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In vitro fertilization related with mutation of mitochondrial COX1 gene in sheep

KEYWORDS: In vitro fertilization , COX1 gene ,Sheep

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ABSTRACT *This study was conducted to investigate the possibility of occurrence genetic mutations in the embryos by in vitro fertilization of oocyte in sheep. The recovery of 450 oocytes from follicles in abattoir , it was incubated in the CO2 incubator , the oocytes maturation 320 .When In vitro fertilization by adult ram semen and follow up cell division of embryos to 2cell stage ,4cells stage and 8cells stage , the number of embryos 30 ,7 ,and 2 respectively . Genomic DNA extraction from 2 cell stages ,4cell stages ,and 8cell stages , followed by PCR to cytochrome oxidase subunit 1 (CXO1) gene , The sequencing of ovis CXO1 gene amplified product from 8cells, Sequence ID: gb|KC669585.1|, shows 99%, compatibility, score 1116 compare with Ovis aries breed Naemi5 (CXO1) gene from 2 cell,and emb|HE577849.1| 93% compatibility ,score 881 compare with Ovis aries, Assaf breed COX1gene .While a 4cells sequence ID lemb|HE577849.1| show 93% compatibility score 880 compare with Ovis aries, Assaf breed COX1 from 2 cell. However, the polymorphism of COX1 genes of 8 cell after fertilizing was found to be compatibly 93% the result appeared conformation of protein CO1 for two , four and eight cells , the lack of similarity of conformation of protein ,the difference in the order of the amino acids and the emergence of site side of acids as in shape and the difference in body protein leads to variation in the function of CXO1gene In conclusion there was variation of mutation in two cells stage compared with four cells and eight cells stage in vitro fertilization of COX1 gene .This study is the first according to the information in Iraq Awassi sheep which were registered as a genius of Awassi sheep in Iraq in NCBI.*

Introduction

IVF involves the fertilization of an egg (or eggs) outside the body. The treatment can be performed using your own eggs and sperm, or using either donated sperm or donated eggs, or both. Mitochondrial respiration accounts for about 90% of cellular oxygen consumption . It is reported that approximately 1% to 5% of the reactive oxygen species (ROS) under physiological condition (1). Mitochondria had an essential role in many cell functions including respiration and ATP production, lipid metabolism, synthesis of steroid hormones, regulation of apoptosis, and calcium signaling (2). Mitochondria are involved in determine early rate of oocyte success of *in vitro* embryonic development in bovine (3). Failure to activate the machinery responsible for mtDNA replication could lead to development of arrest due to the inability to replenish the mtDNA stores required around the time of blastulation. This could be a factor in poor-quality oocytes (e.g., infertility, cytoplasmic immaturity, or follicular atresia) (4-5). Mitochondria play a pivotal role in cell/oocyte function (6). The pattern of mitochondrial distribution within the oocyte has been shown to correlate with oocyte morphological characteristics and developmental competence (7). In line with these results, manipulation of mtDNA copy number in a female germ line did not affect fertilization outcome (8) and was not associated with maternal age (9).Recent work has suggested that a DNA-based identification system, founded on the mitochondrial gene, cytochrome *c*oxidase subunit 1 (COI), can aid the resolution of this diversity. While past work has validated the ability of COI sequences to diagnose species in certain taxonomic groups, the present study extends these analyses across the animal kingdom (10)

Materials and methods

Oocytes collected from ewe genitalia in abattoir ,in vitro maturation . Mature oocytes were mixed with proper sperm in the petri dish contain TCM 199 (11). Fertilizing oocytes were incubated at 39C°, 5% in CO_2 incubator and kept until they development at stage 2, 4 and 8 cells (12).

After extraction of genomic DNA, gel electrophoresis was used to detect to the presence and integrity of the extracted DNA and presence of PCR product(13).

Two conserved primers (forward) 5-GCTGGTATCACAATACTACT--3 and (reverse): 5-TAGTCCTAGGAAATGCTGTG-3 (14).

Sequencing of COX1 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 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.

Results and discussion

A total of 450 cultured oocytes were collected from ovarian follicles from Baghdad slaughter house ,only 320 oocytes were reached to maturation ,Table 1 . *In vitro* fertilization and follow up cell division of embryos to 2cell stage ,4cells stage and 8cells stage Figure 1 , in the number 30 ,7 ,and 2 respectively Table 2 .

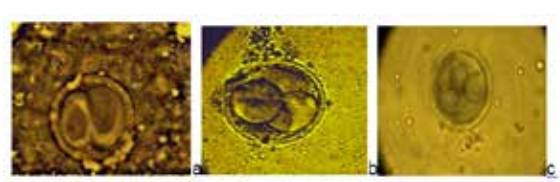

Figure 4-2: The a- 2 cells , b- 4 cells and c- 8 cells (10X).

