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Molecular Study of Mitochondrial Cytochrome Oxidase Subunit 1 Gene in the Oocyte in Vitro Maturation in the Iraqi Ewes

KEYWORDS

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ABSTRACT *This study was conducted on 668 ovary from abattoir of adult ewes. Three types oocytes aspirated from mature follicles (Oocyte with cumulus cells , partial surrounded by cumulus cells ,and oocytes without cumulus cells) it is incubated in the Co 2 incubator to maturation in TCM199 .The step of oocyte maturation previously the in vitro fertilization, therefore the investigation of oocyte in the molecular level very important for detection the oocyte quality ,so the defect in the mitochondrial gene Cytochrome Oxidase subunit 1 (CO1) may take place through the oocyte cultured in the tissue culture media. Subsequent by genomic DNA extraction by (Bioneer Kit), to investigation the goal gene, samples yielded evidence of amplified CO1 gene on the agarose gel electrophoresis. No mutation in the CO1 sequence compared with the Al-naemi sheep breed and Assaf sheep breed . The conclusion , via PCR and sequencing of CO1 gene, no differences in the oocytes through the oocyte in vitro maturation in the age 3-6 old of ewes.*

Introduction

The oocyte is surrounded by layers of cumulus cells and attached to the granuloma cells by a stalk of cumulus cells, it is made up of an oocyte enclosed by a glycoprotein coat, the zona pellucida, and is surrounded by tight, compact layers of cumulus cells (**1-2**). Eukaryotic cells contain two distinct genomes, one is located in the nucleus (nDNA) and is transmitted in a mendelian fashion, whereas the other is located in mitochondria (mtDNA) and is transmitted by maternal inheritance **(3).** A group of mitochondrial diseases are caused by mutations of the mtDNA and inherited **(4).** Human oocytes and early embryos from aging, clinically infertile women contain mitochondria with ultra structural features similar to those senescing or degenerating somatic cells including vaculation swelling and mineral deposition (**5).** Mitochondria are organelles that play an essential role in cellular energetic metabolism, homeostasis, and death **(6).** ATP depletion causes a series of cell injuries including plasma membrane permeabilization, lipid degradation, phospholipase A2 activation, intracellular calcium elevation, calpain activation, loss of membrane-cytoskeleton linkage and alteration of function aggregation of membrane proteins. Among these pathological changes plasma membrane become permeable to macromolecules the injury of the cells become irreversible (**7).** Mitochondrial genome very important for fertility in the ewes, the mitochondria energy house of cell, therefore any defect in the mitochondrial genomes led to low quality of oocyte with the infertility of ewes. Mitochondrial DNA (mtDNA) has been used to study the domestication history of many species **(8),** and the complete mtDNA sequence of sheep has been determined 660 nucleotides base pairs (bp) **(9).** Mitochondrial DNA content is critical to fertilization outcome and serves as an important marker of oocyte quality **(10-11)**, The mean mtDNA copy number for the fertilized oocytes was 250,454, whereas for the unfertilized group it was 163, 698. There were significant differences for mtDNA copy number between the male factor infertility female factor infertility unfertilized oocytes and between unexplained infertility and female factor infertility group (**12**). Mitochondrial DNA copy number is an important indicator of oocyte developmental competence (**13**) COXI (NADH uniquinone oxidoreductase), this complex consist of 42 or 43 sub unit (**14).** Complex 1 of the respiratory chain consists of 41 subunits, 7 of which are encoded by mtDNA **(15)**.

