T.C.

AYDIN ADNAN MENDERES UNIVERSITY

GRADUATE SCHOOL OF HEALTH SCIENCES

HISTOLOGY AND EMBRYOLOGY (MEDICAL)

MASTER'S PROGRAM

ANALYSIS OF SPERM CYTOPLASMIC mRNA CONTENT WITH SPECIAL REFERENCE TO THE STORAGE TEMPERATURE AND ITS POSSIBLE IMPACT ON EARLY EMBRYONIC DEVELOPMENT

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MASTER THESIS

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LIST OF ABBREVIATIONS

ART : Assisted Reproductive Technology

CDS : Coding Sequence

DNA : Deoxyribonucleic Acid

EGA : Embryonic Genome Activation

ICSI : Intracytoplasmic Sperm Injection

IVF : In-vitro Fertilisation

PGT : Preimplantation Genetic Testing

RNA : Ribonucleic Acid

RNA Pol : DNA-directed/dependent RNA polymerase

ZGA : Zygote Genome Activation

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ÖZET

FARKLI SAKLAMA SICAKLIĞINA BAĞLI OLARAK SPERMATOZOONLARDA SİTOPLAZMİK mRNA İÇERİĞİNDEKİ DEĞİŞİMLERİN ERKEN EMBRİYONİK GELİŞİM ÜZERİNE MUHTEMEL ETKİLERİ

Özdemir M. Aydın Adnan Menderes Üniversitesi, Sağlık Bilimleri Enstitüsü, Histoloji ve Embriyoloji (Tıp) Programı, Yüksek Lisans Tezi, Aydın, 2022.

Amaç: Yardımcı üreme teknikleri, infertil çiftlerin gebe kalmaya yönelik tedavisinde kullanılan teknikler bütünüdür. Infertilite ise, bir yıl süren korumasız ve düzenli cinsel ilişkilerden sonrasında gebe kalmama durumudur. Yardımcı üreme teknikleri IVF, ICSI, kriyoprezervasyon, ilaç tedavisi gibi tıbbı uygulamaları kapsamaktadır.

Yardımcı üreme tekniklerinin tarihi MÖ 2200 antik Mısır zamanına kadar uzanmaktadır. Tıbbi bir müdahale olan suni tohumlama ise 1784 yılında Spallanzani tarafından geliştirilmiştir. İnsanlarda ilk ‘tüp bebek’ olan IVF bebeği 25 Temmuz 1978'de İngiliz Oldham General Hastanesi'nde dünyaya gelmiştir. Bu başarıyı elde eden jinekolog Dr. Patrick Steptoe ve fizyolog Robert Edwards sonra da Nöbel Ödülüne layık görülmüştür. ICSI tekniği ise Gianpiero D. Palermo tarafından Brükseldeki Reprodüktif Tıp Merkezinde şans eseri olarak bulunmuştu. ICSI tedavisinden sonra ilk bebek 14 Ocak 1992’de doğmuştur. Türkiye’de ise ilk tüp bebek 02.05.1989 yılında Ege Üniversitesi Tüp Bebek Merkezinde doğmuştur.

O yıllardan bu güne her yıl giderek artan sayıda çift infertilite nedeniyle tedavi olmaya başlamıştır. Günümüzde her 100 çiftten 20 si gebe kalma sorunlarını yaşamaktadır. 2015 yılında sadece Avrupa Birliğinde Yardımcı üreme tekniklerini kullanarak 157.500 bebek dünyaya gelmiştir. Bu rakamlar, yardımcı üreme tekniklerinin gittikçe artan önemini göstermektedir.

Tıbbı teknolojinin gelişmesine rağmen Yardımcı üreme teknoloji alanında başarı oranları hala yüksek değildir. Teşhis konan infertilite vakaların yanı sıra 4 vakada 1 gibi yüksek oranda bilinmeyen başarısızlık söz konusudur. Yani, muhtemel unsurların (ovaryum, oviduct, uterus,

endometrium, oosit, sperm) sağlıklı görünmesine rağmen ortaya çıkan infertilite olguları görülmektedir.

Son çalışmalar embriyo genomunun aktifleşmesine kadar zigot gelişiminde rol oynayan sitoplazmik mRNA ların önemine dikkat çekmektedir. Günümüze kadar bu mRNA ların oosit kaynaklı olduğunu, spermde hiç bulunmadığını var sayılmaktaydı. Fakat yeni bulgularına göre, sağlıklı spermatozoitlerin az miktarda sitoplazmaya sahip olduğu fertilizasyon sırasında da bunları zigotun içine getirdikleri belirlenmiştir (Miller, 2010). Bu durumda, maternal mRNA havuzuna az miktarda paternal mRNA da dahil olmaktadır. Yeni çalışmalara göre, sitoplazması olmayan sperm ile ICSI yapıldığında embriyonun arest olması, bu paternal mRNA havuzunun önemini göstermektedir.

Nedeni bilinmeyen infertilite olgularında paternal mRNA havuzunun araştırması oldukça büyük önem taşımaktadır. Bu araştırmaların sonuçlarına göre ICSI mediumlarının geliştirilmesi, ICSI ve ART teknolojilerinin geliştirilmesine ve başarı oranlarının yükseltilmesine katkı sağlayabilir.

Proje, spermatozoon sitoplazmasındaki mRNA stoğu üzerinde odaklanmaktadır. Zigot gelişiminde önemli rol oynadığı bilinen 5 adet mRNA'nın (TET3, NPM2, CNOT1, CNOT8, XRN1) Oryctolagus cuniculus sperm sitoplazmasında araştırılmış ve saklama koşullarının (4 veya 15 °C) bu mRNA içerikleri üzerine etkileri araştırılmıştır.

Bu mRNA'ların fonksiyonları aşağıda özetlenmiştir.

NPM2: paternal kromatinin yeniden modellenmesi (protamin-histon değişimi),

TET3: totipotensi kazanımı (DNA demetilasyonu),

CNOT1, CNOT8 ve XRN1: mRNA yıkımı.

Bu çalışmanın amacı, yukarıda bahsedilen erken embriyonik gelişimine katılan 5 gene ait mRNA'ların Oryctolagus cuniculus sperm sitoplazmasında tespiti ve farklı saklama koşullarının bunlar üzerine etkisinin belirlenmesidir.

Gereç ve Yöntem: Çalışmada kullanılan beş Yeni Zelanda ırkı erkek tavşan her biri 4-5 kg canlı ağırlığa sahiptir. Fertilitesi bilinen tavşanlar rutin bakım-besleme koşullarında (16-28°C sıcaklık ve %55-60 nisbi nem oranı) bireysel kafeslerde barındırılmış, ad libitum su ve arpa pelleti ile beslenmiştir. Tez çalışmasında, erkek tavşanlardan suni vajen ile toplanan sperma örnekleri kullanılmıştır. Hayvanlar, ortamda herhangi bir yapay ışık düzenlemesi yapılmaksızın gün ışığına maruz bırakılmıştır. Deney sonunda hiçbir tavşanlar öldürülmemiştir. Spermatolojik

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1. Spermanın alınması ve ön muayenesi

Suni vajen yardımıyla alınan spermaların mikroskobik kontrollerinde kitle hareketi 3 ve üstü (0-5 arası skalada), motilitesi %70 ve üstü, anormal spermatozoon oranları ise %20'nin altında olan ve yoğunluğu 250x106 spermatozoa/ml olmalarına özen gösterilmiştir. Tavşanlar arasındaki bireysel farklılıkları yok edebilmek amacıyla yukarıdaki koşullarını sağlayan ejakulatlar 37°C su banyosunda birleştirilip pool edilerek kullanılmıştır.

2. Spermanın Sulandırılması ve Deneysel Grupların Oluşturulması

Birleştirilmiş ejekülatlar iki eşit kısma ayrıldı ve önceden 37°C dereceye ısıtılmış Tris-citric acid-glukoz medyumu kullanılarak spermatozoon yoğunluğunun 50x106/ml olacak şeklinde sulandırıldı. Sulandırma sonrası örnekler 2 kısma ayrılıp ilk bölümü 4°C de ve ikinci bölümü de 15°C de 72 saat süresince saklandı. Taze olarak alınan bir ejekülat da kontrol grubu olarak bu iki gruba eklenip, 3 grup ayrı ayrı olarak %50-75 lik percoll-TCG kolonu üzerine yerleştirildikten sonra, 1200 RPM de 10 dk santrifüj işlemiyle ölü ve yabancı hücrelerin (lipid damlacıkları) ayrılmaları sağlandı. Tüplerdeki supernatant uzaklaştırılıp tabanda kalan sperm pelleti 2ml TCG solüsyonu içinde sulandırıldı.

3. mRNA izolasyonu

TCG solüsyonundaki sperm örneklerinden total RNA izolasyonu Trizol ile gerçekleştirildi, RNA örnekleri -80 ˚C'de derin dondurucuda saklandı.

4. RT-PCR çalışmaları

-cDNA sentezi

cDNA sentez kiti kullanılarak cDNA sentezi Applied Biosystems thermal cycler'da gerçekleştirilmiştir.

-qRT-PCR

qRT-PCR reaksiyonları SYBR Green PCR Master Mix ve TET3, NPM2, CNOT1, CNOT8, XRN1 primerleri kullanılarak Analizler Applied Biosystems Step One RT-PCR Sistemi ile gerçekleştirilmiş, kontrol gen olarak GAPDH kullanılmıştır.

Normalizasyon işleminde örnekler arasında ilgili genin ifadesinin karşılaştırılma amacıyla ΔC_T metodu kullanılmıştır. Karşılaştırmalı ifade seviyesi, $2^{-\Delta\Delta C_T}$ formülü kullanılarak hesaplanmıştır.

5. İstatistiksel Analizler

Gruplarda veriler mean±SEM olarak sunulmuş olup grupların parametreler yönünden karşılaştırılma maksadıyla T testi kullanılmıştır.

Bulgular: Araştırma sonucuna göre, yukarıda belirtilen beş gen transkriptleri Oryctolagus cuniculus sperm sitoplazmasında tespit edilmiş olup onlardan miktar olarak en çok CNOT8 ve CNOT1, en az ise XRN1 ve NPM2 gen transkriptleri bulunmuştur. Ayrıca, araştırmada TET3 transkriptlerinin saklama sıcaklığına duyarlı olup 15°C'de saklanan grupta 4°C'de saklanan gruba göre daha yüksek miktarda tespit edilmiş ve gruplararası bu farkın istatistiksel olarak anlamlı ($P<0.05$) olduğunu saptanmıştır.

Sonuç ve Öneriler: Bu çalışma, sperm sitoplazmasındaki mRNA araştırma alanındaki henüz yetersiz olan literatüre, ayrıca da Oryctolagus cuniculus alanındaki literatür havuzuna önemli katkıda bulunmaktadır.

Spermatozoonların saklanma sıcaklıklarının hücre içindeki mRNA havuzunu değiştirebildiği kanısına varılmıştır ($P<0.05$).

Ayrıca, sonraki araştırmalarda primer hazırlığı ve pipet seçimi gibi konularda dikkatli olunması tavsiye olunmaktadır.

Anahtar kelimeler: sperm sitoplazması, paternal mRNA, embriyo gelişimi, Oryctolagus cuniculus.

ABSTRACT

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ANALYSIS OF SPERM CYTOPLASMIC mRNA CONTENT WITH SPECIAL REFERENCE TO THE STORAGE TEMPERATURE AND ITS POSSIBLE IMPACT ON EARLY EMBRYONIC DEVELOPMENT

Ozdemir M. Aydın Adnan Menderes University, Graduate School of Health Sciences, Histology and Embryology (Medical) Program, Master's Thesis, Aydın, 2022.

Aims: The current study aims to investigate five mRNA necessary for chromatin decompaction and demetilation and mRNA degradation in Oryctolagus cuniculus sperm cytoplasm. The investigation was implemented on three groups, one fresh control and two stored at different temperatures such as 4°C and 15°C to investigate also an impact of storage temperature on possible mRNA self decay.

The results would be beneficial for understanding of maternal – paternal genes interplay during the very first hours of the embryonic development and the impact of paternal genes on the basic tasks implementation just before the embryonic genome activation (EGA). This knowledge would help to improve ART success rates by development better medium, storage and ICSI techniques for the cases with unexplained infertility.

Materials and Methods: Sperm for the current project was collected once or twice a week (during 7 weeks) using artificial vagina from adult male rabbits known to be fertile. After dilution, the samples were divided into two equal groups. The first group of the samples was stored at 4°C and the second one was stored at 15°C for 72 hours. At the end of this period, a freshly taken ejaculate after examinations was also added as a control group (without previous storage). The mRNA was isolated, cDNA synthesized and qRT-PCR performed. For the gene expression comparison among the groups the ΔC_T method was used as a normalization method, and the Independent Samples one-sided T Test was used to decide on the significance of the intergroups differences with the 0.05 level of significance. Statistic analyses were performed using SPSS (Statistical Packag for Social Sciences) for Windows 22.

