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RESEARCH ARTICLE

CORRELATION BETWEEN CELLFREE MITOCHONDRIAL DNA CONTENT OF EMBRYO CULTURE MEDIUM AND HUMAN EMBRYO FRAGMENTATION AND GRADING.

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Manuscript Info *Abstract*

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Background: -During In vitro fertilization technology, embryo potential for implantation and successful pregnancy is usually evaluated depending on mostly morphological criteria. Embryo fragmentation is often used as the main criterion for qualifying the human embryo grade and its implantation potential. Fragments of human embryoblastomeres are apoptotic bodies and or anuclear cytoplasmic pieces that release mitochondrial DNA (mtDNA) into the embryo secretome. Cell freemtDNA content of human embryo secretome can be used as a noninvasive tool to evaluate the fragmentation of the embryo cells and thus its grade and implantation potential.

Aim: the aim of the present research was to evaluate the correlation between Mitochondrial DNA content of embryo culture medium and human embryo blastomeres fragmentation as an objective noninvasive criterion for embryo quality.

Materials and Methods:-50 spent embryo culture mediawere collected from17 Intracytoplasmic sperm injection (ICSI) cycles.After extraction and purification of the free circulating DNA, mitochondrial DNA (mtDNA) was profiled by specific semi quantitative method (Agarose gel electrophoresis).Agarose gel electrophoresis revealed the amplification of the mtDNA from many samples but with different concentrations (the intensity of the bands were vary greatly) and some other samples failed to give a positive amplification.

Results: - mtDNA was semi quantified in 50 spent media samples collected from 17 female patients. In 12 samples (24%) mtDNA was undetectable. 6 samples showed mild mtDNA profile, 21 samples with moderate mtDNA profile and 11 samples showed severe mtDNA profile. Insignificant correlation was found between mtDNA profiling in D3 of embryonic development and embryo fragmentation, but when correlating mtDNA profile on D3 and embryo fragmentation and grade; highly significant correlation was found with embryos of G2 or lower quality.

Conclusion: -Results of the work shown in the present paper are suggestive of a significant correlation between the human embryo culture media mtDNA content and the degree of embryo blastomeres fragmentation especially in bad quality embryos; that may constitute a promising noninvasive objective criterion for human embryo quality grading and for selecting single embryo transfer policy.

Introduction:-

Assessment of embryoswith optimumdevelopment potential is one of the major challenges in human IVF technology. Routinely, embryo selection for transfer in utero is mainly based on morphological and developmental characteristics such as the degree of fragmentation, blastomere size and multinucleation, embryo symmetry and cleavage rate, which are assessed at few predeterminedmicroscope evaluations on Day 2 or 3 post-insemination (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

However, to date, there is no non-invasive platform that has been proven to be of true clinical predictive value or been examined in prospective randomized control trials to be better than current morphology-based selections methods (Hardarson et al., 2012; Vergouw et al., 2012).

It was previously discovered that both genomic DNA (gDNA)and mitochondrial DNA (mtDNA) are detectable in the secretome of human cleavage-stage embryos (Stigliani et al., 2013).

Since widespread fragmentation is often associated with genetic abnormalities, i.e. aneuploidy and mosaicism (Sathananthan et al., 1990; Pellestor et al., 1994a,b; Munne´ et al., 1995; Ebner et al., 2001; Chavez et al., 2012), it has been hypothesized that fragmentation is the manifestation of a defective embryogenesis.

Alternatively, fragmentation could result in loss of whole blastomeres, loss of cytoplasm from individual blastomeres or both, and deplete the embryos of essential organelles (Pereda and Croxatto, 1978; Lehtonen, 1980; Ebner et al., 2001), such as mitochondria. Consistent with this, fragmented embryos may have lower mitochondrial DNA (mtDNA) levels (Lin et al., 2004) and a different mitochondrial distribution pattern compared with non-fragmented embryos (Wilding et al., 2001). This pattern may be linked to reduced adenosine triphosphate content and reduced developmental potential (Van Blerkom et al., 1995).