Materials and Methods

Ewes' genitalia collected from abattoir, the age of ewes between 3-6-years old. The best age of oocyte quality between 3-6 years **(16),** the study included oocyte recovery from 668 ovaries, the ovaries transmitted by cooler container at 22 C washed with preserved by sterile normal saline. Oocyte aspirated by needle gage 18. The oocyte recovery mixed with TCM 199 in pitry dish. The procedure was done in humidified incubators maintained at 38.5 C in air with 5% CO2 and incubated 24 hour in the Co2 incubator. In the Biotechnology of Baghdad Center of Al–Nahrian University, oocytes recovered from tissue culture media in the petri dish and washing with normal saline, mechanically rid out the cumulus cells many times aspiration until to denude the oocyte. Subsequent by Genomic DNA extracted from oocytes after oocytes maturation. Genomic DNA was amplified using the polymerase chain reaction (PCR) **(17-18-19)**. Detection of cytochrome oxidase subunit 1 gene was conducted by using primers for amplification of Ovis CO1 gene. A fragment 660 bp of Ovis CO1 **(20)** was amplified using a forward primer (Ovis CO1-F: 5-GCTGGTATCACAATACTACT-3 and a reverse primer (Ovis CO1-R: 5-TAGTCCTAGGAAATGCTGTG-3. These primer sets were supplied by IDT (Integrated DNA Technologies) company, Canada. Sequencing of *CO1* gene was performed by national instrumentation center for environmental management (nicem) online at (http://nicem.snu.ac.kr/main/?en_skin=index.html), biotechnology lab, machine is DNA sequencer 3730XL, Applied Biosystem), Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and BioEdit program.

Result and Discussion

Oocyte Collected from ewes ovaries obtained from abattoir, it is aspirated from mature follicle (**21**). There are

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three types of oocytes complete surrounded by cumulus cells, partial surrounded, and oocyte denuded from cumulus cells (Figure 1) . Grading of the oocytes was done on the basis of cumulus cells investment and homogeneity of cytoplasm according to **(22).** Grade A (Good): Those with over 3 layers of cumulus cells encompassing the oocyte and uniform cytoplasm. Grade B (Fair): Those with less than 3 layers of cumulus cells encompassing the oocyte and uniform cytoplasm. Grade C (Poor): Those with no cumulus cells surrounding the oocyte.

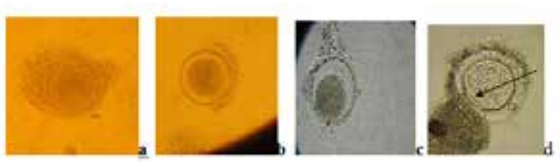

Figure 1: a -Show oocytes with complete surrounded by cumulus cells; b-partial surrounded by cumulus cells; c-denuded from cumulus cells; d-mature oocyte observe the first polar body .

The number of cumulus cells in the table 1 , appear 1250, (450; 36%) with completely surrounded by cumulus cells, (480; 38.4%) with partially surrounded by cumulus cells and (320; 25.6%) denuded. presence of cumulus cells may minimize the release of cortical granules and prevent premature zona reaction for zona hardening, so it improved the fertilization rates (**23**). Suggested that the oocytes partially denuded from cumulus cells before vitrification may be beneficial to subsequent fertilization and embryonic development **(24).**Bovine matured oocyte without cumulus cells had a higher survival rate after vitrification **(25).** Moreover, the rates of embryo development to the 8-cell stage in cumulus cells free group were significantly higher than that of cumulus cells intact group.

Table 1: appear the number of cumulus cells with the matured oocyte

No. of ovary	No. of follicles	No. of oocyte recovery	cumulus cells			No. of cultured oocyte	No. of mature oocyte
			Oocytes completely surrounded by cumulus cells	Oocytes partially surrounded by cumulus cells	Oocytes not surrounded by cumulus cells		
668	1800	1250	450	480	320	450	320
			36%	38.4%	25.6%	36%	71.1%

The observation of oocyte recovery with cumulus cells in Table **2**, appeared 405 aspirated , 45 slicing .Completely surrounded by cumulus cells 425 aspirated , 55 clicing partially surrounded by cumulus cells **,**and 300 aspirated , 20 clicing denuded oocytes **.** These result similar to **(26)** in sheep, **(27)** in goat, It could be cause this difference is the stage the ovary in estrus cycle **(28)**, or presence of CL on the surface of the ovary **(29)**. These results do not agree with the researcher **(30,31)**because that the method of slicing for us to get oocytes from small follicles either in a way the aspiration cannot pull oocytes of small follicles because the larger diameter needle .The oocyte after maturation appeared the first polar body in the **figure 1, d.**