Results: The study has confirmed that sperm cytoplasm indeed contains paternally produced mRNA, and this study could determine five gene transcripts in Oryctolagus Cuniculus sperm cytoplasm: CNOT1, CNOT8, TET3, NPM2 and XRN1. Moreover, CNOT8 and CNOT1 were present the most, XRN1 and NPM2 were present the least. According to the Independent Samples T Test, TET3 transcripts' sensitive to low storage temperatures was claimed with 87% of statistical power at 0.95 significance.

Conclusion: The current study has met it's research goals succesfully. It has added valuable research data to the field of research in sperm cytoplasmic content and to the whole field of embryology. This research contribution has also added to the field of knowledge on Oryctolagus cuniculus species.

Keywords: sperm cytoplasm, paternal mRNA, embryo development, Oryctolagus cuniculus.

1. INTRODUCTION

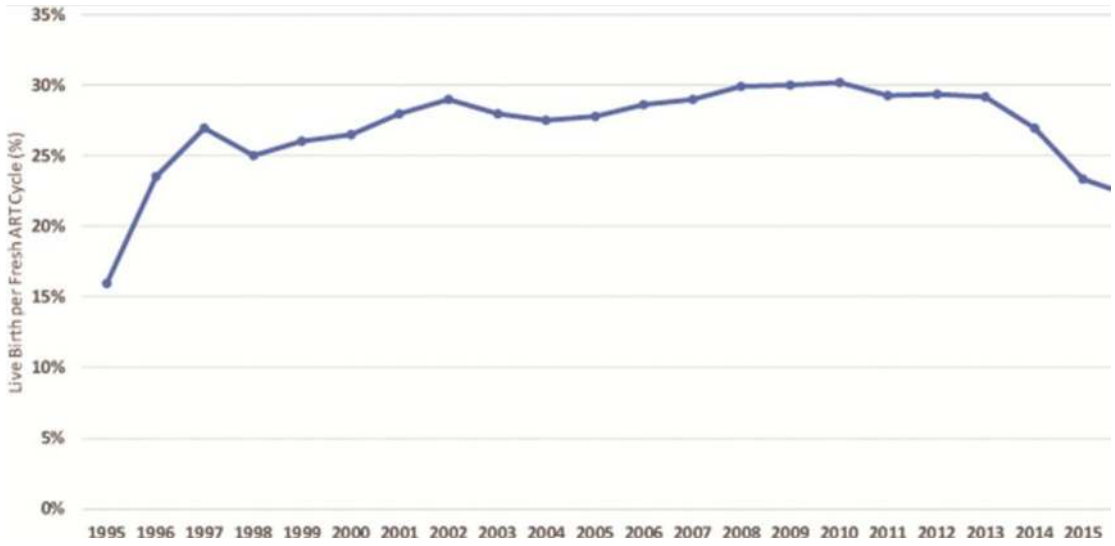
1.1. ART Global Overview and Infertility Problems

Assisted Reproductive Technology (ART) is a whole set of medical treatments for the condition of infertility (Erol, 2011). According to Center for Disease Control and Prevention (CDC), Infertility is a condition of inability to conceive during 1 year of unprotected sexual intercourse (CDC, 2022). As an infertility treatment, ART includes a wide range of procedures such as drug therapy, ICSI, IVF, PGT, cryopreservation. The main aim of ART is to help infertile people to conceive (Karaca, 2012). To be considered an ART procedure, the treatment must include the removal of the oocytes from the body for their processing in a laboratory environment. Treatments such as artificial insemination or just drug therapy are not considered as ART (CDC, 2022).

ART history had started with the birth of the first child following a successful IVF treatment on 25 July 1978. The IVF technique was developed and implemented by gynaecologist Patrick Steptoe and physiologist Robert Edwards in Dr Kershaw's Cottage Hospital. ICSI technique was discovered by chance when Gianpiero D. Palermo in the Center for Reproductive Medicine in Brussels put a spermatozoid into an oocyte cytoplasm by mistake. The first child was born following a successful ICSI treatment on 14 January 1992 (Palermo et al., 1992).

Since then a number of IVF and ICSI children were born. More and more people use ART every year worldwide. According to Research and Markets, infertility affects 8%-10% of couples all around the globe therefore, the IVF global market is estimated to reach $51.51 billion by 2030 (Research and Markets, 2021).

Despite an increasing in IVF demand, the success rates are not increasing, with an absolute maximum of around 30% (Picture 1). The success of the treatment is defined as live birth rate per IVF or ICSI cycle (Gleicher et al., 2019).



Picture 1. Live birth rates (Gleicher et al., 2019).

The probable causes of infertility traditionaly includes age, gamet quality, lifestyle, physical conditions such as endometriosis but in 1 out of 4 infertility cases the cause can not be identified (NHS, 2022). Sperm evaluation is a promising research area in investigation of unidentified infertility. The routine tests focus on appearance and motion of spermatozoa. However, there are invisible factors that are normally outside of consideration (Lambard, 2004). One of this promising factors is a sperm cytoplasm. Its existence was even not considered until very recent times. The spermatozoon cytoplasm enters oocyte upon fertilisation and contributes to oocyte cytoplasm with mRNA playing important roles in the early embryo development (Cooper, 2004; Park, 2013).

1.2. Fertilisation Events and Early Embryonic Development

Fertilisation may be defined as a fusion of two haploid cells into a diploid one. The cells participating in the fusion are an oocyte and a sperm. The fusion forms a new cell that is called a zygote or one cell embryo. Upon the fusion the newly formed cell has to performe some vital tasks in order to develop into a multicellular organism. These tasks logically should include preparing both sets of chromosomes for transcription and replication (protamine-to-histone change, chromatin archytecture establishment, chromatin movement), synthesis, processing and transport of transcripts, proteins and other molecules. The great challenge is that while ordinary cells use a well established cycle of moleculer production, the newly formed zygote is yet to create the very basis for it. Until that moment the zygot does not even have a chromatin

in form available for transcription. Therefore, the only source of production is an available cytoplasmic stock of necessary materials. This stock has to include a minimum set of ingredients at a minimum required concentration level. The stock comes with oocyte and sperm cytoplasm but mostly with oocyte's cytoplasm. Until recently, sperm cytoplasm was ignored but the recent research points to a number of unexplained embryo arrests bounded to sperm non-chomosomic issues (Zafar, 2021). An increasing number of studies concentrate on sperm cytoplasmic droplet (Cooper, 2004).

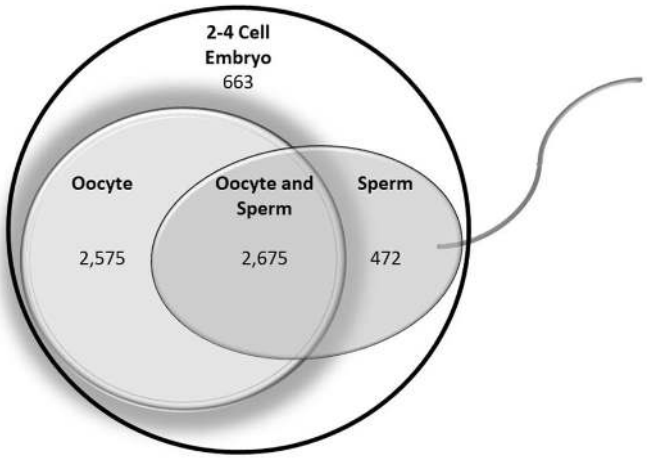
In order to establish a minimum required ingredients set one should take a look into the necessary events performed by the cell.

Upon fertilisation the newly arrived paternal DNA undergoes decondensation, protamine-to-histone change, demetilation, epigenetic reprogramming and chomatid archytecture reformation (Gerrard, 2020). At the same time pronuclei form and get closer to each other. Later on, pronuclear membranes dissolve, mitotic spindles form and reorganize chromosomes getting them ready for the first mitotic division. In mice zigote, this reorganisation is the first step toward zigote genome activation (ZGA), in the human zigote, ZGA occures much later. ZGA in mice occures as early as at the 2-cell stage, it means, embryo's newly formed chromatids archytecture has reached the level of maturation enough for the beginning of producing its own transcripts. Once the embryo starts its own mRNA production and processing, the old cytoplasmic stock of foreign mRNA must be rapidly degraded, as any delay will cause fatal upregulation and disruptions in embryo development. At this point, the story of maternal-paternal mRNA is ended.

Summarising mRNA adventures, the initial cytoplasmic stock consists of mRNAs produced in ovary and testicles during gametogenesis, maternal stock acquires in the folicular and oocyte cytoplasm with a later addition of paternal mRNAs from the sperm cytoplasm. One should keep in mind that although the fraction of paternal mRNA is small, deficiencies cause heavy disruptions in development and even embryonic arrests (Zafar, 2021; Lambard, 2004). This phenomenon may be explained by underlying genetic mechanisms of the early embryonic events explained above.

The early embryonic events require expression of hundreds of genes, and mRNA stock may be considered as a direct parental gene expression. Recent studies (Gross, 2019) revealed 472 sperm-born, 2575 oocyte-born, 2675 both sperm- and oocyte-born, and 663 embryo-exclusive RNAs (Picture 2). Although it is impossible to cover all of these genes in a single

study, a few of them may throw some light on the interplay of maternal-paternal mRNAs during the early embryonic development.



Picture 2. The origin of RNA in 2-4 cell Bovine embryo (Gross, 2019)

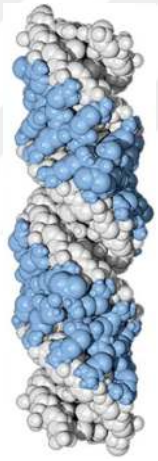
For the current study, a few genes participating in two important events of the early embryonic development were chosen. They are protamine-to-histone change and mRNA degradation mechanisms. The main reason for this choice is a possible requirement of mRNA being sperm born or oocyte born for specific parental or maternal targets. These two mechanisms are examples of successful maternal-paternal genomes teamwork.

1.3. Protamine-to-histone change and reprogramming

In normal cells the DNA molecule called chromatid consists of a double strain DNA wrapped around histone octamers linked to each other by linker histone protein. A histone octamer with wrapped DNA is called a nucleosome. It consists of four types of histone proteins each of which has a tail used for regulation of DNA wrapping tension by addition of certain groups (Me, Ac) to certain positions. Sperm DNA is packed mostly with protamines. The histone-to-protamine reorganisation takes place during spermiogenesis starting from the round spermatids (Wang et al., 2019). During this process the histones are gradually replaced firstly by transitional proteins and then by protamines P1 and P2 (Gou et al., 2020). Although histones are essential elements of the normal functional chromatin archetecture, they are unwanted in compact sperm head structures as they cause fragility in transcriptionally silent sperm chromosomes. Smaller protamines are proved to work better for cromatid compaction preventing chromosome damage in harsh environment (Miller et al., 2010). The logic behind

this is the reduction of the possibility of chromosom damage by compacting them into a small space.The problem with protamines is that they cannot carry epigenetic informations such as tail metilation and acetylation. That is why sperm chromatids still have to have some amount of histones in conserved epigenetic regions. The histone/protamine ratio is variable among species and even among individuals, counting for epigenetic differences and sperm quality. In humans 10% histone ratio is canonically accepted (Xiaoyang et al., 2006).

Upon fertilisation this compact chromatin structure has to become decompacted for normal cell cycle functions such as replication and transcription. It is achieved by reverse protamine-to-histone change. Protamines are short proteins 50-110 amino acids long (Picture 3), consisting of Arginine rich major groove DNA binding domain and Cystein containing domain that is responsible for inter-protamines disulfide bonds formation. Therefore, the protamine scaffolded DNA structure may be imagined as a tight ribbon braided hair for the sole purpose of tight and compact holding (Balhorn, 2007).

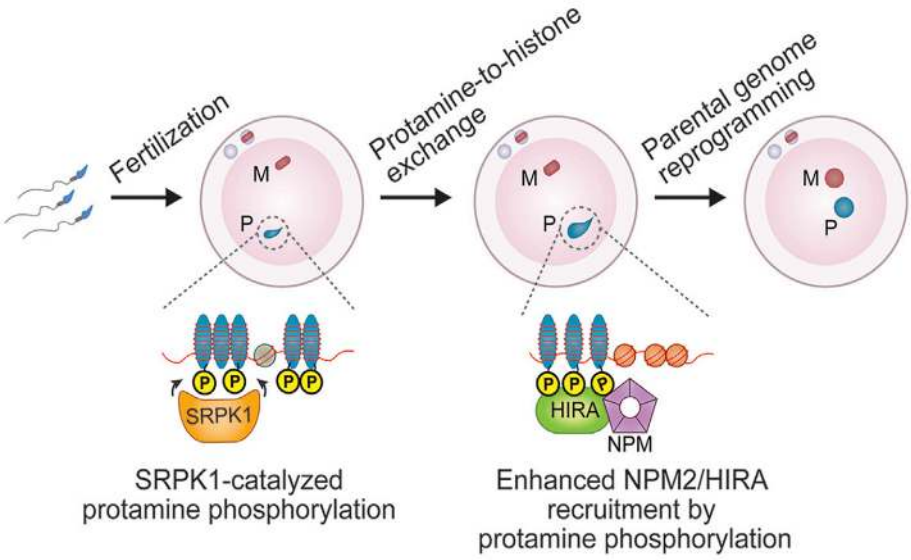


Picture 3. The protamine structure (Balhorn, 2007).