Patients, Materials and Methods:-

The study is a prospective cohort observational study, evaluating the possible clinical use of the amount of cell-free mtDNA released by human embryos into culture medium as a correlated criterion with embryo morphological features. The population of this study comprised fifty embryo spent culture media samplestaken from 17 patients undergoing ICSI treatment cycles. Written informed consent was obtained for theuse of embryo culture media samples at D3.The local Institutional Review Board approved the study.Cryopreserved gametes and all oocytes inseminated by conventional IVF were excluded from the study, in order to avoid potential biases due to cooling/defrosting procedures and potential contaminating DNA from spermatozoa, respectively.

Patients' Characteristics:-

The women's age mean ± SD: was (30.3 ± 6.0years) and their body mass index (BMI) mean ± SD: was (27.5 ± 4.6kg/m2). In this study, female infertility was the cause of theconsultation for 64% of couples; male factors for 42%; mixed infertility for 43% and unexplained infertility for 5%. The baseline hormonal status was evaluated in each patient at Day 3 of the cycle.(Table 1)

On Day 3, following removal of the embryos in preparation for transfer into the blastocyst medium or into the uterine cavity, 20 microliter spent culture media from each embryo were collected into sterile, DNA-, DNase-, RNase-, polymerasechain reaction(PCR)inhibitors-freetubes (Eppendorf,Hamburg, Germany) and immediately stored at -20C until nucleic acid purification. Appropriate precautions were taken to prevent contamination of samples by extraneous cells or DNA. During each experimental step, laboratory personnel wore gloves and coat, and physical isolation was guaranteed by working in clean air hoods. Specifically, culture medium collection and further analytical procedures were performed using sterile, PCR-clean filter tips with a two-phase filter for contamination protection from aerosol and biomolecules for both pipette and sample.

Materials:-

Extraction and purification of the free circulating DNA:-

QIAamp Circulating Nucleic Acid Kit (Qiagen cat # 55114)
Absolute ethanol –for HPLC, gradient grade, ≥99.8% (Fluka Cat # 02854)

Amplification of human mitochondrial DNA from:-
REPLI-g Mitochondrial DNA Kit (Qiagen cat # 151023)

Buffers and reagents used for agarose gel electrophoresis:-
(Sambrook et al., 1988)
Ethedium bromide (EBr):

It was prepared in stock solution, 10mg/ml by dissolving one tablets (10mg/tab) in 1ml-distilled water, vortex, stored in dark at +4oC. To be used at 0.5 ug/ml final concentration.

50X electrophoresis buffer:
(Tris-acetate EDTA, pH 8)
Tris-base.....……..……... 242.0 g
Glacial acetic acid...…..…… 57.1 ml
EDTA (0.5 M pH 8).....…..……. 100 ml
Bidist.water……..... to ……...……1000 ml

6X gel loading buffer:
Bromophenol blue..... ……. 0.25 g
Xylene cyanol FF..... ……... 0.25 g
Glycerol..... ……. 30 ml
Bidist.water..... to ..... …… 100 ml

1% agarose gel:
Agarose..... ……. 1 g
1X TAE..... ……100 ml

The solution was heated but not boiled in a microwave till dissolving, left to cool to 60oC, and then 5ul of EBr solution was added. The solution was poured in the casting tray of electrophoresis apparatus and left to solidify.

Equipment:-

- Micro centrifuge tubes
- Micro centrifuge
- Water bath or heating block
- Vortexer
- Pipets and pipet tips
- Nuclease-free water
- T professional thermal cycler (Biometra – Germany)

Methods:-

Extraction and purification of the free circulating DNA:-
It was done according to the manufacture instructions. All the sample volumes were adjusted to 1000 µl with nuclease free water. Proteinase K was then added (100µl/sample) and 800µl of the buffer ACL (containing 1.0 µg carrier RNA), the whole mixture was incubated in water bath at 60 °C/30 min. To the lysate 1.8 ml of buffer ACB was added and mixed thoroughly by pulse-vortexing for 15–30 s. The whole lysate was then transferred to the QIAamp Mini column and subjected to vacuum at a rate of –800 to –900 mbar , after the whole fluid have been drawn off , the columns were washed once with 600µl of ACW1 then with 750 µl of ACW2 buffer and finally with 750 µl of absolute ethanol. The column was then dried by centrifugation at 14000 rpm/5 min and then left at 60 °C for further 5 min to insure complete dryness. The DNA was then eluted with 20 µl of AVE buffer by centrifugation at 14000 rpm/1 min. eluted DNA was stored at -20 °C till used.

