*) gene. *AKAP4* is highly expressed in spermatids and mature spermatozoa. Some variants in the *AKAP4* gene have been reported as causes of male in/subfertility in humans and mice with different male infertility phenotypes ranging from asthenozoospermia, and multiple morphological abnormalities of the sperm flagellum to NOA. The variant is predicted as pathogenic supporting by SIFT. The Q785R amino acid replacement was studied via the NetSurfP-2.0 web service. The mutant protein stability is decreased when the solvent accessibility is increased. Also, absolute solvent accessibility has increased from 40-59 Å and relative solvent accessibility has increased from 22-26%. Another variant was a homozygous ultra-rare variant (c.1472C>T; p.Thr491Met) in the ring finger protein 220 (*RNF220*) gene. It is expressed in the spermatozoa head and flagellum and plays a vital role in chromatin condensation. The detected c.1472C>T variant has not been reported in a homozygous state previously. Based on NetSurfP-2.0 web service result, absolute solvent accessibility has increased from 25-26 Å and relative solvent accessibility has decreased from 18 to 13% regarding the T491M amino acid replacement. It was shown that *Sox30*-null male mice are sterile; however, no variant in this gene has been reported related to infertility in humans so far. For the first time, we reported a homozygous variant in the *SOX30* gene as a cause of NOA in humans. We also reported a novel hemizygous variant in the *AKAP4* gene plus a homozygous variant in the *RNF220* gene in one NOA patient unraveling the cause of spermatogenic failure.

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Keywords: *Infertility, Nonobstructive azoospermia, Whole-genome sequencing.*

A-5
Development of bioengineered human artificial testis using 3D bioprinted gelatin methacrylate/ human testicular extracellular matrix scaffold

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Background: The advancement of in vitro 3D culture techniques for human spermatogonial stem cells has been demonstrated to be an effective approach for the investigation of spermatogenesis. This may potentially provide a viable therapeutic intervention for male infertility. Tissue decellularization represents a potential alternative procedure for enhancing in vitro culture conditions, whereby natural 3D and extracellular matrix (ECM) conditions are provided to support cellular growth. Gelatin methacrylate (GelMA) is an engineered gelatin derivative that has been employed extensively in 3D bioprinting over the past decade due to its exceptional mechanical properties, biocompatibility, and favorable cell adhesion characteristics.
Objective: The objective was to develop a novel 3D bioprinted microenvironment using GelMA and human testicular ECM (htECM) in order to evaluate the

in vitro spermatogenesis process in a human bioartificial testis.

Materials and Metheds: This experimental study was done during 2023-2024 yr at Iran University of Medical Sciences, Tehran, Iran. At first, 4 human testicular tissues were decellularization and the removal of cellular debris from the tissue samples was confirmed by the hematoxylin and eosin staining and DNA content investigation. The confirmation of the presence of the htECM compound was achieved by the utilization of alcian blue, orcein, and masson's trichrome staining. The synthesized GelMA was subjected to characterization by fourier transform infrared spectroscopy and nuclear magnetic resonance spectroscopy. The htECM was used in the fabrication of a bioprinted scaffold in combination with GelMA. A comprehensive assessment of the mechanical and biological properties was conducted using a range of analytical techniques, including fourier transform infrared spectroscopy, scanning electron microscope, degradation testing, swelling rate, MTT assay and in vivo biocompatibility analysis for GelMA and GelMA/htECM groups. 10 wk after the proliferation and differentiation of human testicular cells on 3D scaffolds, the presence of pre-meiotic and post-meiotic cells in the study groups (GelMA and GelMA/htECM) were assessed by molecular techniques.

Results: The decellularization protocol for human testicular tissue was effective in eliminating cellular debris while conserving the ECM compounds ($p = 0.001$). The GelMA/htECM formulation exhibited a distinctive surface morphology, augmented mechanical properties, and superior performance compared to the GelMA group in terms of MTT and in vitro biocompatibility. The cell viability increased in GelMA/htECM compared to GelMA ($p = 0.002$). The expression of pre-meiotic markers in cultured testicular cells on GelMA/htECM scaffolds were significant in comparison with GelMA group ($p = 0.001$). Moreover, the culture of human testicular cells onto GelMA/htECM scaffolds resulted in the generation of post-meiotic haploid cells.