The exact process of protamine-to-histone change is not yet very clear but logically it should include a disulfide bonds reduction so, the chaperone can induce exchange of protamines with core histones. Recent studies propose that the phosphorylation of the protamines promotes binding of NPM2 chaperone for the protamine removal (Picture 4).

At the same time, paternal histones and newly incorporated maternal histones with epigenetic marks have to be demetilated to convert the chromatins of terminally differentiated haploid germ cells into totipotent ones (Gerrard, 2020). Totipotency is the state of ability to give raise

of all tissue specific cell types of the embryo including extraembryonic tissues. This demetilation job is done mainly by Ten eleven translocation (TET) proteins.



Picture 4. Paternal genome reprogramming (Gou et al., 2020).

In the current study TET3 and NPM2 mRNAs were chosen for investigation of their presence in the sperm cytoplasm.

1.4. Parental (maternal) mRNA degradation

The fusion of two haploid gametes creates a unique diploid organism genetically different from each of its parents, this is the main logic of the sexual reproduction. Thus, any parental mRNA is actually a foreign agent for the new embryo. That is why it is necessary to destroy them as soon as the embryo's genome is activated and able to produce its own mRNAs. So, the other important problem for the early embryo is the parental mRNA degradation.

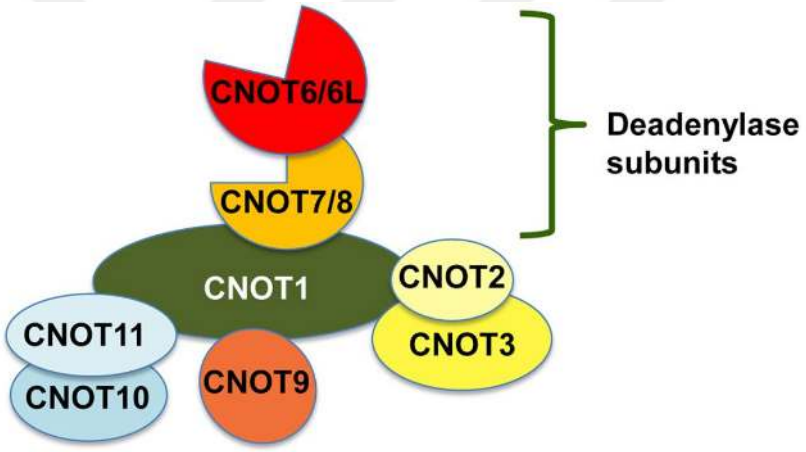
Protein production is a vital celular process so its regulation is well tuned at different stages. The first step of protein production is a creation of mRNA that is the future protein template. This step is called transcription and implemented by RNA Pol II that reads DNA and produces RNA copies. Free RNA may pose a danger to the cell so, it is rapidly degraded by exonucleases. Thus, mRNA gets protective 5'-cap and poly(A) tail upon its production by RNA Pol II (Picture 5). When in cytoplasm, mRNA is stabilised by poly(A) binding proteins (PABP) (Siwaszek et al., 2014).

Picture 5. mRNA structure (Siwaszek et al., 2014).

Logically, the degradation process will be started upon mRNA protection removal. Therefore, mRNA degradation pathways include 5'-cap and poly(A) tail removal.

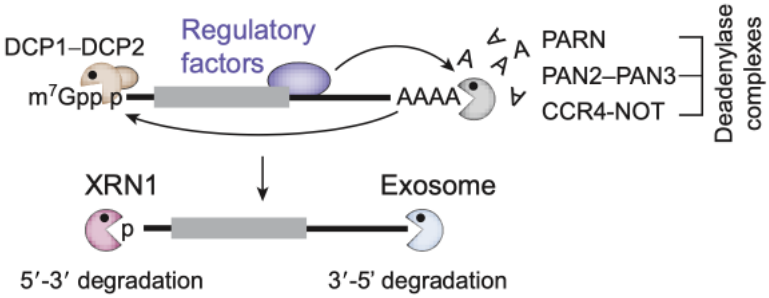
Cytoplasmic mRNA's poly(A) tail removal called Deadenylation is performed in two steps: initially by PAN2–PAN3 and subsequently by the CCR4-NOT complex.

Carbon catabolite repression 4 (CCR4)–negative on TATA-less (NOT) complex is consists of CNOT subunits with CNOT1 as a scaffold for the others (Shirai et al., 2014) (Picture 6).



Picture 6. CCR4-NOT complex (Shirai et al., 2014).

Deadenilation is followed by decapping or 3'>5' decay and subsequent 5'>3' decay or cap hydrolisis. Decapping is implemented by the Dcp2 enzyme and is followed by 5'>3' mRNA degradation performed by by Xrn1 exonuclease (Picture 7) (Siwaszek et al., 2014).



Picture 7. Degradation scheme of mRNA (Despic and Neugebauer, 2018).

1.5. Study Aims and Benefits

In summary, the two processes above require a teamwork of a number of genes. At the very first moments of the new embryo's life, it is not these genes themselves but their parental homologs' mRNA transcripts that have to be used. The early zygot's mRNA pool consists of maternal and paternal mRNAs, and they have to have a precise interplay mechanism. Maternal mRNA complexes must be 'ignited' by the incomining paternal mRNA for the magic to start. The recent studies determined different sperm-born and oocyte-born mRNAs in zigot and 2-cell bovine embryo. Interestingly, transcripts of different parts of the same complex were found to belong to different parents. For example, CCR4-NOT complex's parts CNOT1 and CNOT8. While the scaffold CNOT1 was oocyte-born, CNOT8 was found to be sperm-born (Fang, 2010). It means, that the complex will be activated only after fertilisation, thus ensuring protection of mRNA in the oocyte. In contrast, mRNAs participating in the protamine-to-histone change process logicaly should be oocyte-born to ensure the proper compaction of paternal chromatids.

The current study aims to investigate five mRNA necessary for chromatin decompaction and demetilation and mRNA degradation in Oryctolagus cuniculus sperm cytoplasm. The investigation was implemented on three groups, one fresh control and two groups stored at different temperatures, 4°C and 15°C respectively, to investigate an impact of storage temperature on possible mRNA self decay.

The results would be beneficial for understanding of maternal – paternal genes interplay during the very first hours of the embryonic development and the impact of paternal genes on the basic tasks implementation just before the embryonic genome activation (EGA). This knowledge would help to improve ART success rates by development of better medium, sperm storage environment and ICSI techniques for the cases with unexplained infertility.

1.6. Hypotheses

In order to investigate a dependence of mRNA to the storage temperatures the significance of internal group variance will be compared to the intergroups' one using T Test for Independent Groups. The Statistical Power found will determine the rejection of the Null Hypothesis.

The working hypotheses for each gene expression are as following:

H_1: There is a significant difference in mRNA quantities between the group stored at 4°C and the group stored at 15°C.

H_0: There is **NO** significant difference in mRNA quantities between the group stored at 4°C and the group stored at 15°C.

2. OVERVIEW

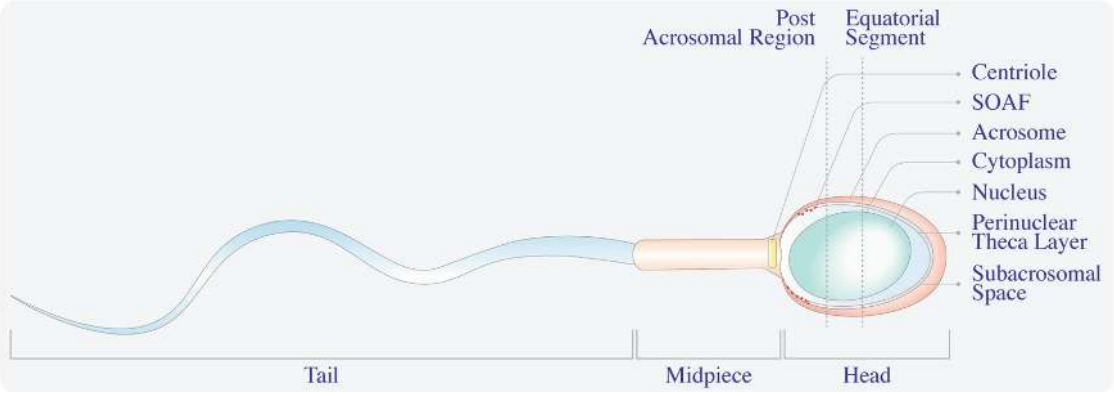
2.1. Biological Material used

The current study is focused on investigation of the mRNA content in the sperm cytoplasm of Oryctolagus cuniculus species.

Rabbits are very valuable in medical research as they are a good model for human reproductive system and also have a high breeding rate. The average litter size is six but the female may become pregnant again imediately after giving birth because rabbits are induced ovulators. The gestation period is 28-31 days, so theoretically a female can give a birth every month. The newborn rabbit has no fur and has his eyes are closed, it can not maintain its own body temperature (Van der Horst, 2022).

2.1.1. Sperm of Oryctolagus cuniculus species

Sperm may be defined as a haploid cell created as a result of spermatogenesis process in male testicles. As the sole purpose of its existence is an oocyte fertilisation, the sperm cell (spermatozoon) has a unique structure. It has a characteristic head with hard acrosome shielding nucleus with tightly packed chromosomes. The sperm is the only cell using small protamine proteins for tight chromosome packing to reduce a possible chromosome harm in the outside environment. Additionally, it has a long tail (flagella) to be able to compete with the fellow sperm for the oocyte, and a mid piece with mitochondria providing the flagella with energy. The recent studies indicated an existence of a cytoplasm that plays an important role in embryonic development by providing paternal mRNAs and other factors (Picture 8).



Picture 8. Spermatozoon structure (Zafar et al., 2021).

Rabbit's ejaculate (0.6 - 1ml of volume) is easily collected with an artificial vagina (Picture 9), thus avoiding any harm to the animals. The semen of Oryctolagus cuniculus consists of two parts, the first part is gelatinous (it creates a copulation plug, so the sperm does not flow out) and the second one is fluid.

Picture 9. Artificial vagina and SEM micrograph of rabbit sperm in oviduct (Van der Horst, 2022).

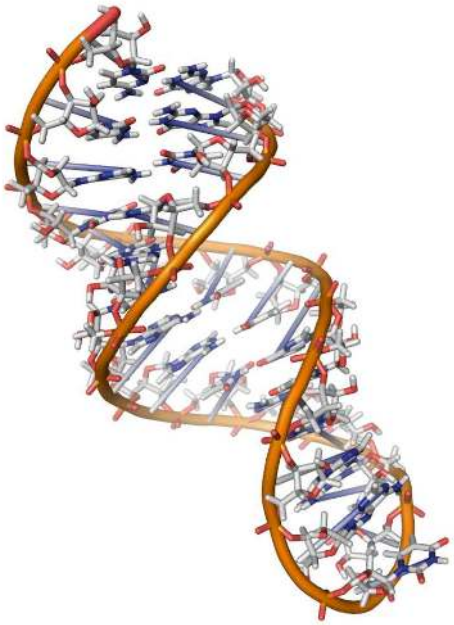
Rabbit's sperm usually has very good characteristics with 200 million sperm per ejaculate, 80% motility and 80% normal forms (Van der Horst, 2022).

2.1.2. mRNA

mRNA is an RNA molecule with a 5'cap and polyA tail that carries a genetic information from the DNA in the nucleus to the cell cytoplasm for ribosomic protein production.

RNA Polymerase enzyme produces a single strand RNA from the DNA template, this process is called transcription. As RNA strand starts to emerge the Capping enzyme associated with RNA Pol adds a 5'cap to the first nucleotide. The polyA tail is added by polyadenylate polymerase after transcription has been terminated. The newly made pre-mRNA then undergoes splicing that is a process of removing non-coding regions by spliceosome complex. Eventually the mature mRNA is ready for the protein syntesis by the ribosome. The process of translation is done by ribosome, such as every amino acid has a coding codone consisting of three nucleotides on mRNA. So, the ribosome reads mRNA 3 by 3 nucleotides. This coding system is called the Genetic Code.

The RNA molecule's structure is unstable, it may form helical structures and loops, its free ends are highly dynamic (Picture 10).



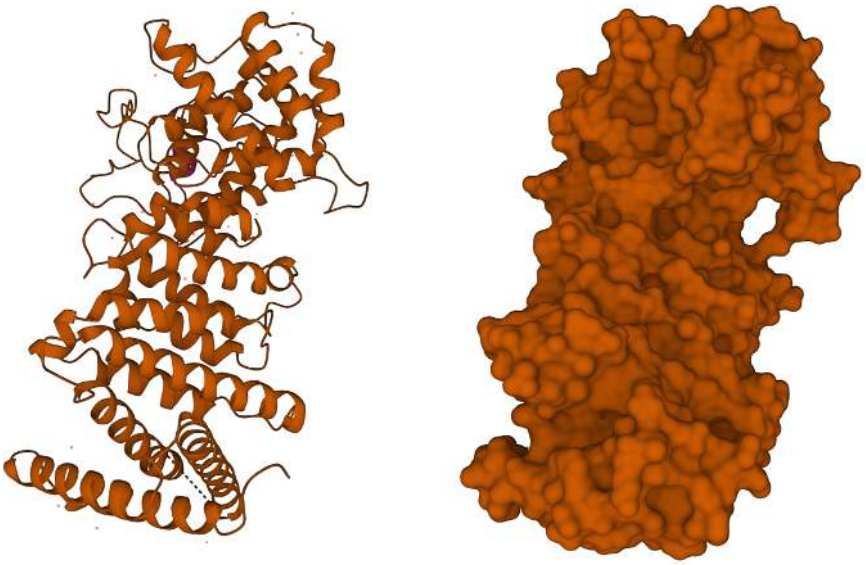
Picture 10. RNA structure (Creative Biolabs, 2022).