Amplification of mitochondrial DNA:-
It was done according to the manufacture instructions. The purified DNA was first thawed on ice then 10 µl of each sample was mixed with equal volume of nuclease free water and 29 µl of the amplification mix (27 µl of reaction buffer and 2 µl of the primer mix) the mixture was then vortexed and centrifuged briefly.

The samples were incubated for 5 min at 75°C and allowed to cool down to room temperature (15–25°C). Then 1 µl REPLI-g Midi Polymerase was added to each sample and incubated at 33°C for 8 h in the thermal cycler. The enzyme was then inactivated at 65 °C/3 min and the reaction mix was then stored at -20°C till used.

Agarose gel electrophoresis:-
According to Sambrook et al. (1989) To assess the PCR product, 4 mm thickness of 1% agarose containing 0.5µg/ml EBr was poured in mini-gel and left till solidify before submarine loading of 8µl of PCR-product mixed with 2µl of DNA loading buffer. Three µl of 100 bp DNA ladder in 1µl loading buffer were used as DNA marker. DNA was electrophoresed at 80 v/15 minutes and finally examined using UV transilluminatore.

Results interpretation:-

Agarose gel electrophoresis revealed the amplification of the mtDNA from many samples but with different concentrations (the intensity of the bands were vary greatly) and some other sample failed to give a positive amplicon. In order to perform the non-parametric statistical analysis; the results were ranked as shown in (Table 2).

Results:-
In this study mtDNA was semi quantified in 50 spent media samples collected from 17 female patients' undergoing ICSI cycles.In 12 samples (24%) mtDNA was undetectable. Thirty eight (76%) samples showed a variable amounts of mtDNA; 6 samples with mild mtDNA profile, 21 samples with moderate mtDNA profile and 11 samples showed severe mtDNA fragmentation profile.(Table 3)

Each embryo was morphologically scored and graded on D3 with blastomeres fragmentation recording. G1 embryos with the least fragmentation amount were 20 embryos (not transferred and allowed to continue development to Day 5). The remaining 30 embryos were scored as follows: 14 embryos were G2, 10 embryos were G3 and 6 embryos were G4. (Table 4)

Insignificant correlation was found between mtDNA profiling in D3 of embryonic development for the whole study population (50 samples) and embryo fragmentation and grading(P value =0.221).Table 5 and 6& Figure 1 and 2).

Highly significant correlation was found when correlating mtDNA profile on D3 and embryo fragmentation and or embryo grade; for embryos of G2 or lower quality (P value=<0.001). (Table 7 and 8)

That is to say; semi quantification of mtDNA profiles of human embryo culture media in relation to embryo grading and fragmentation revealed those high levels of mtDNA fragmentation with poor lower embryo quality.

In twentygood quality grade 1 embryos culture samples which allowed continuing in culture to reach blastocyst stage; significant correlation was found between mtDNA fragmentation profiling and blastocyst formation in D5.(Table 9) Also, a highly significant correlation was detected between mtDNA fragmentation profiling on D3 and blastocyst grading in D5. (Table 10)

Significant correlation was also found between the following patients' variables and mtDNA profile of embryo culture media on D3: age of the patient, base line E2, previous ICSI trials, numbers of gonadotropins stimulation days and fertilization rate. (Table 11)

Receiver operating characteristic (ROC) curve derived from the multivariable binary logistic regression model for prediction of the producing of grade 1 or 2 embryos reveled sensitivity of 79.4 and specificity 93.7 (Figure 3).

Tables:-

Table (1):-Characteristics of the study population.

Variable	Value
Age (years)	30.3 ± 6.0
BMI (kg/m^2)	27.5 ± 4.6
Male factor	
Nil	29 (58.0%)
Oligospermia	6 (12.0%)
Azospermia	15 (30.0%)
Female factor	
Nil	18 (36.0%)
Tubal factor	8 (16.0%)
PCO	18 (36.0%)
Endometriosis	2 (4.0%)
Old age	4 (8.0%)
Baseline FSH (mIU/l)	6.0 ± 2.6
Baseline LH (mIU/l)	6.1 ± 4.5
Baseline E2 (pg/ml)	45.4 ± 21.2
Follow-up time	
Up to D3	30 cases (60.0%)
Up to D5	20 cases (40.0%)

Data are mean ± SD or number (%)

Table (2):-Rank of the non-parametric statistical analysis of the results.