Conclusion: Therefore, GelMA/htECM represents a promising avenue for the development of in vitro spermatogenesis procedures, with the potential to facilitate future sperm production from human spermatogonial stem cells.

Keywords: *Gelatin, Extracellular matrix, Testis, Bioprinting.*

A-6

Comparison of double blastocyst transfer versus sequential transfer on pregnancy outcomes in individuals with frozen embryo transfer and a history of recurrent implantation failure: An RCT

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Background: Recurrent implantation failure poses a significant challenge in assisted reproductive technology despite the transfer of high-quality embryos over multiple cycles.

Objective: This study aimed to compare the clinical outcomes between double blastocyst transfer and sequential single cleavage-stage and blastocyst transfer in individuals undergoing frozen embryo transfer and those with a history of repeated implantation failure.

Materials and Methods: This randomized clinical trial was conducted at the Research and Clinical Center for Infertility, Yazd, Iran from February to November 2024 and included 125 women (< 45 yr) with a history of more than 2 implantation failures. Participants were randomized into 2 groups: one receiving double blastocyst transfer and the other receiving sequential single cleavage-stage and blastocyst transfer. The primary and secondary outcomes included clinical pregnancy, chemical pregnancy, early abortion, multiple pregnancy, and implantation rates.

Results: Baseline characteristics were similar between the 2 groups. Chemical pregnancy rates were comparable (51.6% for double blastocyst transfer vs. 49.2% for sequential transfer, $p = 0.790$), as were clinical pregnancy rates (46.9% vs. 44.3%, $p = 0.769$). Early abortion rates showed no significant difference (27.3% vs. 20%, $p = 0.498$). Multiple pregnancy rates were similar (23.3% vs. 25.9%, $p = 0.820$), and implantation rates did not differ significantly (28.9% vs. 27.86%, $p = 0.889$).

Conclusion: This study demonstrated that sequential single cleavage-stage and blastocyst transfer does not significantly improve assisted reproductive technology outcomes compared with double blastocyst transfer in individuals with recurrent implantation failure. Both methods had similar efficacy rates in terms of chemical pregnancy rates, clinical pregnancy rates, early abortion rates, multiple pregnancy rates, and implantation rates.

Keywords: *Assisted reproductive technology, Repeated implantation failure, Blastocyst transfer, Sequential embryo transfer, Pregnancy rates.*

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A-7

Evaluation of the effect of lecithin and nanolecithin in repairing membrane damage, maintaining membrane integrity, and improving human sperm function in the freezing–thawing process

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Background: Sperm cryopreservation is a routine procedure used in in vitro fertilization clinic. However, cryopreservation is associated with detrimental damaged to spermatozoa. Plasma membrane is one importamt target that could be damaged during cryopreservation. It was shown that lecithin may reduce sperm cryo-damage.
Objective: To evaluate protective effects of lecithin and nano-lecithin on human sperm during sperm freeze-thaw process.
Materials and Methods: This study was performed in 2 phases. In phase one, human sperm samples were divided to 10 groups to evaluate cryoprotective effects of lecithin and nano-lecithin on sperm parameters during cryopreservation. Sperm freezing media were formulated using lecithin at concentrations of 0.5%, 1%, and 2%, with nanoparticle sizes of 50-100 nm, 100-200 nm, and ≥ 200 nm, while a control group received no lecithin. Following thawing, assessments were performed on sperm motility, viability, mitochondrial membrane potential, lipid peroxidation (via malondialdehyde levels), and DNA fragmentation. In the second phase, the acrosomal reaction was evaluated in the highest and lowest performing groups identified in phase one. Dil labeling was used to investigate the interaction between lecithin nanoparticles and the sperm membrane. Additionally, field emission scanning electron microscopy analyzed the surface structure and binding sites on the sperm membrane, while atomic force microscopy measured surface height variations in the top-performing group.
Results: The group treated with 1% lecithin nanoparticles (50-100 nm) demonstrated a significantly higher post-thaw sperm viability compared to all other groups ($p < 0.0001$), along with lower levels of DNA fragmentation and lipid peroxidation (malondialdehyde). Although all groups experienced a decline in motility after freezing, better outcomes were observed with lower lecithin concentrations and smaller nanoparticle sizes. Mitochondrial membrane potential decreased significantly in all groups, with no notable differences among them. The acrosomal reaction was markedly reduced in the 1% 50-100 nm group compared to the 2% ≥ 200 nm group. Dil labeling and field emission scanning electron microscopy analysis showed that lecithin nanoparticles predominantly attached to and penetrated the sperm membrane, especially in the head and postacrosomal regions.
Conclusion: Lecithin nanoparticles efficiently attach to the sperm membrane, providing protection during the freeze-thaw process and enhancing sperm viability.
Keywords: *Cryopreservation, Lecithin, Nanoparticles, Cryoprotectant agents, Plasma membrane.*