Table 2: appear the method of recovery oocyte with cumulus cells

Method of collection	No. of follicles	No. of oocyte recovery		cumulus cells						No of cultured oocyte	No. of mature oocyte	
				Oocytes completely surrounded by cumulus cells		Oocytes partially surrounded by cumulus cells		Oocytes not surrounded by cumulus cells				
Aspiration	1500	1130	75.3%	405	35.8%	425	37.6%	300	26.5%	405	300	74%
Slicing	300	120	40%	45	37.5%	55	45.8%	20	16.6%	45	20	44.4%

The results shown in **Figure (2)** indicated that a yield of single band of the desired product with a molecular weight of 660 bp for Ovis CO1 gene of oocytes maturation was obtained **,** with the protein architecture of COX1 in vitro oocyte maturation **Figure (3**). The sequencing of Ovis CO1 gene amplified product from adult ewes appeared 100% compatibility with standard Ovis aries breed Naemi5 cytochrome

oxidase subunit 1 (CO1) gene of Gene Bank results as shown in Figure (4A), (Sequence ID: gb|KC669585.1|, there was no any polymorphism in cytochrome oxidase subunit 1 (CO1) gene, however, the cases of oocytes maturation, showed 100% compatibility as shown in Figure (4B), ID: emb|HE577849.1| appeared 100% compatibility with standard Ovis aries complete mitochondrial genome, Assaf breed and have number score (1351) bits. The bit Score is defined as statistical measure of the moral similarity and the higher value indicates that the high degree of similarity, and if dropped from the class of 50 points, the sense that there is no similarity, shown in Table (3).

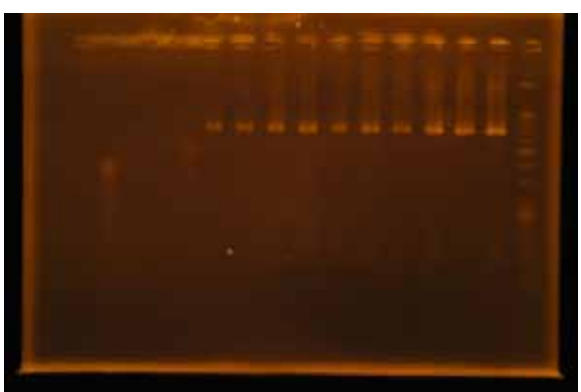

Figure 2: Agarose gel electrophoresis for amplified Ovis CO1 gene of oocytes maturation. Bands were fractionated by electrophoresis on a 1.5 % agarose gel (2 h., 5V/cm2, 0.5X TBE buffer) and visualized under U.V. light after staining with ethidium bromide staining. Lane: M:100bp ladder; Lane: 1,2,3, product for Ovis CO1 gene (660 bp).

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Figure 3 : Show Protein architecture of COX1 in vitro oocyte maturation .

Figure 4 - A: Ovis aries breed Naemi5 cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial, Sequence ID: gb|KC669585.1| (Mature oocyte)

Score	Expect	Identities	Gaps	Strand
1134 bits(614)	0.0	614/614(100%)	0/614(0%)	Plus/Plus