2.1.3. Genes of Interest and their Transcripts

The current study is focused on five genes CNOT1, CNOT8, NPM2, TET3 and XRN1. The genes described below are highly similar (>95%) Homo Sapience orthologs as there are no crystal structures and not enough research data available on Oryctolagus Cunuculus genes.

2.1.3.1. CNOT1

CNOT1 (CCR4-NOT Transcription Complex Subunit 1) gene is located on 16th Chromosome at 16q21 (Uniprot entry A5YKK6). The precise location is from 58,519,951 to 58,629,826 bp, that gives its total length of 109,876 nt. The coding sequence (CDS) length is 7,131 nt and after transcription and splicing it leaves 3572 bp mRNA. Post-translational protein's length is 2,376 aa and molecular mass is 266939 Da, that means, the protein is very big (Picture 11). The size is in line with it's function as CNOT1 is a central scaffold protein of the major cellular mRNA deadenylase participating in bulk mRNA degradation (GeneCards, 2022; NCBI, 2022).

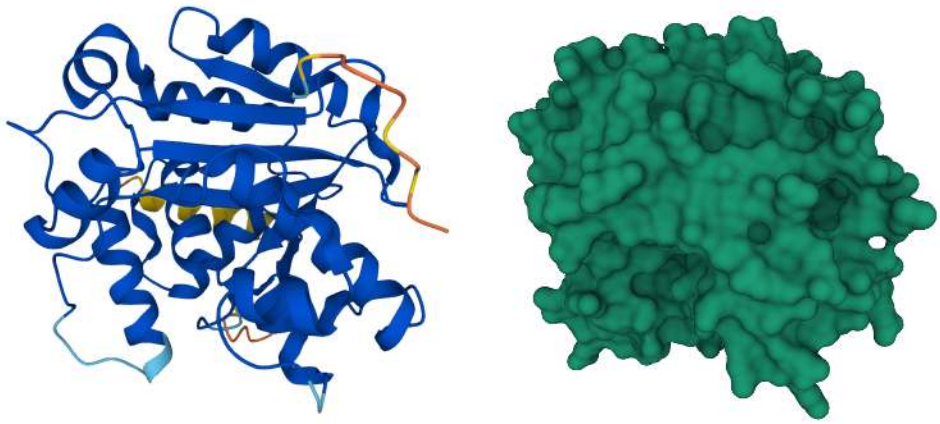


Picture 11. CNOT1 (CCR4-NOT Transcription Complex Subunit 1) structure: PDB entry 4CQO (RCSB, 2022).

CNOT1 gene's mRNA transcript investigated in this study is a linear RNA molecule 3572 nt long with unstable folded structure, ENA entry AL117492 (ENA,2022).

2.1.3.2. CNOT8

CNOT1 (CCR4-NOT Transcription Complex Subunit 8) gene is located on 5th Chromosome at 5q33.2 (Uniprot entry Q9UFF9). The precise location is from 154,858,627 to 154,876,792 bp, that gives its total length of 18,166 nt. The coding sequence (CDS) length is 879 nt and after transcription and splicing the mRNA's length is 2265 bp. Post-translational protein's length is 292 aa and molecular mass is 33540 Da (Picture 12). CNOT8 is a part of CCR4-NOT Transcription Complex and it binds to central scaffold protein of this major cellular mRNA deadenylase participating in bulk mRNA degradation (GeneCards, 2022; NCBI, 2022).

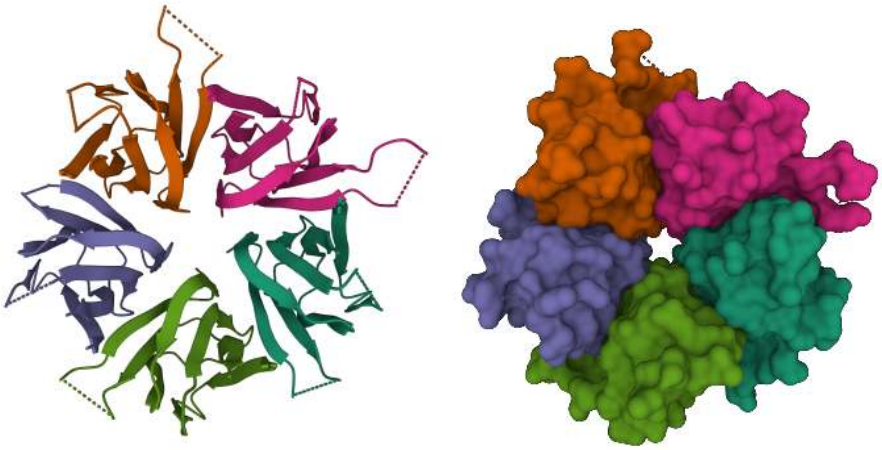


Picture 12. CNOT8 (CCR4-NOT Transcription Complex Subunit 8) structure: Uniprot entry Q9UFF9 (UniProt, 2022).

CNOT8 gene's mRNA transcript investigated in this study is a linear RNA molecule 2265 nt long with unstable folded structure (NCBI, 2022).

2.1.3.3. NPM2

NPM2 (nucleophosmin/nucleoplasmin 2) gene is located on 8th Chromosome at 8p21.3 (Uniprot entry Q86SE8). The precise location is from 22,024,134 to 22,036,897 bp, that gives its total length of 12,764 nt. The coding sequence (CDS) length is 645 nt and after transcription and splicing the mRNA's length is 2265 bp. Post-translational protein's length is 214 aa and molecular mass is 24152 Da (Picture 13). NPM2 is a core histone chaperone participating in sperm chromatin decondensation (GeneCards, 2022; NCBI, 2022).

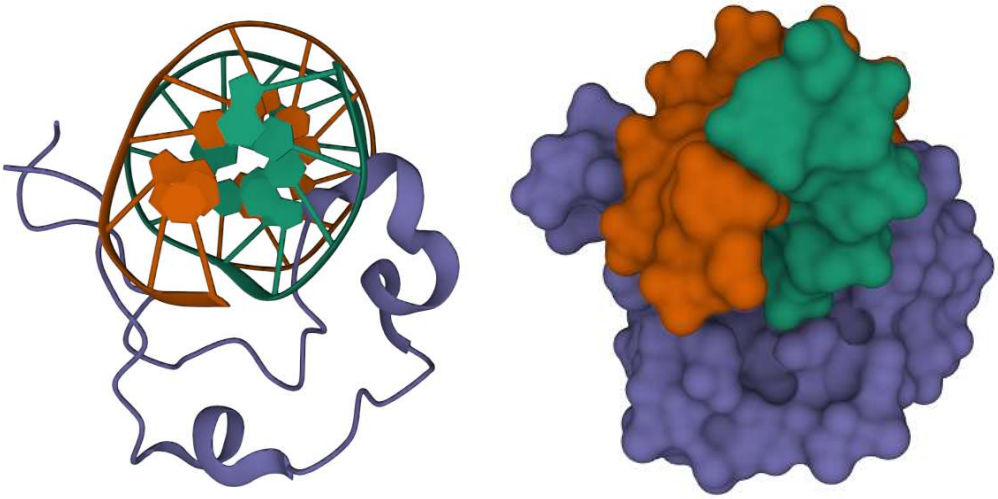


Picture 13. NPM2 (nucleophosmin/nucleoplasmin 2) structure: PDB entry 3T30 (RCSB, 2022).

NPM2 gene's mRNA transcript investigated in this study is a linear RNA molecule 1208 nt long with unstable folded structure, ENA entry AY262113 (ENA,2022).

2.1.3.4. TET3

TET3 (tet methylcytosine dioxygenase 3) gene is located on 2nd Chromosome at 2p13.1 (Uniprot entry O43151). The precise location is from 73,984,910 to 74,108,176 bp, that gives its total length of 123,267 nt. The coding sequence (CDS) length is 5,388 nt and after transcription and splicing the mRNA's length is 5388 bp. Post-translational protein's length is 1,795 aa and molecular mass is 193705 Da that means, the protein is very big (Picture 14). TET3 dioxygenase catalyzes conversion of 5mC into 5hmC participating in paternal DNA base cytosine demethylation and paternal epigenetic reprogramming (GeneCards, 2022; NCBI, 2022).



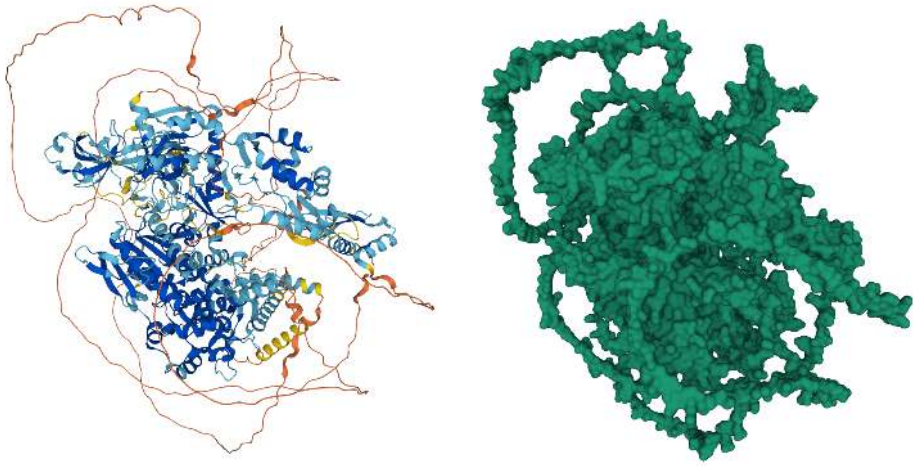
Picture 14. TET3 (tet methylcytosine dioxygenase 3) structure: PDB entry 4HP3 (RCSB, 2022).

TET3 gene's mRNA transcript investigated in this study is a linear RNA molecule 5388 nt long with unstable folded structure, ENA entry HQ220209 (ENA,2022).

2.1.3.5. XRN1

XRN1 (5'-3' Exoribonuclease 1) gene is located on 3rd Chromosome at 3q23 (Uniprot entry Q8IZH2). The precise location is from 142,306,610 to 142,448,037 bp, that gives its total length of 141,428 nt. The coding sequence (CDS) length is 5,085 nt and after transcription and

splicing the mRNA's length is 5121 bp. Post-translational protein's length is 1706 aa and molecular mass is 194107 Da, that means, the protein is very big (Picture 15). XRN1 participate in mRNA 5'-3' degradation (GeneCards, 2022; NCBI, 2022).

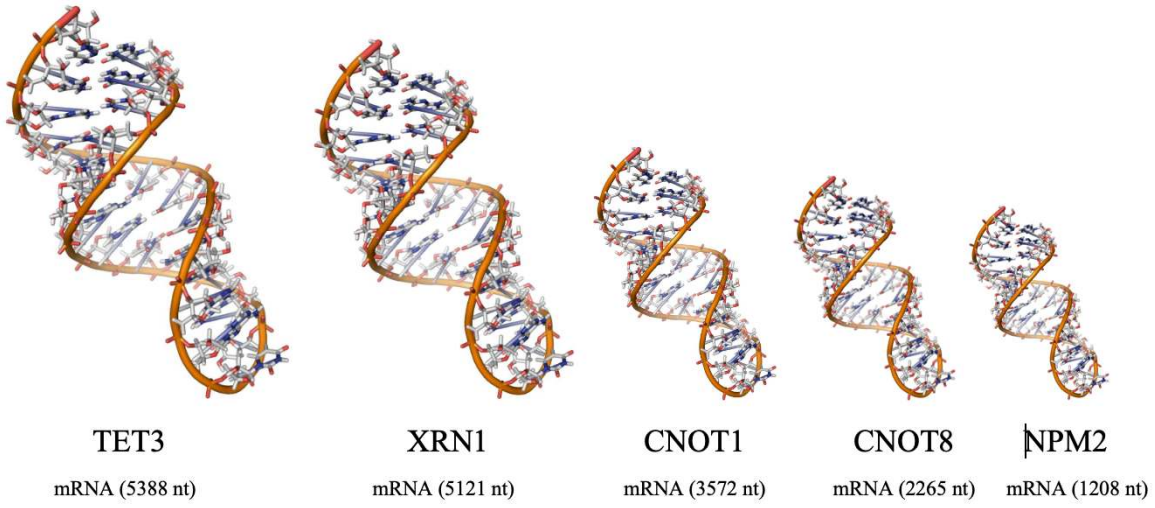


Picture 15. XRN1 (5'-3' Exoribonuclease 1) structure: Uniprot entry Q8IZH2 (UniProt, 2022).