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A-8
Cell phone waves disturbed the morphokinetics, and apoptosis in mouse preimplantation embryos

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Background: Due to the increasing use of smart mobile phones, the impact of radiofrequency electromagnetic radiation (RF-EMR) on reproductive health has become a serious concern.
Objective: This study investigated the effect of mobile phone RF-EMR with frequency 900-1800 MHZ on the mouse embryo morphokinetics and genotoxic effect in laboratory conditions.
Materials and Methods: In this experimental study, after ovarian stimulation in mice, the metaphase II oocytes were collected and underwent by in vitro fertilization method. The generated zygotes were divided into control and exposed groups. Then, the zygotes with 30 min of exposure to mobile phone RF-EMR, and the control zygotes without exposure, were incubated in the time-lapse for 5 days. The intracellular reactive oxygen species (ROS) level, morphokinetic, embryo viability rate, and gene expression were evaluated.
Results: Exposure of zygotes to RF-EMR by inducing ROS caused a significant decrease in blastocyst viability (87.85 ± 2.86 vs. 94.23 ± 2.44, $p = 0.0001$), delay in cleavage development (t3-t12) and also increased the time (in hours) to reach the blastocyst stage (97.44 ± 5.21 vs. 92.56 ± 6.7, $p = 0.04$) compared to the control group. A significant increase observed in mRNA levels of Hsp70 in exposed group; while *SOD* gene expression showed a significant down-regulation in this group compared to the controls, respectively. However, there was no significant change in the transcript level of proapoptotic and antiapoptotic genes in embryos of the exposed group compared to the controls.
Conclusion: RF-EMR emitted by mobile phone with a frequency of 900-1800 MHZ, through inducing the production of ROS and oxidative stress, could negatively affect the growth and development as well as the transcript levels of oxidative stress associated genes in the preimplantation embryos of mice.

Keywords: *Radiofrequency, Electromagnetic radiation, Cell phone, Reactive oxygen species, Apoptosis.*

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A-9
Effect of endometrial thickness compaction on pregnancy outcomes in frozen-thawed embryo transfer cycles using hormone replacement therapy: A cross-sectional study