Query 1 GCAGGAGGAGGAGACCCTATCCTATATCAACACCTATTCTGATTCTTTGGGCACCCTGAA 60

Sbjct 31 GCAGGAGGAGGAGACCCTATCCTATATCAACACCTATTCTGATTCTTTGGGCACCCTGAA 90

Query 61 GTATATATTCTTATTTTACCTGGGTTTGGGATAATCTCCCATATTGTGACCTACTATTCA 120

Sbjct 91 GTATATATTCTTATTTTACCTGGGTTTGGGATAATCTCCCATATTGTGACCTACTATTCA 150

Query 121 GGaaaaaaaGAACCATTCGGATATATAGGAATAGTATGAGCCATAATATCAATTGGGTTC 180

Sbjct 151 GGAAAAAAAGAACCATTCGGATATATAGGAATAGTATGAGCCATAATATCAATTGGGTTC 210

Query 181 CTAGGATTCATTGTATGAGCCCACCATATATTCACAGTCGGAATAGACGTCGATACACGG 240

Sbjct 211 CTAGGATTCATTGTATGAGCCCACCATATATTCACAGTCGGAATAGACGTCGATACACGG 270

Query 241 GCTTACTTCACGTCAGCTACTATAATTATCGCCATCCCAACAGGAGTAAAAGTATTCAGT 300

Sbjct 271 GCTTACTTCACGTCAGCTACTATAATTATCGCCATCCCAACAGGAGTAAAAGTATTCAGT 330

Query 301 TGACTAGCAACGCTTCATGGGGGTAATATCAAATGATCTCCTGCCATAATATGAGCCCTA 360

Sbjct 331 TGACTAGCAACGCTTCATGGGGGTAATATCAAATGATCTCCTGCCATAATATGAGCCCTA 390

Query 361 GGTTTCATCTTTCTTTTCACAGTCGGAGGCTTAACTGGAATTGTTCTAGCCAACTCCTCC 420

Sbjct 391 GGTTTCATCTTTCTTTTCACAGTCGGAGGCTTAACTGGAATTGTTCTAGCCAACTCCTCC 450

Query 421 CTTGACATTGTCCTCCATGACACATATTATGTAGTAGCACATTTCCACTACGTATTATCA 480

Sbjct 451 CTTGACATTGTCCTCCATGACACATATTATGTAGTAGCACATTTCCACTACGTATTATCA 510

Query 481 ATAGGAGCTGTATTTGCTATTATAGGAGGATTTGTACATTGATTTCCCCTATTCTCAGGC 540

Sbjct 511 ATAGGAGCTGTATTTGCTATTATAGGAGGATTTGTACATTGATTTCCCCTATTCTCAGGC 570

Query 541 TATACTCTCAATGATACATGAGCCAAAATCCACTTTGCAATTATATTTGTAGGTGTTAAC 600

Sbjct 571 TATACTCTCAATGATACATGAGCCAAAATCCACTTTGCAATTATATTTGTAGGTGTTAAC 630

Query 601 ATGACTTTCTTTCC 614

Sbjct 631 ATGACTTTCTTTCC 644

B: Ovis aries complete mitochondrial genome, Assaf breed, isolate 5502, Sequence ID: emb|HE577849.1|

Score	Expect	Identities	Gaps	Strand
1134 bits(614)	0.0	614/614(100%)	0/614(0%)	Plus/Plus

Query 1 GCAGGAGGAGGAGACCCTATCCTATATCAACACCTATTCTGATTCTTTGGGCACCCTGAA 60

Sbjct 6000 GCAGGAGGAGGAGACCCTATCCTATATCAACACCTATTCTGATTCTTTGGGCACCCTGAA 6059

Query 61 GTATATATTCTTATTTTACCTGGGTTTGGGATAATCTCCCATATTGTGACCTACTATTCA 120

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Sbjct 6060 GTATATATTCTTATTTTACCTGGGTTTGGGA-TAATCTCCCATATTGTGACCTACTATTCA 6119