-	**No amplification corresponding to no DNA fragmentation**
+	**Mild DNA fragmentation (profile)**
++	**Moderate DNA fragmentation (profile)**
+++	**Severe DNA fragmentation (profile)**

Table (3):-DNA profile on D3 in the study population

Variable	Value
DNA profile on D3	
Nil	12 (24.0%)
Mild	6 (12.0%)
Moderate	21 (42.0%)
Severe	11 (22.0%)

Data are number (%).

Table (4):-Primary outcome measures in the whole study population

Variable	Value
Degree of fragmentation on D3	
Nil	20 (40.0%)
Mild	14 (28.0%)
Moderate	10 (20.0%)
Severe	6 (12.0%)
Embryo grade on D3	
Grade 1	20 (40.0%)
Grade 2	14 (28.0%)
Grade 3	10 (20.0%)
Grade 4	6 (12.0%)

Data are number (%).

Table (5):-Relation between the DNA profile and degree of fragmentation on D3.

	DNA profile on D3						
Variable	**Nil (n=12)**	**Mild (n=6)**	**Moderate (n=21)**	**Severe (n=11)**	**χ^2**	**DF**	**p-value¶**
Degree of fragmentation on D3					1.498	1	0.221
Nil	3 (25.0%)	1 (16.7%)	11 (52.4%)	5 (45.5%)			
Mild	8 (66.7%)	5 (83.3%)	1 (4.8%)	0 (0.0%)			
Moderate	1(8.3%)	0 (0.0%)	6 (28.6%)	3 (27.3%)			
Severe	0 (0.0%)	0 (0.0%)	3 (14.3%)	3 (27.3%)			

Data are number (%).
χ^2, Chi-squared statistic;
DF, degree of freedom.
¶Chi-squared test for trend.

Table (6):-Relation between the DNA profile and embryo grade on D3.

	DNA profile on D3						
Variable	**Nil (n=12)**	**Mild (n=6)**	**Moderate (n=21)**	**Severe (n=11)**	**χ^2**	**DF**	**p-value¶**
Embryo grade on D3					1.772	1	0.183
Grade 1	3 (25.0%)	1 (16.7%)	11 (52.4%)	5 (45.5%)			
Grade 2	2 (16.7%)	1 (16.7%)	3 (14.3%)	1 (9.1%)			
Grade 3	5 (41.7%)	1 (16.7%)	3 (14.3%)	4 (36.4%)			
Grade 4	2 (16.7%)	3 (50.0%)	4 (19.0%)	1 (9.1%)			

Data are number (%).
χ^2, Chi-squared statistic;
DF, degree of freedom.
¶Chi-squared test for trend.

Table (7):-Relation between the DNA profile and embryo grade on D3 in the subgroup of patients with G2 or lower quality embryos on D3.

	DNA profile on D3						
Variable	Nil (n=9)	Mild (n=5)	Moderate (n=10)	Severe (n=6)	**χ^2**	**DF**	**p-value¶**
Embryo grade on D3					16.461	1	<0.001
Mild	8 (88.9%)	5 (100.0%)	1 910.0%)	0 (0.0%)			
Moderate	1 (11.1%)	0 (0.0%)	6 (60.0%)	3 (50.0%)			
Severe	0 (0.0%)	0 (0.0%)	3 (30.0%)	3 (50.0%)			

Data are number (%).
χ^2, Chi-squared statistic;
DF, degree of freedom.
¶Chi-squared test for trend.

Table(8):-Correlation between the DNA profile on D3 and the main outcome measures in the subgroup of patients with G2 or lower quality embryos on D3

	DNA profile on D3		
Variable	**Number**	**Spearman rho (ρ)**	**p-value¶**
Degree of fragmentation on D3	30	0.707	**<0.0001**
Embryo grade on D3	30	0.707	**<0.0001**

¶Spearman rank correlation.

Table (9):-Relation between the DNA profile on D3 and blastocyst formation on D5

	DNA profile on D3						
Variable	**Nil (n=3)**	**Mild (n=1)**	**Moderate (n=11)**	**Severe (n=5)**	χ^2	**DF**	**p-value¶**
Blastocyst formation onD5					3.280	1	0.007
No blastocyst formation	0 (0.0%)	0 (0.0%)	6 (54.5%)	3 (60.0%)			
Blastocyst formation	3 (100.0%)	1 (100.0%)	5 (45.5%)	2 (40.0%)			

Data are number (%).
χ^2, Chi-squared statistic;
DF, degree of freedom.
¶Chi-squared test for trend.