XRN1 gene's mRNA transcript investigated in this study is a linear RNA molecule 5121 nt long with unstable folded structure, ENA entry AY137776 (ENA,2022).

2.1.3.6. mRNA size comparison

According to the length of the RNA molecule the sizes may be inferred as following (Picture 16).



Picture 16. Comparative size analysis of the transcripts.

RNA molecule is unstable due to the reactive hydroxil group in it’s base structure. It constantly folds and unfolds forming loops, helices and other tetriary structures. Thus, it may be inferred, molecular instability increases with the length.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Equipment

The current study was performed using the follow equipment at the laboratory of Adnan Menderes University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination and at the laboratory of Adnan Menderes University, Faculty of Medicine.

The following equipment was used during the study:

Hettich centrifuge universal 320R,

Vortex Nüve NM 110,

Samsung Deep freezer/ Fridge,

Applied Biosystems thermal cycler,

Applied Biosystems Step One RT-PCR System,

Thermo Scientific NanoDrop 2000 spectrophotometer.

3.1.2. Chemicals

The following chemicals were used during the current study for different purposes.

- *For the semen dilution:*

Tris – Citric acid – Glucose (TCG) medium (Sigma-Aldrich): 250mM Tris (hydroxtmethylaminomethan), 79mM Citric acid, 69mM glucose, 75 IU streptomycin and 166.20 IU penicillin G (299mOsm/kg and pH 7.14)

- *For the mRNA extraction:*

Trizol (ABP Biosciences)

Chloroform (AppliChem)

Glycogen (Sigma-Aldrich)

Isopropanol (Sigma-Aldrich)

75% ethanol (Merck)

RNase-free distilled water (GeneAll)

- *For the cDNA synthesis:*

A.B.T.TM cDNA Synthesis Kit (High Capacity) 50 Rxn

- *For qRt-PCR reaction:*

Primers (Sentebiolab)

A.B.T.TM 2X qPCR SYBR-Green MasterMix, w/ROX, 5ml

3.1.3. Animal Model

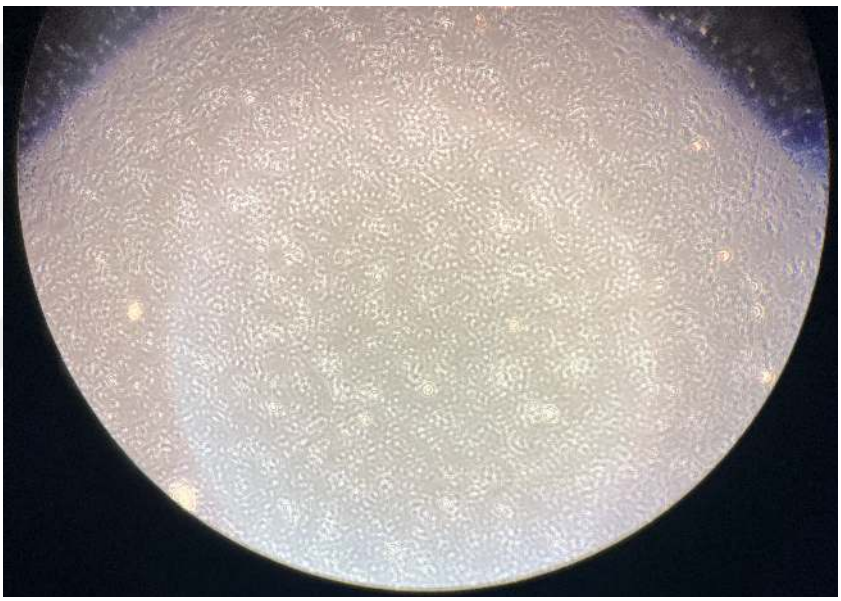
Rabbit is considered to be good model organism for andrologic research because of its low cost, quite big size and genital organs accesability. Five New Zealand male rabbits (Oryctolagus cuniculus) with a live weight of 4-5 kg were used in the current study. During the study, animals were fed *ad libitum* with tap water and barley pellet. The semen samples for the experiment were collected from male rabbits by artificial vagina method. Adult and fertile males were housed in individual cages under the routine conditions applied in Adnan Menderes University, Faculty of Veterinary Medicine with a room temperature of 16-28°C and a relative humidity of 55-60%. Animals were exposed to daylight, no artificial light was used. All spermatological procedures were done in the laboratory of Adnan Menderes University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination. Rabbits were not sacrified at the end of the Project (no any rabbit was harmed).

3.2. Method Details

3.2.1. Semen collection and pre-examination

Ejaculates were collected once or twice a week (during 7 weeks) using artificial vagina (Picture 17) from adult male rabbits known to be fertile. After all necessary microscopic controls of the semen collected (Picture 18), only spermatozoa with mass motility of 3 and above (in the scales between 0-5), motility of 70% and above, abnormal sperm ratios below 20% and density of 250x106 spermatozoa/ml were included to the experimental pool. In order to eliminate individual differences, the suitable samples were combined into a single pool in a 36.5°C water bath (Picture 19).

Picture 17. Artificial vagina preparation steps: internal membrane placement, warm water filing, spreading vaseline to the anterior part, ready for use device.

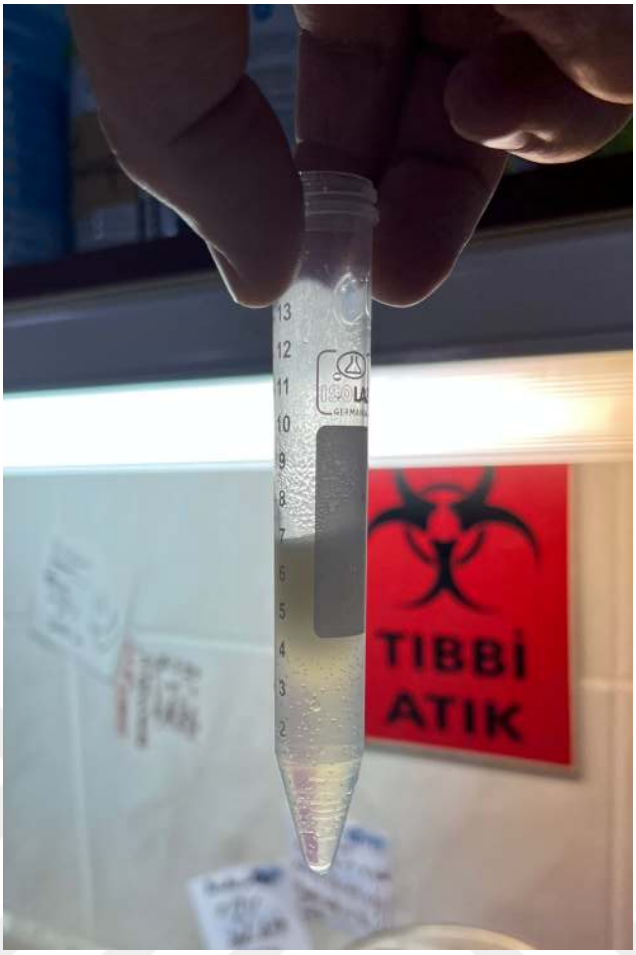


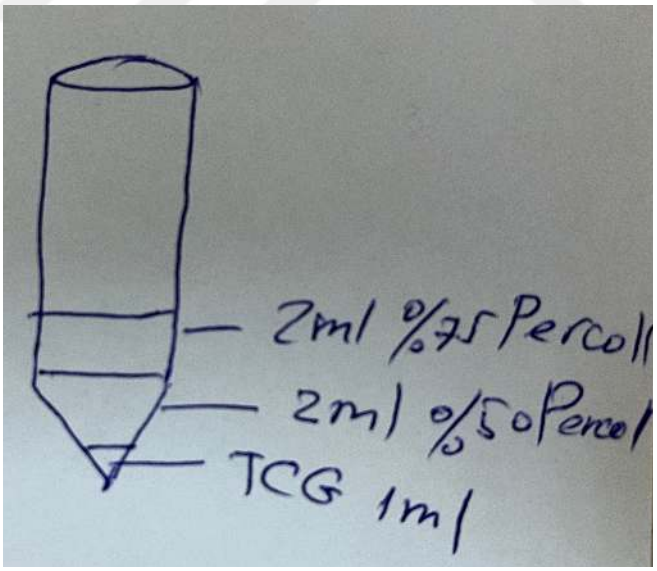
Picture 18. Microscopic control of the semen collected.

Picture 19. Water bath.

3.2.2. Dilution of Sperm and Formation of Experimental Groups

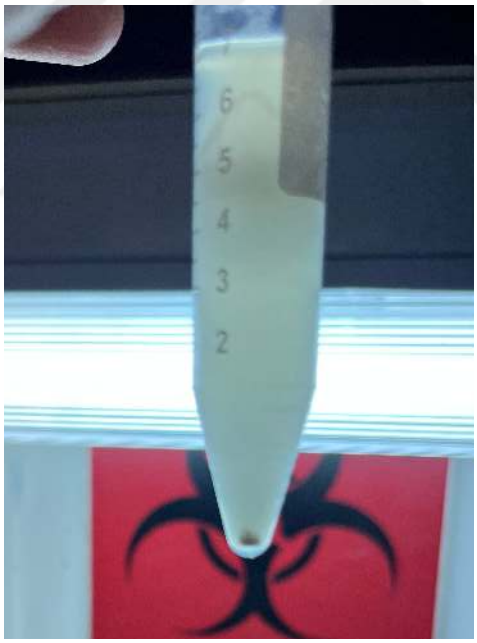
The pool of samples that met the above-mentioned parameters was diluted using preheated to 37°C Tris – Citric acid – Glucose (TCG) medium (1) till the spermatozoon concentration reach 50x106/ml. After dilution, the samples were divided into two equal groups. The first group of the samples was stored at 4°C and the second one was stored at 15°C for 72 hours. At the end of this period, a freshly taken ejaculate after examinations was also added as a control group (without previous storage); then, all 3 groups were separately placed on a 50% percoll-TCG column (Picture 20), and centrifuged at 1200 RPM for 10 minutes (Picture 21) so, the dead cells and the other debris (lipid droplets) other than sperm cells were eliminated (Picture 22). The top substance in the tubes was removed and the bottom part was placed in 2ml of TCG solution for storage.





Picture 20. Semen on percoll-TCG column and the column's scheme.

Picture 21. Centrifugation at 1200 RPM for 10 minutes.



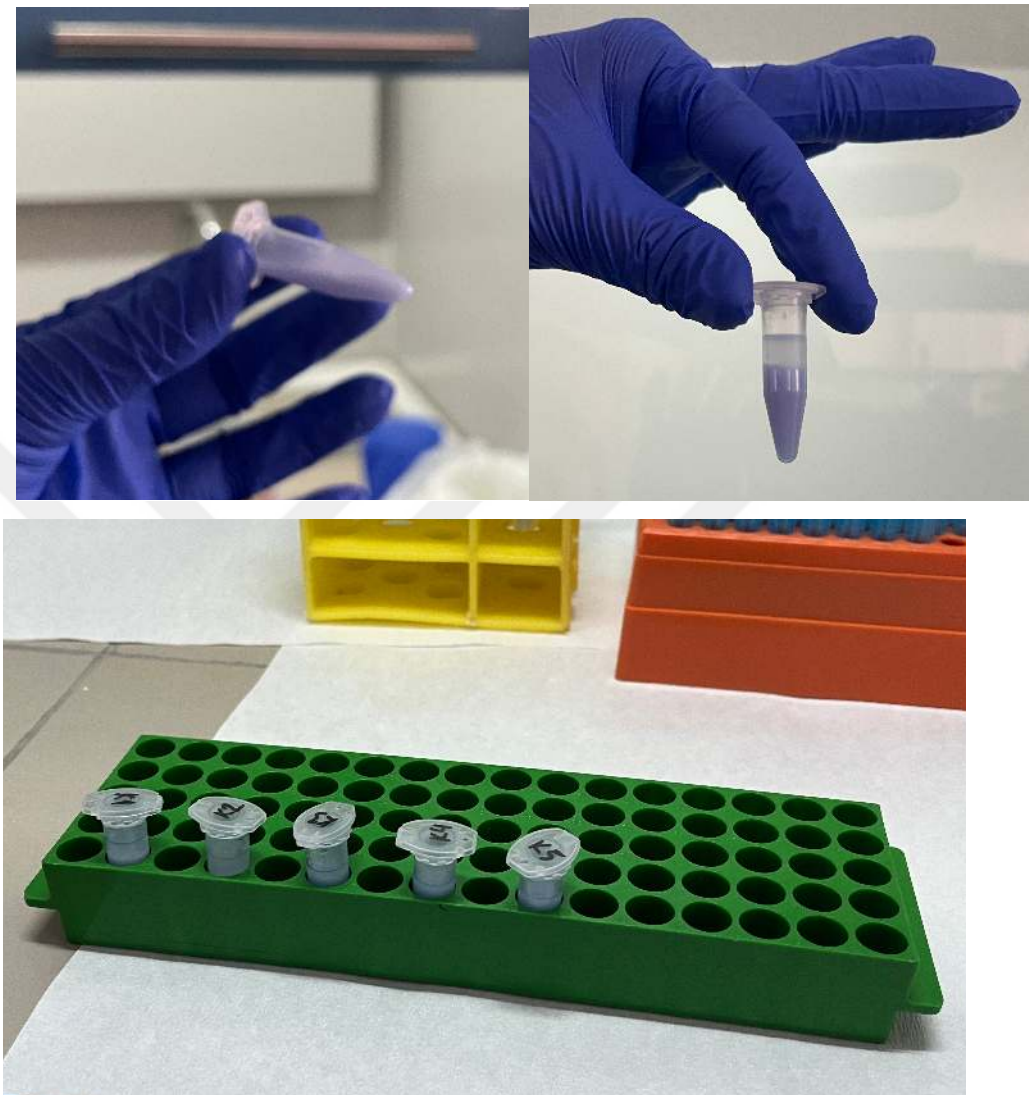
Picture 22. Pellet of spermatozoa after centrifugation.