The experiment another includes two major parts. The first to study the DNA extraction from deferent embryonic development with the PCR for goal gene Cytochrome Oxidase subunit 1 (COX1) by (Bioneer Kit), to investigate the

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COX1 gene on the agarose gel electrophoresis. While the second one was to investigate the genetic variations between each cell division of embryonic development

So the defect in the mitochondrial gene of (COX1) may take place through the oocyte cultured in the tissue culture media. Tissue culture induced genomic changes (15).

The metabolic properties of mitochondria make them highly mutagenic environment for the naked , circular mtDNAs that lie within them (16). Mitochondrial DNA is susceptible to damage more. One reason for this is that mtDNA is under much stronger oxidative stress than is nuclear DNA . mtDNA is close to the respiratory chain generates ROS . An oxidized guanine , 8oxoguanine (8-oxoG) , accumulates more and increases more rapidly in mtDNA than in nuclear DNA .Another reason is that chemical damage of mtDNA is also stronger than that of nuclear DNA (17). these mutations may accumulate in part because mitochondria lack protective histones and the highly efficient DNA repair mechanisms that are seen in the nucleus (18). followed:

The experiment showed that mutation or genetic variations in the COX1 sequence in two cells stage compared with four cells and eight cells Table 3. Many factors on the expected evolution of the ova and such as age (19), nutrition (20-21), stage of estrous and high temperature (22) , environment (23).

Table 1: Effect of the aspiration and slicing techniques on the number of culture, matured and divided oocytes.

Number of cultured oocyte	Number and percentage of mature oocyte		Number and percentage of divided Oocytes (2-8)	
450	320	71.11	39	12.18

Table 2: Total number of fertilized oocytes with different stages of growing embryos.

Number of divided Oocytes (2-8)	Number %	%
2 cell	30	76.9%
4 cell	7	17.9%
8 cell	2	5.1%
Total	39	100%

The results showed in Figure. 2 indicated that a yield of single band of the desired product with a molecular weight of 700 bp for Ovis CO1 gene of 2 Cell, 4 cell and 8 Cell and Ovis CO1 gene. The results of CO1 size 700pb ,Figure 2 , however (Mohammad ***et al.***, 2013) Amplified COX1from ewe 607bp Cause of this difference in molecular weight may be due to variation of breed ewe in different countries (24).

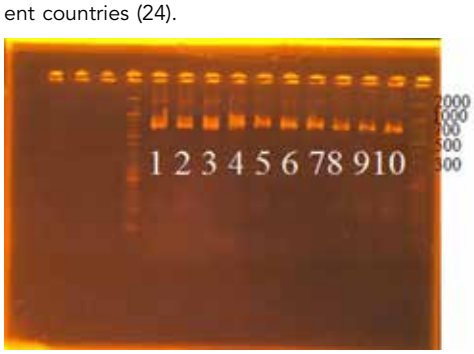

Figure 2: Agarose gel electrophoresis for amplified Ovis CO1 gene of oocytes maturation. Bands were fractionated by electrophoresis on a 1.5 % agarose gel (2 h., 5V/cm², 0.5X TBE buffer) and visualized under U.V. light after staining with ethidium bromide staining. Lane: M:25bp ladder; Lane: 1,2,3,4 product for Ovis CO1 gene (700 bp), Lane 5,6,7,8 product for for Ovis CO1 gene of 4 Cell, Lane 9,10 product for for Ovis CO1 gene of 8 Cell. Query represent of sample; Sbject represent of database of National Center Biotechnology Information (NCBI).

The sequencing of ovis CO1 gene amplified product from five samples of four cells after fertilizing appeared 93% compatibility with standard ovis aries breed Assaf cytochrome oxidase subunit 1 (CO1) gene of Gene Bank results as showed in Figure. (3), (Sequence ID: emb|HE577849.1|, and have low score (880) bits than the control cases (1134), and bits and have the same expected action value (0.0), showed in table (3).