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Background: Endometrial thickness compaction (ETC) is a new ultrasound evaluation method that may predict pregnancy outcomes.
Objective: The objective of this study is to determine whether there is an association between the impact of ETC and pregnancy outcomes in frozen embryo transfer (FET) cycles involving hormone replacement therapy (HRT).
Materials and Methods: This cross-sectional study includes 100 HRT-FET cycles designed at the in vitro fertilization unit of Yazd Institute of Reproductive Medicine, Yazd, Iran from June to October 2024. The women were admitted for their transvaginal ultrasound examination on the second day and either the 12th or 13th day of the menstrual cycle. Then in the cases of endometrial thickness ≥ 7 mm, high-grade cleavage embryo transfer was performed 3 days following progesterone administration. Participants were divided into 2 groups of compaction and non-compaction groups. Biochemical, clinical, and ongoing pregnancy were assessed as outcomes.
Results: Statistical analyses demonstrated significant differences in biochemical, clinical, and ongoing pregnancy outcomes between the compaction and non-compaction groups. The findings of the logistic regression analysis indicated that pregnancy rates were significantly higher in ETC 10-15% and above 15%. We found a significant influence of ETC 10-15% (OR, 5.57; CI, 1.27-24.42; p = 0.02), (OR, 6.66; CI, 1.47-30.10; p = 0.01), (OR, 6.66; CI, 1.47-30.10; p = 0.01) and ETC above 15% (OR, 6.81; CI, 2.06-22.45; p = 0.002), (OR, 8.14; CI, 2.38-27.87; p = 0.001), (OR, 6.66; CI, 1.95-22.73; p = 0.002) on biochemical, clinical and ongoing pregnancy rates, respectively.
Conclusion: ETC after progesterone administration in HRT-FET cycles may increase biochemical, clinical, and ongoing pregnancy rates. However, the percentage of ETC changes may also influence the outcomes of these cycles.
Keywords: *Assisted reproductive technology, Compaction, Endometrium, Embryo transfer, Pregnancy outcome.*

A-10
A study of genotype-phenotype correlation in idiopathic infertile Iranian men by a family-based exome-sequencing

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Background: Infertility affects ~15% of couples globally, with male factors contributing to nearly 50% of cases. While over 400 genes are linked to infertility in mice, few causal genes are confirmed in humans. Next-generation sequencing has advanced genotype-phenotype studies in disease research.
Objective: This study investigated genetic causes of male infertility using clinical genomics and transcriptomics.
Materials and Methods: 1st phase (genomics approach): 150 infertile men from Royan Institute (Tehran, Iran; 2020-2022) underwent clinical evaluations (sperm analysis, karyotyping, AZF microdeletion screening, hormone tests). 3 familial cases were found and (2 with asthenoteratozoospermia, one unexplained) were selected for whole-exome sequencing. Candidate mutations were validated using bioinformatics, co-segregation, and functional assays (population genetics, protein modeling, quantitive reverse transcription polymerase chain reaction, Western blot, electron microscopy (scanning electron microscopy and transmission electron microscopy), physiological and functional sperm tests, including hyperactivation assessments and staining with Papanicolaou, Chromomycin A3, aniline blue and FITC-PSA). 2nd phase (Transcriptomics): meta-analyses of microarray/RNA-seq data identified dysregulated kinases, epigenetic, cytoskeletal, and apoptosis-related genes in teratozoospermia and non-obstructive azoospermia. Systems biology approaches (network analyses, ROC analysis, gene ontology, pathway enrichment, siRNA/ASO design) were applied for further investigation of candidate genes were utilized.
Results: The genomics strategy identified mutations in 2 genes, *CATSPERB* and *PDCL2*, for the first time in cases of unexplained infertility and asthenoteratozoospermia, respectively. NM_024764.s4: c.2126G>A: p.R709G is a homozygous recessive mutation in the *CATSPERB* gene, one of the subunits of the CATSPER calcium channel, which plays a crucial role in sperm hyperactivation. NM_152401.3:c.128-1G>A was a homozygous recessive splicing mutation in the *PDCL2* gene, which likely acts as a chaperone to facilitate protein folding during spermatogenesis. Additionally, in another family, a novel mutation (NM_031907.2:c.A1205C: p.N402T) in the *USP26* gene -an enzyme involved in ubiquitin homeostasis- was

reported related with asthenoteratozoospermia. Individuals with mutations in the *CATSPERB* gene were normal in all sperm parameters except hyperactivation. Individuals with the *PDCL2* mutation lacked sperm with normal intracellular sperm organelles, such as axonemal components. During the transcriptomics strategy, 34 kinases, 63 epigenetic factors, 67 apoptosis factors, and 6 cytoskeleton related factors were identified among the differentially expressed genes.

Conclusion: This study identified novel mutations/genes in *CATSPERB*, *PDCL2*, and *USP26* genes associated with male infertility, revealing their roles in sperm hyperactivation, axonemal integrity, and ubiquitin homeostasis. Transcriptomics analysis further uncovered dysregulated pathways in spermatogenesis, including kinase signaling and epigenetic regulation. These findings enhance our understanding of male infertility genetics and provide potential targets for future diagnostic and therapeutic approaches.