Query 121 GGaaaaaaaGAACCATTCGGATATATAG-GAATAGTATGAGCCATAATATCAATTGGGTTC 180

Sbjct 6120 GGAAAAAAAGAACCATTCGGATATATAG-GAATAGTATGAGCCATAATATCAATTGGGTTC 6179

Query 181 CTAGGATTCATTGTATGAGCCCACCATA-TATTCACAGTCGGAATAGACGTCGATACACGG 240

Sbjct 6180 CTAGGATTCATTGTATGAGCCCACCATA-TATTCACAGTCGGAATAGACGTCGATACACGG 6239

Query 241 GCTTACTTCACGTCAGCTACTATAAT-TATCGCCATCCCAACAGGAGTAAAAGTATTCAGT 300

Sbjct 6240 GCTTACTTCACGTCAGCTACTATAATTATCGC-CATCCCAACAGGAGTAAAAGTATTCAGT 6299

Query 301 TGACTAGCAACGCTTCATGGGGGTAATAT-CAAATGATCTCCTGCCATAATATGAGCCCTA 360

Sbjct 6300 TGACTAGCAACGCTTCATGGGGGTAATAT-CAAATGATCTCCTGCCATAATATGAGCCCTA 6359

Query 361 GGTTTCATCTTTCTTTTCACAGTCGGAG-GCTTAACTGGAATTGTTCTAGCCAACTCCTCC 420

Sbjct 6360 GGTTTCATCTTTCTTTTCACAGTCGGAGGCT-TAACTGGAATTGTTCTAGCCAACTCCTCC 6419

Query 421 CTTGACATTGTCCTCCATGACACATATTATG-TAGTAGCACATTTCCACTACGTATTATCA 480

Sbjct 6420 CTTGACATTGTCCTCCATGACACATATTATG-TAGTAGCACATTTCCACTACGTATTATCA 6479

Query 481 ATAGGAGCTGTATTTGCTATTATAGGAG-GATTTGTACATTGATTTCCCCTATTCTCAGGC 540

Sbjct 6480 ATAGGAGCTGTATTTGCTATTATAGGAG-GATTTGTACATTGATTTCCCCTATTCTCAGGC 6539

Query 541 TATACTCTCAATGATACATGAGCCAAAATC-CACTTTGCAATTATATTTGTAGGTGTTAAC 600

Sbjct 6540 TATACTCTCAATGATACATGAGCCAAAATC-CACTTTGCAATTATATTTGTAGGTGTTAAC 6599

Query 601 ATGACTTTCTTTCC 614

Sbjct 6600 ATGACTTTCTTTCC 6613

Figure (3): A: Sequencing of sense flanking the partial cytochrome oxidase subunit 1 gene, for cases of adult ewes as compared with standard *CO1* gene of Ovis aries breed Naemi5, obtained from Gene Bank. B: Sequencing of sense flanking the partial cytochrome oxidase subunit 1 gene for cases of adult ewes as compared with standard Ovis aries complete mitochondrial genome, Assaf breed obtained from Gene Bank. Query represents of sample; Sbject represent of database of National Center Biotechnology Information (NCBI). The bit Score: Statistical measure of the moral similarity and the higher value indicates that the high degree of similarity, and if dropped from the class of 50 points, the sense that there is no similarity mention. Expectation value: Give an estimate of the number of times the expected to get the same similarity coincidental and the lower the value of E whenever this indicates that the degree of similarity high between sequences which gives greater confidence that this relay views already follow under study, as the value of a very close to zero means that these sequences are identical.

Table (3): Sequencing ID in gene bank, score, expect and compatibility of DNA sequences obtained.

	Organism	Sequence ID	Score	Expect	compatibility	No.Nucleotide
1	Ovis aries breed Naemi5	gb|KC669585.1|	1134	0.0	100	31-644
2	Ovis aries Assaf breed	emb|HE577849.1|	1134	0.0	100	6000-6613

Animal mitochondrial DNA (mtDNA) is the marker of choice for a wide range of applications such as phylogeonography, phylogenetics and population genetics (**32-33**). The compare between genomic DNA from ovarian tissue it is like from the mature oocytes and no differences between three types mature oocyte in the PCR product. Many factors induced alterations in oocyte mitochondrial DNA, because tissue culture media induced genomic changes (**34**). Mention the mutation which affect only some copies of mtDNA are known as heteroplasmic and will vary between different mitochondria in the same individual. (**35**) Therefore the limitation of embryos continues growing, depending on the number of mitochondrial DNA mutation (heteroplasmy), and the type of gene that is included with the damage. That means heteroplasmy of mitochondria reflects the quality of ova, then the severity of mitochondrial disorder in mature egg cell (oocyte) is depending on the number of the diseased mitochondria inherited from the mother, when 80% mutation causes severe disease, 50% gives mild disease while 20% no disease (**36**). There are no deferences between deferent three types of oocyte in the COX1 sequence. There may be the healthy with the 3-6 age of ewes give good quality of oocytes. The aged ewes give oocyte low quality (**33-37**). The conclusion , no defect of oocytes that is matured in the CO2 incubator at environment (PH, humidity ,Temperature, and tissue culture media) in the age 3-6 of ewes. And no defect at the molecular level, via PCR product and sequencing of CO1 gene ,

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