The expectation value is defined to give an estimate of the number of times expected to get the same similarity coincidental and the lower the value of E. This indicates that the degree of similarity was high between sequences which give greater confidence. The value of a very close to zero means that these sequences are identical. There was polymorphism in the promoter region, where as three transversion mutation and 43 transitions. The sequencing of ovis CO1 gene amplified product from two samples of 8 cells after fertilizing appeared 99% compatibility with standard Ovis aries breed Naemi5 cytochrome oxidase subunit 1 (CO1) gene of Gene Bank results as shown in Figure. (3) , (Sequence ID: gb|KC669585.1|, shows 99% compatibility, score 1116 compare with Ovis aries breed Naemi5 cytochrome oxidase subunit 1 (CO1) gene from 2 cell. However, the polymorphism of COX1 genes of 8 cell after fertilizing was found to be compatibly 93% and score 881Table 3 compared with the wild type sequences of gene bank, under number emb|HE577849.1| from 6003-6614 number of nucleotide from Ovis aries complete mitochondrial genome, Assaf breed gene of Gene Bank had 41 transitions and 3 transversions mutation as shown inTable (4).

Animal DNA barcoding is mainly focused on the mitochondrial *cox1* gene because mitochondrial DNA is highly abundant in the cell; its amplification is comparably reliable; and *cox1* is often variable from populations to higher taxonomic levels (25). In the 8- cell stage the mutations of COXIII gene gave 11(73%) *in vitro* fertilization (26).

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

	Type of cells	Organism	Sequence ID	Score	Expect	Identities	No.nucleotide
3	2 cell	Ovis aries breed Naemi5	gb|KC669585.1|	1142	0.0	100	27-644
4	2 cell	Ovis aries. Assaf breed	emb|HE577849.1|	1142	0.0	100	5996-6613
5	4 cell	Ovis aries. Assaf breed	emb|HE577849.1|	880	0.0	93	6001-6614
6	8cell	Ovis aries breed Naemi5	gb|KC669585.1|	1116	0.0	99	35-641
7	8cell	Ovis aries. Assaf breed	emb|HE577849.1	881	0.0	93	6003-6614

Table 4: Comparison between 2cells, 4 cells and 8 cells through proliferation of cell and Embryo formation.

	Compare between 2cell and 4 cell				Compare between 4cell and 8 cell		
1	17	T>C	Transition	1	102	C>T	Transition
2	21	C>T	Transition	2	225	T>C	Transition

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	Compare between 2cell and 4 cell				Compare between 4cell and 8 cell		
3	51	C>T	Transition	3	388	T>C	Transition
4	89	G>A	Transition	4	403	T>C	Transition
5	104	T>C	Transition	5	429	C>T	Transition
6	116	T>C	Transition	6	451	G>C	Transversion
7	122	A>G	Transition	7	510	C>T	Transition
8	123	a>A	Transition	8	561	G>C	Transversion
9	134	A>G	Transition	9	573	T>C	Transition
10	137	C>T	Transition				
11	139	G>A	Transition				
12	140	A>G	Transition				
13	143	T>C	Transition				
14	203	C>T	Transition				
15	227	C>T	Transition				
16	233	T>C	Transition				
17	238	G>A	Transition				
18	245	C>T	Transition				
19	269	C>T	Transition				
20	281	A>T	Transversion				
21	239	A>C	Transversion				
22	311	G>A	Transition				

	Compare between 2cell and 4 cell				Compare between 4cell and 8 cell		
23	317	T>C	Transition				
24	323	T>C	Transition				
25	338	T>C	Transition				
26	341	T>C	Transition				
27	344	C>T	Transition				
28	362	T>C	Transition				
29	368	C>T	Transition				
30	371	T>C	Transition				
31	377	C>T	Transition				
32	405	C>T	Transition				
33	410	C>T	Transition				
34	437	T>C	Transition				
35	470	C>T	Transition				
36	497	T>C	Transition				
37	500	T>C	Transition				
38	512	T>C	Transition				
39	524	T>C	Transition				
40	527	C>T	Transition				
41	542	T>C	Transition				
42	548	C>T	Transition				
43	563	C>G	Transversion				
44	605	T>C	Transition				

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

Score	Expect	Identities	Gaps	Strand
1116 bits(604)	0.0	606/607(99%)	0/607(0%)	Plus/Plus

Query 1 GAGGAGGAGACCCTATCCTATATCAACACCTATTCTGATTCTTTGGGCACCCTGAAGTAT 60

Sbjct 35 GAGGAGGAGACCCTATCCTATATCAACACCTATTCTGATTCTTTGGGCACCCTGAAGTAT 94

Query 61 ATATTCTTATTTTACCTGGGTTTGGGATAATCTCCCATATTGTGACCTACTATTCAGGaa 120

Sbjct 95 ATATTCTTATTTTACCTGGGTTTGGGATAATCTCCCATATTGTGACCTACTATTCAGGAA 154

Query 121 aaaaaGAACCATTCGGATATATAGGAATAGTATGAGCCATAATATCAATTGGGTTCCTAG 180

Sbjct 155 AAAAAGAACCATTCGGATATATAGGAATAGTATGAGCCATAATATCAATTGGGTTCCTAG 214

Query 181 GATTTATTGTATGAGCCCACCATATATTCACAGTCGGAATAGACGTCGATACACGGGCTT 240

Sbjct 215 GATTCATTGTATGAGCCCACCATATATTCACAGTCGGAATAGACGTCGATACACGGGCTT 274

Query 241 ACTTCACGTCAGCTACTATAATTATCGCCATCCCAACAGGAGTAAAAGTATTCAGTTGAC 300

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

Score	Expect	Identities	Gaps	Strand
881 bits(477)	0.0	567/612(93%)	0/612(0%)	Plus/Plus