Keywords: *Exome sequencing, Infertility, Male, Genetic association studies, Microarray analysis, Sequence analysis, RNA.*

A-11
Prenatal kisspeptin antagonist exposure prevents polycystic ovary syndrome development in prenatally-androgenized rats in adulthood: An experimental study

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Background: Increased levels of kisspeptin are associated with hypothalamus-pituitary-ovary axis dysfunction. It may lead to the development of polycystic ovary syndrome (PCOS).

Objective: We aimed to investigate the effect of prenatal kisspeptin antagonist exposure on the development of PCOS in prenatally androgenized rats in adulthood.

Materials and Methods: In this experimental study, pregnant rats were injected with free testosterone (T, 5 mg/day) or T+P271 (kisspeptin antagonist) on the 20th day of the pregnancy period (n = 5/each), while rats in the control group received solvent. Female offspring were examined in terms of anogenital distance (AGD), anovaginal distance (AVD), vaginal opening, serum total testosterone (TT) levels, ovarian follicles, and the regularity of estrous cycles in adulthood. AGD and AVD were measured using a vernier caliper. TT levels were measured by the ELISA method. Ovaries fixed in 10% formalin, processed by a standard protocol, and embedded in paraffin. Ovaries were serially sectioned at 5 µm thickness; sections were mounted on a glass slide, deparaffinized, and stained using Harris's Hematoxylin and Eosin Y.

Results: AGD, AVD ($p < 0.001$), TT levels ($p = 0.02$), and the numbers of preantral and antral follicles ($p < 0.001$) in the ovaries were significantly decreased in prenatally T-P271-exposed rats compared to prenatally T-exposed rats. The age of vaginal opening was early in T-P271-exposed rats compared to prenatally T-exposed rats ($p < 0.001$). The number of corpora lutea was significantly increased in T-P271-exposed rats ($p < 0.001$). No cystic follicles were observed in the ovaries of prenatally T-P271-exposed rats. Prenatally T-P271-exposed rats had regular estrous cycles compared to prenatally T-exposed rats.

Conclusion: Prenatal exposure to Kisspeptin antagonist can prevent PCOS development in prenatally androgenized rats in adulthood.

Keywords: *Androgen, Kisspeptin antagonist, Polycystic ovary syndrome, Rat.*

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A-12
Investigation of gene mutations in men with oligoasthenoteratozoospermia through whole genome sequencing

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Oligoasthenoteratozoospermia (OAT) is diagnosed when there is a reduction in sperm count, decreased sperm motility, and structural abnormalities in sperm heads. The genetic causes of spermatogenetic failure in cases with OAT is still not fully understood. 2 male cases with OAT, who referred to Yazd Research and