3.2.3. mRNA isolation

Total mRNA isolation from the semen pool was performed using Trizol according to the following protocol.

1. 1000 µl Trizol was added to 500 µl sperm cells in microcentrifuge tubes.

2. Then 200 µl of chloroform was added to the microcentrifuge tubes and the tubes were turned end over end for 15 seconds (Picture 23).

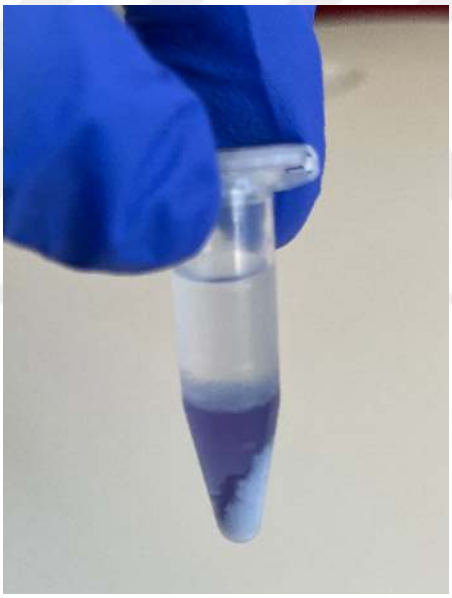


Picture 23. Tubes with Trizol – Chloroform addition.

3. After incubation for 3 minutes at room temperature, the tubes were centrifuged at 12000 g for 15 minutes at 4 ˚C (Picture 24).

Picture 24. Centrifugation.

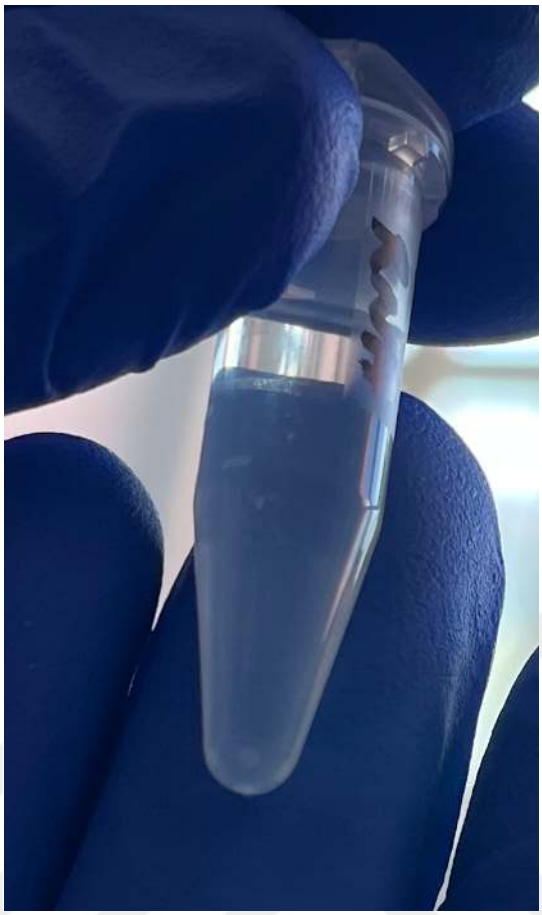
4. After centrifugation, the supernatant (Picture 25) was carefully taken into a new microcentrifuge tube.



Picture 25. Supernatant with nucleotides.

5. 0.5 µl of glycogen (20 µg/µl) and 500 µl of isopropanol were added, mixed and incubated for 10 minutes at room temperature.

6. After incubation, the tubes were centrifuged at 12000 g for 10 minutes at 4 ˚C (Picture 26).



Picture 26. White pellet containing RNA after centrifugation.

7. The supernatant was removed and 1 ml of cold 75% ethanol was added to the pellets; then, the pellets were washed. The tubes were then centrifuged at 7500 g for 5 minutes at 4 ˚C.

8. The supernatant was removed and the mRNA pellets (Picture 27) were left to dry for 5-10 minutes.

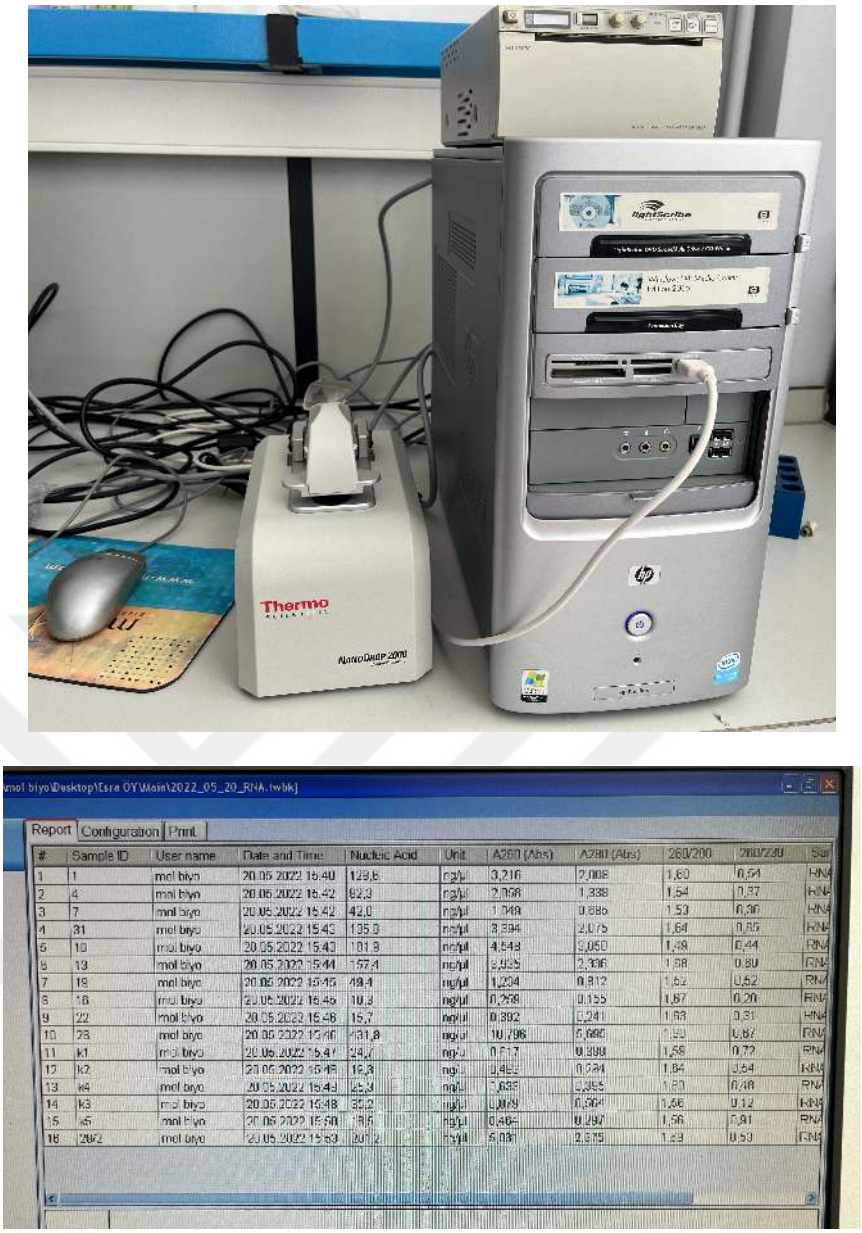
Picture 27. White pellet of RNA after the washing.

9. 30 µl of RNase-free distilled water was added to the pellet and incubated for 10 minutes at 55-60 ˚C (Picture 28).

Picture 28. Incubation.

10. Dissolved mRNA samples were stored in a deep freezer at -80 ˚C.

Before the samples were put into the deep freezer, the RNA concentration (ng/µl) was checked using Thermo Scientific NanoDrop 2000 spectrophotometer (Picture 29).



Picture 29. The RNA concentration (ng/µl) by NanoDrop 2000.

3.2.4. RT-PCR

3.2.4.1. cDNA synthesis

In order to determine the mRNA expressions of the relevant genes by RT-PCR, first of all, cDNA synthesis was performed using the cDNA synthesis kit. The reaction ingredients used in cDNA synthesis is given in Table 1, and the cDNA synthesis reaction conditions are given in Table 2. Reactions were performed in an Applied Biosystems thermal cycler.

Table 1. cDNA synthesis reaction mix

Reaction ingredients	Quantity
10X Reaction Buffer	2 µl
RNase Inhibitor (40 U/µl)	0,5 µl
20X dNTP mix (2.5 mM each)	1 µl
Random hexamer (50 µM)	2 µl
Reverse Transcriptase (200 U/µl)	1 µl
RNase free water	3,5 µl
RNA template	10 µl

Table 2. cDNA synthesis conditions

RT phases	Temperature (◦C)	Time	Number of cycles
1	25	10 min	1
2	37	120 min	1
3	85	5 min	1
4	4	∞	1

3.2.4.2. qRT-PCR

qRT-PCR reactions were performed using SYBR Green PCR Master Mix and primers TET3, NPM2, CNOT1, CNOT8, XRN1, with GAPDH as the control gene (Table 3). Reactions were performed on the Applied Biosystems Step One RT-PCR System.

The content of the RT-PCR reaction mix is given in Table 4, the reaction conditions are given in Table 5.

Table 3. Real-Time PCR primers

TET3	(F) 5'- GGGAGGAAGCAGTGACAGAG-3' (R) 5'- ACCAGTCCTTTCCACCACAG -3'
NPM2	(F) 5'- CTCTCACGGTAGGCTGGAAG -3' (R) 5'- GGACCTTTCGTGAGAAGCTG -3'
CNOT1	(F) 5'- GTGTTCCTGGTTGGCATTCT -3' (R) 5'- AAAGGACTCGGGACACACAC -3'

CNOT8	(F) 5'- CATTGTTGCATCTGCCAATC -3' (R) 5'- CTTCTAAGGCAAGCCCACTG -3'
XRN1	(F) 5'- CAGCAGTATGCCACTCTCCA -3' (R) 5'- CTGTGCTCTGTCAAGGGTGA -3'
GAPDH	(F) 5'- GTCGGAGTGAACGGATTTGG -3' (R) 5'- AAAGCAGCCCTGGTGACCA -3'

Table 4. RT-PCR reaction mix

2X qPCR SYBR-Green MasterMix	10 µl
ROX Dye (50X)	0.4 µl (0.04µl)
Forward Primer (10 µM)	0.2 - 2 µl
Reverse Primer (10 µM)	0.2 - 2 µl
cDNA template	1 µl
RNase-free distilled water	Up to 20 µl total

Table 5. RT-PCR reaction conditions

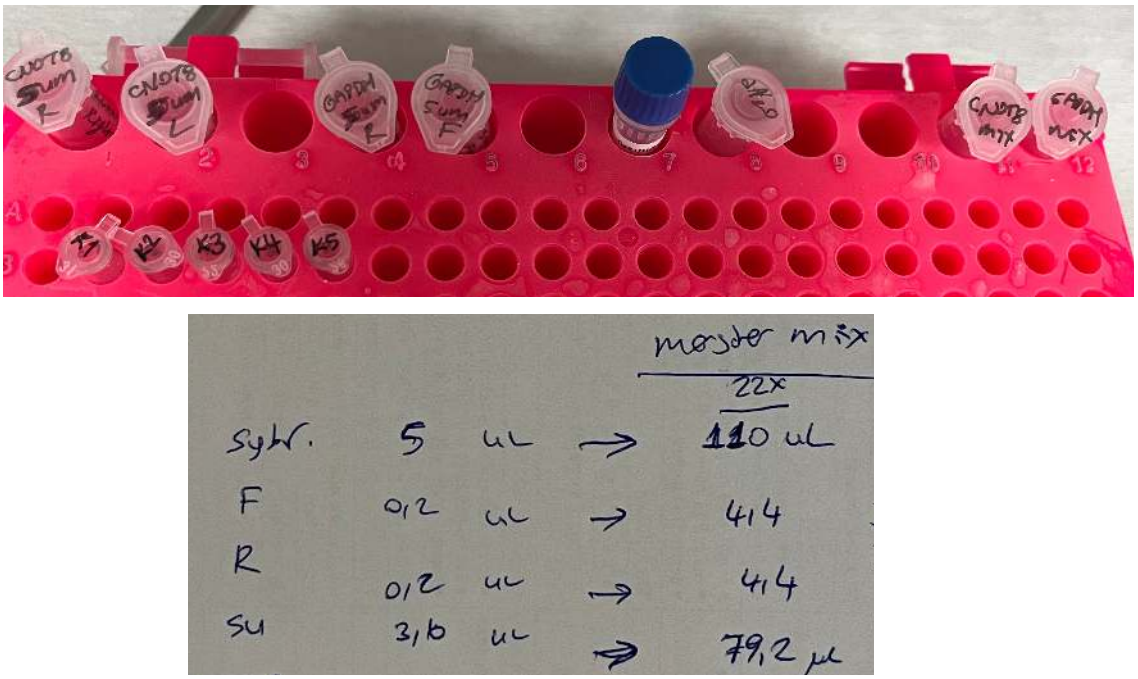
qRt-PCR phases	**Temperature (◦C)**	**Time**	**Number of cycles**
1st Denaturation	95	5 min	1
Denaturation	95	10-30 sec	40
Primer binding and elongation	55-68	10-60 sec	
Melting curve	65-95	2-5 sec/step	1

The total number of performed qRT-PCR reactions was 15 (three groups x 5 genes).