Query 1 GGAGGAGGAGACCCCATCTTATATCAACACCTATTCTGATTCTTTGGGTACCCTGAAGTA 60

Sbjct 6003 GGAGGAGGAGACCCTATCCTATATCAACACCTATTCTGATTCTTTGGGCACCCTGAAGTA 6062

Query 61 TATATTCTTATTTTACCTGGGTTTGGAATAATCTCCCATATTGTGACCTACTACTCAGGG 120

Sbjct 6063 TATATTCTTATTTTACCTGGGTTTGGGATAATCTCCCATATTGTGACCTACTATTCAGGA 6122

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Query 121 AAAAAAGAACCGTTTGAGTACATAGGAATAGTATGAGCCATAATATCAATTGGGTTCCTA 180
Sbjct 6123 AAAAAAGAACCATTCGGATATATAGGAATAGTATGAGCCATAATATCAATTGGGTTCCTA 6182

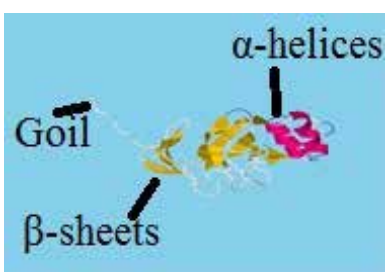
Query 181 GGATTTATTGTATGAGCCCATCATATATTCACAGTCGGAATAGACGTCGACACACAGGCT 240
Sbjct 6183 GGATTCATTGTATGAGCCCACCATATATTCACAGTCGGAATAGACGTCGATACACGGGCT 6242

Query 241 TATTTCACGTCAGCTACTATAATTATTGCCATCCCAACTGGAGTAAAAGTCTTCAGTTGA 300

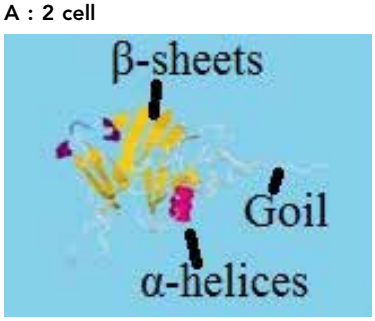
Figure (3): A: Sequencing of sense flanking the partial cytochrome oxidase subunit 1 gene, for two cases of 8 cell after fertilizing as compared with standard *CO1* gene of Ovis aries breed Naemi5, obtained from Gene Bank. B: Sequencing of sense flanking the partial cytochrome oxidase subunit 1 gene for two cases of 8 cell after fertilizing 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 results were compared with data obtained from Gene Bank published ExPASY program which is available at the NCBI online at www.ncbi.nlm.nih.gov. Translation of CXO1 gene of all groups to a amino acid sequence was performed, then Raptorx software was used (http:reptox.uchicago.eDu/predict) for drawing structure protein (helixes, B-Sheets and coils) of all groups.

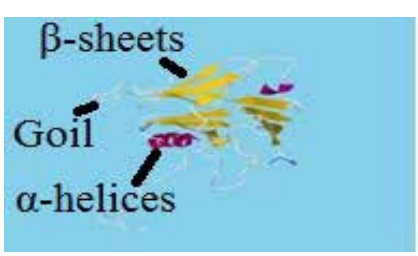
The result appeared conformation of protein CXO1 for two , four and eight cells , the lack of similarity of conformation of protein as in Figure (4), the difference in the order of the amino acids and the emergence of site side of acids as in shape and the difference in body protein leads to variation in the function of CXO1



A : 2 cell



B : 4 cell



C: 8 cell

Figure (4): Conformation of protein co1 from Ovis aries, used Raptorx software (http:reptox.uchicago.eDu/predict) for drawing structure protein (helixes, B-Sheets and coils) of amino acid (A: 2cell, B: 4 cell and C: 8cell).

In conclusion there was variation of mutation in two cells stage compared with four cells and eight cells stage in vitro fertilization of COX1 gene . This study is the first according to the information in Iraq Awassi sheep which were registered as a genius of Awassi sheep in Iraq in NCBI.

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