Clinical Center for Infertility, Yazd, Iran between 2023 and 2024, were enrolled in this study. Cases with OAT were initially identified by an andrologist. Blood samples were collected from the cases. Karyotype analysis was conducted, and the cases were screened for Y-chromosome microdeletions. DNA was extracted from the blood of cases with normal karyotypes. For this study, only OAT cases with normal karyotype results and no Y-chromosome microdeletions were selected. whole-genome sequencing was performed as a collaborative project with Institute of Human Genetics Polish Academy of sciences, Gdansk, Poland, using Illumina HiSeq X. The quality of raw data was evaluated using FastQC. Reads were aligned to the reference human genome GRCh38 using BWA aligner. Single nucleotide variants and small indels were called using FreeBayes v0.9.21. Variant files obtained from whole genome data were annotated using Ensembl Variant Effect Predictor. Variants were filtered based on allele frequency < 0.01 in gnomAD, variant impact (high and moderate), recessive inheritance model, testis expression, and potential relevance to fertility and spermatogenesis. We identified potential causative c.*4+1G>T hemizygous splice donor variant in the synovial sarcoma X1 (*SSX1*) gene. *SSX1* is specifically expressed in the testis and is mainly located in germ cells from spermatogonia to spermatocytes according to the Human Protein Atlas database. This gene has a role in spermatogenesis-sperm motility and abnormal sperm morphology. The variant had not been previously reported in homozygous state in any allele frequencies databases. We also found a homozygous missense variant (c.1190A>G) in the copine1 (*CPNE1*) gene. *CPNE1* is expressed in the flagella of mature sperm and plays a role in sperm motility. In addition, *CPNE1* may represent a target of Ca^{2+} channel inhibitors and may therefore be implicated in the regulation of Ca^{2+} signaling and sperm motility. We also uncovered homozygous missense variants in the coding protein of the *MT-ATP6* (c.334A>G) and *MT-CYB* (c.85G>A) genes. Genetic alterations in the mitochondrial DNA (mtDNA) have been linked to certain types of male infertility and abnormal sperm function. Both genes are involved in sperm motility and male infertility. According to the results of the present study, the identified genes can play a role in spermatogenesis, sperm function, morphology, motility, ATP synthesis, store-operated calcium (Ca^{2+}) entry, and calcium signaling. Moreover, their interacting proteins may also be involved in the pathophysiology of germ cell abnormalities and male infertility.

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Keywords: *Male infertility, Oligoasthenoteratozoospermia, Whole-genome sequencing.*

A-13
Selection of sperm with the highest quality and fertilizing potential using a novel microfluidic sperm sorter

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Background: Sperm selection as a critical step prior to assisted reproductive cycles can affect outcomes during infertility treatment. Conventional methods in assisted reproduction are incompetent to select high-quality sperm and may cause sperm damage. Microfluidic sperm sorting (MSS) as a simple, non-invasive, and fast method showed a potential to overcome these issues.

Objective: This study aimed to access the efficiency of a novel MSS to sort high-quality sperm compared to the routine density gradient centrifugation (DGC) and swim-up (SU) methods.

Materials and Methods: In the present comparative experimental study, semen samples collected from 50 men with normal semen analysis and 50 infertile men to sort using MSS, DGC, and SU techniques and to compare sperm parameters and fertilizing potential. To determine sperm fertilizing potential expression level of 2 sperm-specific factors, including phospholipase C zeta (PLCζ) and post-acrosomal sheath WW-domain-binding protein were evaluated using flow cytometry and sperm DNA integrity was assessed by chromatin dispersion test.

Results: In both groups, the sperm selected by MSS showed the highest progressive motility ($p < 0.0001$), expression of PLCζ ($p < 0.0001$) and the highest fluorescence intensity of PLCζ and post-acrosomal sheath WW-domain-binding protein ($p < 0.0001$ and $p = 0.014$ respectively), and the minimal percentage of non-progressive motility ($p < 0.0001$) and DNA fragmentation index ($p < 0.0001$) compared to DGC and SU techniques.

Conclusion: All sperm parameters including motility, DNA integrity, and fertilizing potential could be affected by sperm sorting method. Our findings demonstrated that microfluidic method for sperm sorting can lead to selection of a subpopulation of sperm with the highest quality from raw semen. This technology can improve clinical outcomes following assisted reproductive technologies in infertile couples.

Keywords: *Spermatozoa, Microfluidics, DNA fragmentation, Sperm-specific proteins, Sperm motility.*

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A-14
Alterations expression of key RNA methylation (m^6A) enzymes in testicular tissue of rats with induced varicocele