The plate for each qRT-PCR reaction cycle had two regions (gene and GAPDH) (Picture 30).

Picture 30. Reaction plate and the scheme of wells' arrangement.

First of all, the Master mixes for gene and GAPDH plate regions were prepared (Picture 31), then, wells were filled according to the protocol (Table 4).



Picture 31. Master mix content and preparation plate.

The plate was loaded into the Applied Biosystems Step One RT-PCR system and the reaction settings were done according to the Table 5 (Picture 32).

Picture 32. RT-PCR run settings.

3.2.5. Statistical Analysis

For the gene expression comparison among the groups the ΔC_T method was used as a normalization method. The most common method for relative quantification is the $2^{-\Delta\Delta C_T}$ method. The normalization process begins with calculations of the difference between the C_T values of the target and the C_T value of the endogenous control:

$\Delta C_T = C_T$ (target gene) - C_T (endogenous control)

This value was calculated separately for each group. The fresh group was selected as a reference. The comparative $\Delta\Delta C_T$ calculation aims to find the difference between the ΔC_T value of each group and the ΔC_T value of the reference group.

$\Delta\Delta C_T = [C_T$ (target gene) - C_T (endogenous control)] – [C_T (gene of reference group) - C_T (endogenous control of reference group)]

Finally, these calculated values were converted into absolute values using the following formula (2) (assuming 100% efficiency or product doubling per cycle):

Comparative expression level = $2^{-\Delta\Delta C_T}$ (Equation 1)

The data for the groups was presented as mean±SEM and the Independent Samples one-sided T Test was used to decide on the significance of the intergroups differences with the 0.05 level of significance.

Statistic analyses were performed using SPSS (Statistical Packag for Social Sciences) for Windows 22 (SPSS Inc., Chicago, IL, USA) software.

4. RESULTS

4.1. Comparative Gene Expression Levels

The genes comparative expression levels (Table 6) were calculated using Formula 1. The calculation was performed according to the groups' C_T mean values obtained from the Applied Biosystems Step One RT-PCR system.

Table 6. Comparative gene expression levels by groups with extreme values highlighted

	CNOT1	CNOT8	NPM2	TET3	XRN1
Fresh	1	1	1	1	1
	0.1159	0.948	0.0545	3.2266	0.0012
	2.9984	0.9292	0.0002	0.0791	0.0017
	0.4004	0.2273	0.0047	0.2993	0.0111
	0.2527	1.4455	0.1446	0.0891	2.1719
Average:	0.95348	0.91	0.2408	0.93882	0.63718
4˚C	1	1	1	1	1
	2.8972	1.5957	0.6165	4.8056	0.1211
	0.9372	3225883.8	0.2794	0.4487	0.2813
	2.2208	3224494.8	0.1881	0.0366	1.2459
	3.7066		1.4719	0.2403	0.0438
Average:	2.15236	1612595.3	0.71118	1.30624	0.53842
15˚C	1	1	1	1	1
	1.1789	2.5473	30.8463	1.1017	0.6165
	1.0226	33674.738	3.5035	1.7112	0.2794
	1.3115	1578.0051	6.7449	17.2234	0.1881
	1.6297	0.0193	0.2091	1.2136	1.4719
Average:	1.22854	7051.262	8.46076	4.44998	0.71118

4.2. Statistical Analysis

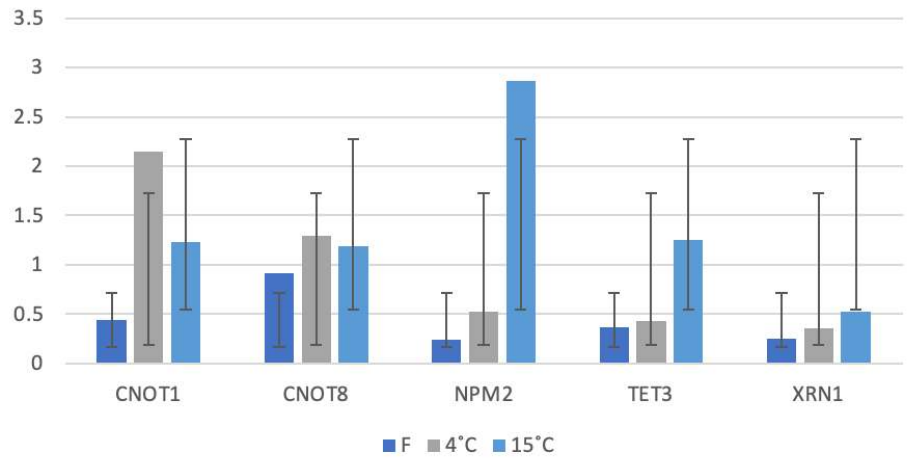
SPSS IBM Software was used for the statistical analysis and interpretation of the results. Extreme values which occured due to possible various errors were eliminated. In order to decrease a possible bias, fresh control groups were further interpreted separately as they were not from the same pool (they were collected later). The groups' descriptive statistics are given below (Table 7, 8) (Graph 1).

Table 7. Descriptive statistics of the groups (SPSS)

	N	Minimum	Maximum	Mean	Std. Deviation
CNOT14	5	.9372	3.7066	2.15236	1.2020483
CNOT115	5	1.0000	1.6297	1.22854	.2573508
CNOT84	2	1.0000	1.5957	1.29785	.4212235
CNOT815	3	.0193	2.5473	1.18887	1.2745387
NPM24	4	.1881	1.0000	.521000	.3686707
NPM215	4	.2091	6.7449	2.86438	2.9435429
TET34	4	.0366	1.0000	.431400	.4147254
TET315	4	1.0000	1.7112	1.25663	.3153557
XRN14	4	.0438	1.0000	.361550	.4369743
XRN115	4	.1881	1.0000	.521000	.3686707
CNOT1	4	.1159	1.0000	.442250	.3895596
CNOT8	5	.2273	1.4455	.910000	.4366784
NPM2	5	.0002	1.0000	.240800	.4283627
TET3	4	.0791	1.0000	.366875	.4341225
XRN1	4	.0012	1.0000	.253500	.4976875

Table 8. Average gene expression levels with extreme values excluded

	CNOT1	CNOT8	NPM2	TET3	XRN1
Fresh	0.44225	0.91	0.2408	0.366875	0.2535
4˚C	2.15236	1.29785	0.521	0.4314	0.36155
15˚C	1.22854	1.18887	2.86438	1.25663	0.521



Graph 1. Average gene expression levels with extreme values excluded.

In order to understand the significance of differences between the groups Statistical Power was determined by Independent Samples T Test (Picture 33).

CNOT1:

	Power[b]	N1	Std. Dev1	N2	Std. Dev2	Mean Difference	Sig.
Test for Mean Difference[a]	.411	5	1.2020483	5	.2573508	.924	.05

a. One-sided test.

b. Based on noncentral t-distribution.

CNOT8:

	Power[b]	N1	Std. Dev1	N2	Std. Dev2	Mean Difference	Sig.
Test for Mean Difference[a]	.062	2	.4212235	3	1.2745387	.109	.05

a. One-sided test.

b. Based on noncentral t-distribution.

NPM2:

	Power[b]	N1	Std. Dev1	N2	Std. Dev2	Mean Difference	Sig.
Test for Mean Difference[a]	.343	4	.3686707	4	2.9435429	-2.343	.05

a. One-sided test.

b. Based on noncentral t-distribution.

TET3:

	Power[b]	N1	Std. Dev1	N2	Std. Dev2	Mean Difference	Sig.
Test for Mean Difference[a]	.867	4	.4147254	4	.3153557	-.825	.05

a. One-sided test.

b. Based on noncentral t-distribution.

XRN1:

	Power[b]	N1	Std. Dev1	N2	Std. Dev2	Mean Difference	Sig.
Test for Mean Difference[a]	.125	4	.4369743	4	.3686707	-.159	.05

a. One-sided test.

b. Based on noncentral t-distribution.

Picture 33. Statistical Power for groups stored at different temperatures for CNOT1, CNOT8, NPM2, TET3 and XRN1 gene expression at 0.05 significance level (SPSS).

5. DISCUSSION

According to the qRT-PCR reaction results, the sperm cytoplasm contains transcripts of all of the genes of interest in different quantities.

The transcripts are RNA molecules of different length with unstable tetriary structures. The sizes of these transcripts were shown above in the following order: TET3 (5388 nt) → XRN1 (5121 nt) → CNOT1 (3572 nt) → CNOT8 (2265 nt) → NPM2 (1208 nt).

As it was also mentioned above, TET3 dioxygenase participates in paternal (but not maternal) DNA base cytosine demethylation and paternal epigenetic reprogramming; XRN1 participate in mRNA 5'-3' degradation; CNOT1 is a central scaffold protein of the CCR4-NOT Transcription Complex that is major cellular mRNA deadenylase participating in bulk mRNA degradation; CNOT8 is a part of CCR4-NOT Transcription Complex and it binds to CNOT1; NPM2 is a core histone chaperone participating in sperm chromatin decondensation.

Logically thinking, TET3 and NPM2 transcripts should not be in close proximity to their target, that is the sperm DNA, to prevent possible early activation, so they should not be present in the sperm cytoplasm. XRN1 is self sufficient for mRNA 5'-3' degradation, so it can be dangerous for the other transcripts in very limited space of the sperm cytoplasm. On the other hand, CNOT1 and CNOT8 are parts of a complex, so they can not act by themselves and are safe for the sperm cytoplasm carriage. Moreover, some CNOT parts should be separated from the others presented in oocyte cytoplasm, so the assembly and activation of the whole complex could be done only after the fertilisation. Being a small part of CNOT mRNA degradation complex, CNOT8 may easily be carried by spermatozoon for integration in CNOT complex presented in oocyte. Previous studies on Bovine embryos (Gross, 2019) indeed provisionally suggested origins of some parts of CCR4-NOT Transcription Complex (Table 9).

Table 9. Paternal origins of the CNOT mRNA in 2-4 cell Bovine embryo (Gross, 2019)

CNOT1	Sperm and oocyte-derived
CNOT10	Sperm and oocyte-derived
CNOT11	Sperm-derived (provisional)
CNOT2	Sperm and oocyte-derived
CNOT3	Oocyte-derived
CNOT4	Oocyte-derived
CNOT6	Sperm and oocyte-derived
CNOT6L	Oocyte-derived
CNOT7	Oocyte-derived
CNOT8	Sperm-derived (provisional)
CNOT9	Sperm-derived (provisional)

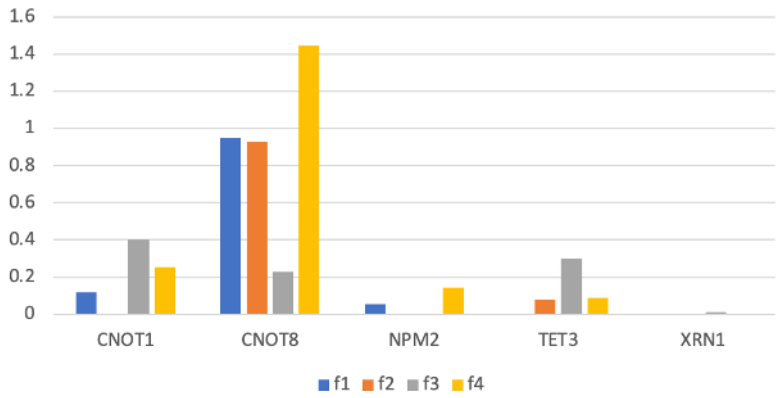
The same study also provisionaly suggested origins of the other mRNA investigated in the current study (Table 10).