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Background: Epigenetics plays a vital role in male fertility and reproductive health. Among RNA modifications, N6-methyladenosine (m^6A) significantly impacts RNA metabolism. Varicocele, a leading cause of male infertility, is associated with oxidative stress, which adversely affects sperm function.
Objective: This study aims to investigate the impact of varicocele-induced oxidative stress on testicular histology, sperm quality, and the expression of RNA modification enzymes including methyltransferase-like 3 (*METTL3*), alkB homolog 5 (*ALKBH5*), fat mass and obesity-associated protein (*FTO*), and YT521-B homology (*YTHDF2*) in a rat model.
Materials and Methods: In this experimental study, 15 male Wistar rats (150-200 gr, 6-8 wk) were randomly assigned to 3 groups: control, sham, and varicocele induction (n = 5/each). Varicocele was surgically induced in the experimental group. After 8 wk, testicular tissues and sperm were collected for analysis. Assessments included histopathological evaluation, sperm parameter analysis, functional tests, and gene expression profiling of key RNA modification enzymes: METTL3 (writer), ALKBH5 and FTO (erasers), and YTHDF2 (reader), using real-time quantitative reverse transcription polymerase chain reaction.
Results: Induction of varicocele resulted in significant histological alterations, including irregular morphology of the seminiferous tubules. Sperm quality was significantly impaired in the varicocele group, evidenced by decreased concentration ($p = 0.006$), and increased abnormal morphology ($p = 0.001$). Markers of oxidative stress such as elevated sperm lipid peroxidation ($p = 0.028$), increased intracytoplasmic reactive oxygen species levels ($p = 0.001$), and heightened DNA damage ($p = 0.006$) were significantly elevated. Gene expression analysis showed downregulation of *METTL3* ($p = 0.001$), *ALKBH5* ($p = 0.006$), *FTO* ($p = 0.008$), and *YTHDF2* ($p = 0.006$) in the varicocele group compared to controls, indicating disrupted m^6A-RNA metabolic processes.
Conclusion: These findings highlight the detrimental effects of varicocele on testicular architecture, sperm quality, and the expression of critical m^6A-RNA modification enzymes. The results contribute to a deeper understanding of the epigenetic modifications underlying varicocele-associated male infertility and may inform future therapeutic strategies.
Keywords: *Chromatin, Oxidative stress, Methylation, Spermatozoa, Varicocele.*

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A-15

Design and implementation of a machine-learning-based method to predict pregnancy loss in women undergoing assisted reproductive technology cycles

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Background: The issue of abortion manifests in approximately 10-15% of natural pregnancies, and this percentage escalates significantly in pregnancies resulting from assisted reproductive methods. Infertile couples experience considerable mental and emotional distress throughout their journey of self-belief therapy. Predicting the outcome of such treatment can alleviate the anxiety of these couples.
Objective: This study aims to construct a machine learning-based method for predicting chemical abortion in infertile women who have conceived through assisted reproductive methods, intending to provide an initial assessment.
Materials and Methods: This retrospective study comprises 2 pivotal phases: 1) data extraction, and 2) model implementation, leveraging the database at the Yazd Reproductive Sciences Institute, Yazd, Iran. The process of addressing duplicate data, variable description discrepancies, and user-generated data entry errors proved to be the most time-intensive aspects of this study. With the invaluable guidance of medical professionals and experts, these challenges were tackled in 2 phases; the extracted data from the study group was meticulously cross-referenced with the registered physical or electronic records to confirm or rectify the information. This process yielded 1234 samples encompassing 32 selected variables. Subsequently, the data pre-processing phase, deemed crucial for model implementation and design, paved the way for the actual modeling. Learning algorithms such as decision tree, random forest, logistic regression, and support vector machine were employed, taking into account the imbalanced nature of the dataset. Balancing techniques, including the synthetic minority over-sampling technique and the adaptive synthetic sampling approach for imbalanced learning algorithms, were explored to enhance the model performance.
Results: In evaluating the models on unbalanced, reduced, and balanced datasets with selected features, the random forest algorithm showed the strongest performance, achieving classification accuracies of 82.2%, 81.18%, and 79.20%, respectively. Among the implemented models, the support vector machine significantly improved recall criteria for miscarriage prediction when applied to the balanced dataset using the adaptive synthetic sampling approach for the imbalanced learning algorithm. This finding emphasizes

the potential impact of data balance strategies in increasing the sensitivity of prediction models, especially for identifying minority class samples. Feature-importance analysis identified 12 key predictors of miscarriage: sperm non-progressive motility, thyroid-stimulating hormone levels, female age, number of previous abortions, body mass index, assisted reproductive technology protocol plan, assisted reproductive technology history, ovarian stimulation protocol, intrauterine insemination history, sperm immotility, normal sperm morphology, and regular menstrual cycles.