Table 10. Parental origins of mRNA in 2-4 cell Bovine embryo (Gross, 2019)

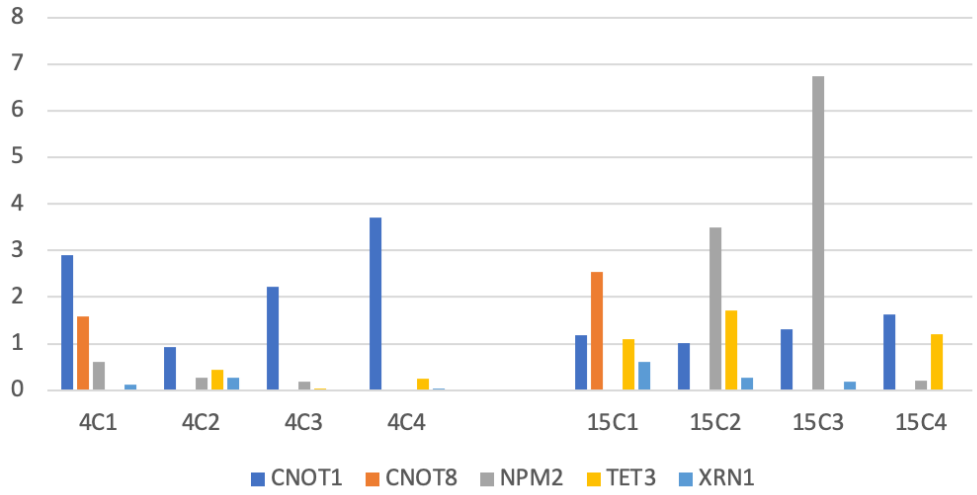
TET3	2-4 cell embryo (provisional)
NPM2	2-4 cell embryo (provisional)
XRN1	Sperm-derived (provisional)

Fresh control group does not belong to the semen pool of two other groups therefore, it was interpreted separately.

In fresh control group CNOT8 presents in the highest quantity, it follows by CNOT1 and TET3 with just a tiny quantity of XRN1 and NPM2 (Graph 2). This pattern is about the same in the stored groups with CNOT1 in higher quantity than CNOT8, they are followed by TET3, XRN1 in a little quantity and just NPM2 present in outstanding high quantity in the group stored at 15˚C (Graph 3).



Graph 2. Fresh control group gene expression pattern

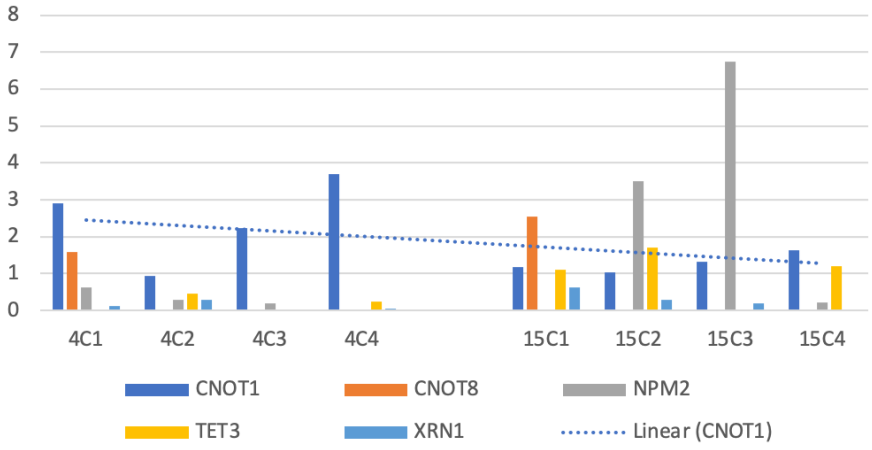


Graph 3. 4˚C and 15˚C groups gene expression pattern

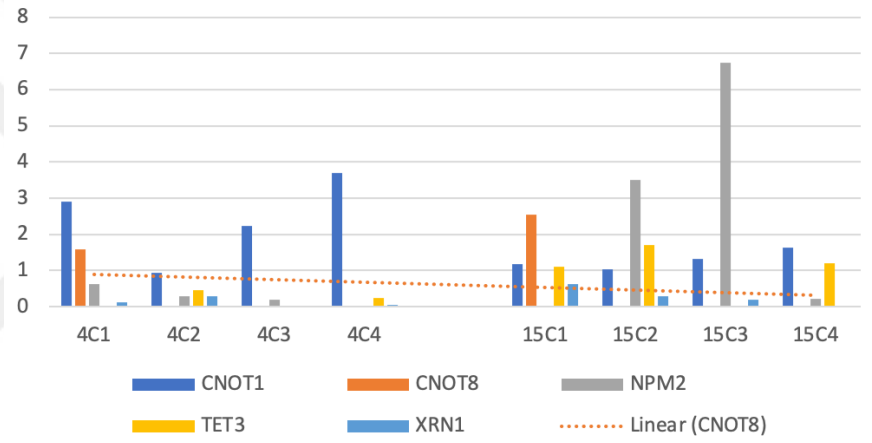
Results of the current study have shown the presence of all five genes in sperm cytoplasm that contradicts previous study findings (Gross, 2019) suggested TET3 and NPM2 not to be sperm-born.

Concentration is very important in biochemistry and it should be taken into consideration rather than the sole existence of a substance. Zygot is very fragile in terms of transcripts concentration, it can not produce it's own ones yet and will arest if their concentration is not sufficient. The presence of the TET3, NPM2, CNOT1 and XRN1 transcripts in the samples in tiny quantities is an evidence of natural backup of spare parts of vital proteins that can not be produced by the embryo until Embryonic Genome Activation.

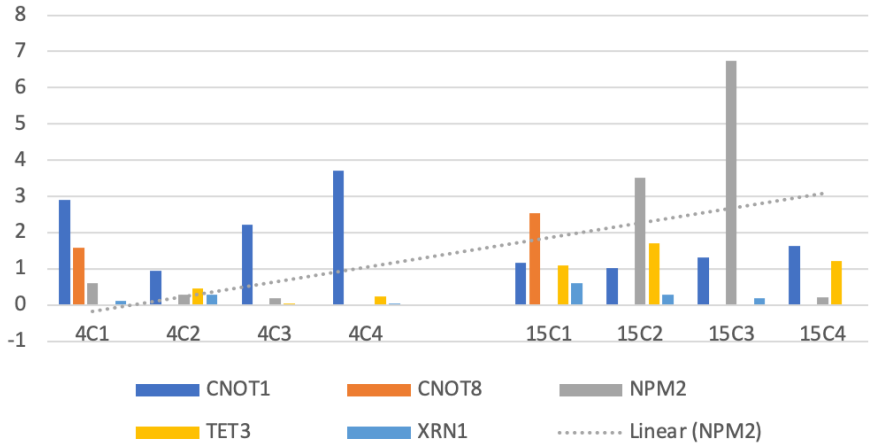
Interpreting the differences between groups stored at different temperatures, according to the trendline MS Excel graphs, while CNOT1 and CNOT8 were present in higher concentration in the group stored at 4˚C, TET3 and NPM2 transcripts' concentration was higher in the other group stored at 15˚C. XRN1 concentration was barely affected by temperature differences (Graphs 4, 5, 6, 7, 8).



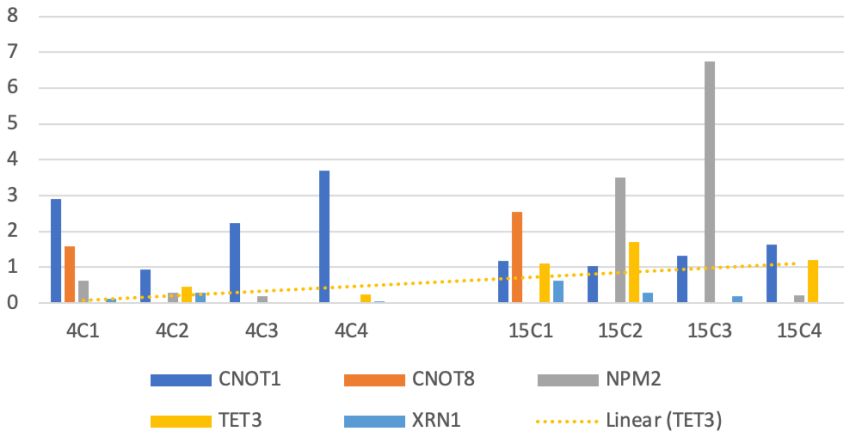
Graph 4. 4˚C and 15˚C groups gene expression pattern with CNOT1 trendline



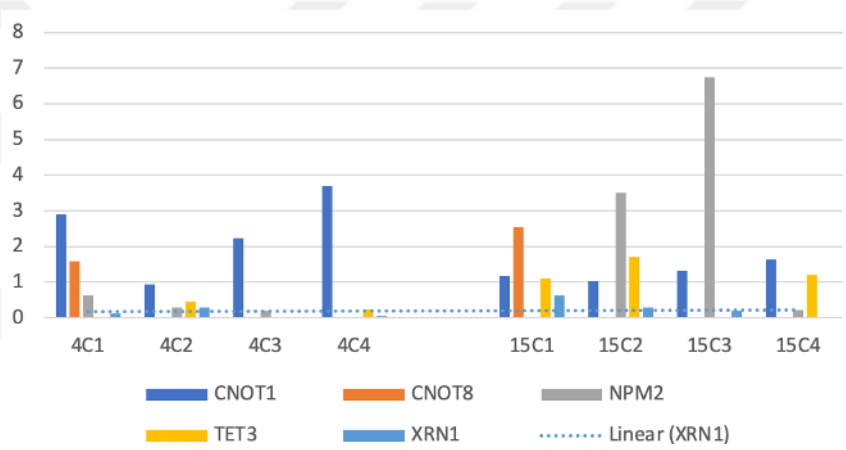
Graph 5. 4˚C and 15˚C groups gene expression pattern with CNOT8 trendline



Graph 6. 4˚C and 15˚C groups gene expression pattern with NPM2 trendline



Graph 7. 4˚C and 15˚C groups gene expression pattern with TET3 trendline



Graph 8. 4˚C and 15˚C groups gene expression pattern with XRN1 trendline

In order to test the confidence of claims above, Hypothesis testing was performed using SPSS Statistical tools. The study Hypotheses were stated as following:

H_1: There is a significant difference in mRNA quantities between the group stored at 4°C and the group stored at 15°C.

H_0: There is **NO** significant difference in mRNA quantities between the group stored at 4°C and the group stored at 15°C.

According to the Independent Samples T Test , the rejection of the null Hypothesis only for TET3 transcripts may be claimed with 87% of statistical power at 0.95 significance therefore, the Hypothesis that the quantity of functional TET3 transcripts has decreased when stored at 4˚C in comparison to those stored at 15˚C has been accepted.

For the other genes, the rejection of the null Hypotheses can not be claimed confidently with 41% of statistical power at 0.95 significance for CNOT1, 34% for NPM2, 13% for XRN1 and 6% for CNOT8.

6. CONCLUSION AND RECOMMENDATIONS

The overall results of the current study may be summarised as following:

- Sperm cytoplasm indeed contains paternally produced mRNA, and this study could determine five gene transcripts in Oryctolagus Cuniculus sperm cytoplasm: CNOT1, CNOT8, TET3, NPM2 and XRN1;
- Out of these genes, CNOT8 and CNOT1 were present the most, XRN1 and NPM2 were present the least;
- TET3 transcripts are sensitive to low storage temperatures.

The current study may have the following limitations and biases due to the following reasons:

- Pipet and pipeting erors,
- Contamination,
- Primer issues.

Based on the results, the following suggestions could be made:

-Culture media development: In line with previous research studies, the current work has determined a few transcripts from mRNA content of sperm cytoplasm. These paternal transcripts are needed for embryo development before Embryonic Genome Activation. Absence or low concentration of parental transcripts lead to embryonic arest and pregnancy loss. While naturally conceived embryos can obtain absent transcript reinforcement from the oviduct, ICSI or IVF embryos do not have this oportunity. The suggestion is to develop embryo culture medium rich of vital mRNAs, also, medium rich of vital paternal mRNAs could be developed for ICSI injections. These mediums should improve embryo quality and success rates.

-Storage temperature: According to the current study, concentration of functional (intact) TET3 transcripts was significantly lower in the group stored at 4°C in comparison to the group stored at 15°C. TET3 dioxygenase participates in paternal epigenetic reprogramming and it's deficiency will result in pronucleus formation fault and embryonic arrest. The suggestion is the semen storage at 15°C but not at 4°C.

-Sperm cytoplasm quality assesment: The current study has clearly shown a presence of paternal mRNAs in sperm cytoplasm. As these transcripts play important role in embryo development, sperm ICSI and IVF assesment should include paternal mRNA counts in repeated cases of unexplained infertility.

-Futher research: The current stage of sperm cytoplasmic research may be defined as neonatal. The most comprehensive study performed in 2019 on Bovine embryos by Gross's team has determined 472 sperm-originated and 2675 either sperm- or oocyte-originated gene transcripts in 2 cell Bovine embryos but most of 472 sperm-originated mRNAs were provisional therefore, this needs to be confirmed. It is clear that much more research similar to the current one with more genes added is needed. Keeping in mind the current study limitation, futher reseachers should pay additional attention to materials such as pipettes used. Also, primer selection is extremely important. In particular, the primer's specificity and melting temperature may cause troubles and even cast doubt on the results reliability. In the current work, the melting curve was pointing to possible issues with NPM2 and XRN1 primers therefore, the run needs to be repeated with improved primers.

In conclusion, the current study has met it's research goals succesfully. It has added valuable research data to the field of research in sperm cytoplasmic content and to the whole field of embryology. This research contribution has also added to the field of knowledge on Oryctolagus cuniculus species.

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