Conclusion: This study successfully developed a machine learning-based approach for predicting chemical pregnancy loss in infertile women undergoing assisted reproductive technologies. The results emphasize the importance of the identified variables, which offer valuable insights for clinical decision-making and future research. While the random forest model performed optimally in predicting outcomes, this research highlights the need for further refinement, including the integration of more comprehensive clinical data. Future studies should focus on improving the model's applicability in diverse clinical settings, enhancing its accuracy by incorporating a broader range of clinical factors, and evaluating alternative machine learning techniques for better prediction accuracy.

Keywords: *Infertility, Pregnancy loss, Machine learning, Prediction, Classification.*

A-16

Ultra-rapid freezing and rapid freezing methods in clinical intracytoplasmic sperm injection program: Effects on sperm biological characteristics, DNA methylation stability, DNA methyltransferase activity, and embryo morphokinetics

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Background: Sperm cryopreservation is one of the routine methods in assisted reproductive technology to preserve fertility in men. Currently, methods suggested for sperm cryopreservation are rapid freezing and ultra-rapid freezing. Compared to the rapid method, ultra-rapid freezing is simple and also, there is no need to use cyclopiazonic acid which can be cytotoxic.

Objective: In this study, differences in sperm function and embryo morphokinetics after sperm cryopreservation using ultra-rapid freezing or rapid freezing methods were evaluated compared to fresh sperm. Moreover, regarding role of sperm cryopreservation on DNA methylation and the concern of transferring methylation changes to children, it is necessary to choose sperm cryopreservation method with the highest efficiency.

Materials and Methods: This study is an experimental trial. A total of 30 samples of normozoospermia were divided equally into three groups of fresh, ultra-rapid freezing and rapid freezing. In rapid freezing, the sperm suspension was placed horizontally on nitrogen vapor. In ultra-rapid freezing, the sperm suspension was directly immersed in liquid nitrogen using a straw-in-straw system. Sperm performance was assessed in terms of motility, morphology, viability, mitochondrial membrane potential, sperm DNA fragmentation, and acrosome reaction status. Also, the effects of the 2 freezing methods on global DNA methylation and DNA methyltransferase activity were compared. In addition, 730 embryos in three groups were cultured using time-lapse imaging up to day 6 to assess embryo morphokinetic activity.

Results: Progressive motility (38.80 ± 4.21 vs. 34.86 ± 4.19; $p < 0.001$) and viability (64.30 ± 6.24 vs. 58.10 ± 8.69; $p < 0.01$) in ultra-rapid freezing were significantly higher than rapid group. DNA fragmentation and acrosome reaction were significantly increased in both cryopreserved groups ($p < 0.001$). However, DNA fragmentation (16.30 ± 1.14 vs. 14.33 ± 2.94; $p < 0.01$) was significantly higher in the rapid than the ultra-rapid freezing group. No significant differences were noted in global DNA methylation ($p > 0.05$) and DNA methyltransferases activity ($p > 0.05$) in fresh compared to cryopreservation groups. The kinetic times, including tPB2, tPNa, tPNf, t2, t3, t4, t5, t6, t7, t8, and tM, showed a significant delay in cell divisions in both cryopreservation groups. Furthermore, tPNa, tPNf, and t8 occurred with a significantly higher delay in embryos fertilized by sperm from the rapid freezing compared to the ultra-rapid freezing group. In addition, blastocysts formation was similar in both cryopreservation groups.

Conclusion: The superiority of sperm progressive motility, viability, DNA integrity and embryo kinetics were observed in the Ultra-rapid freezing compared to the rapid method. Ultra-rapid freezing preserved the sperm biological integrity and lead to better embryo morphokinetics compared to the rapid freezing method. However, both methods of sperm cryopreservation were epigenetically safe.

Keywords: *Human spermatozoa, Ultra-rapid freezing, Rapid freezing, Sperm DNA fragmentation, DNA methylation, DNA methyltransferases, ICSI, Time-lapse, Embryo development.*

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