Table (10):-Relation between the DNA profile on D3 and the blastocyts grade on D5.

	DNA profile on D3						
Variable	**Nil (n=3)**	**Mild (n=1)**	**Moderate (n=5)**	**Severe (n=2)**	χ^2	**DF**	**p-value¶**
Blastocyst grade on D5					8.420	1	**0.004**
Grade 1	3 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)			
Grade 2	0 (0.0%)	1 (100.0%)	1 (20.0%)	0 (0.0%)			
Grade 3	0 (0.0%)	0 (0.0%)	4 (80.0%)	2 (100.0%)			

Data are number (%).
χ^2, Chi-squared statistic;
DF, degree of freedom.
¶Chi-squared test for trend.

Table (11):-Correlation between the Embryo grade on D3 and other relevant variables.

	Embryo grade on D3		
Variable	**Number**	**Spearman rho (ρ)**	**p-value¶**
Age (years)	50	-0.336	**0.017**
BMI (kg/m2)	50	0.054	0.710
Baseline FSH (mIU/l)	50	0.159	0.270
Baseline LH (mIU/l)	50	-0.014	0.921
Baseline E2 (pg/ml)	50	-0.288	**0.043**
Number of previous IVF trials	50	-0.296	**0.037**
Number of HMG ampoules	50	-0.221	0.123
Number of stimulation days	50	-0.346	**0.014**
Number of MII oocyte	50	-0.022	0.877
Number of fertilized oocytes	50	-0.211	0.141
Fertilization rate	50	-0.336	**0.017**
DNA profile on D3	50	-0.195	0.174
Blastocyst grade on D5	11	0.000	-

¶Spearman rank correlation.

Figures:-

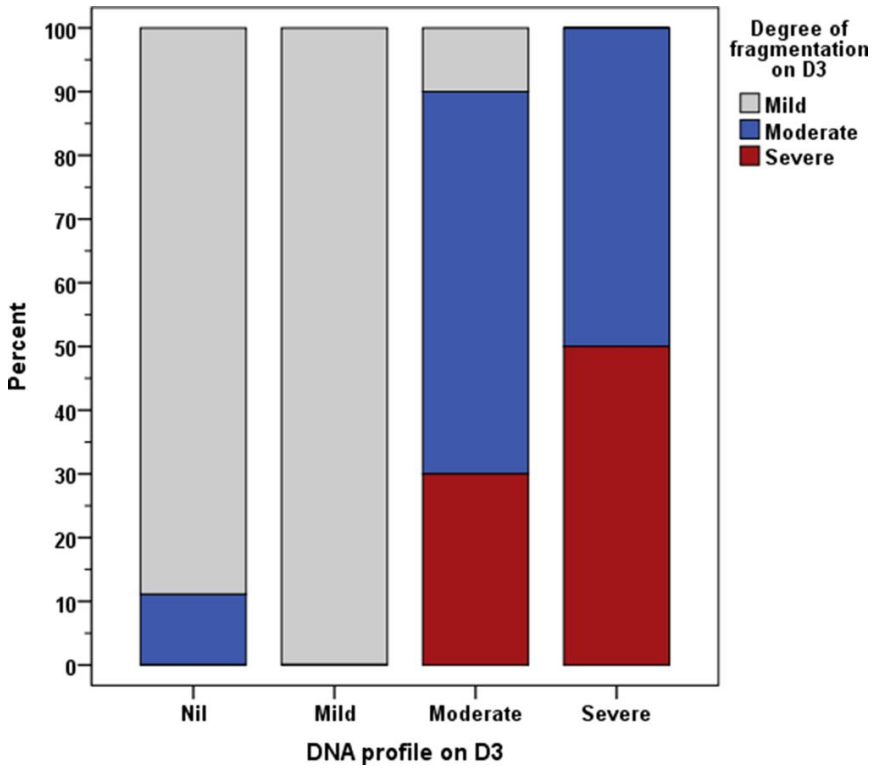

Figure (1):-Relation between the DNA profile and degree of fragmentation on D3.

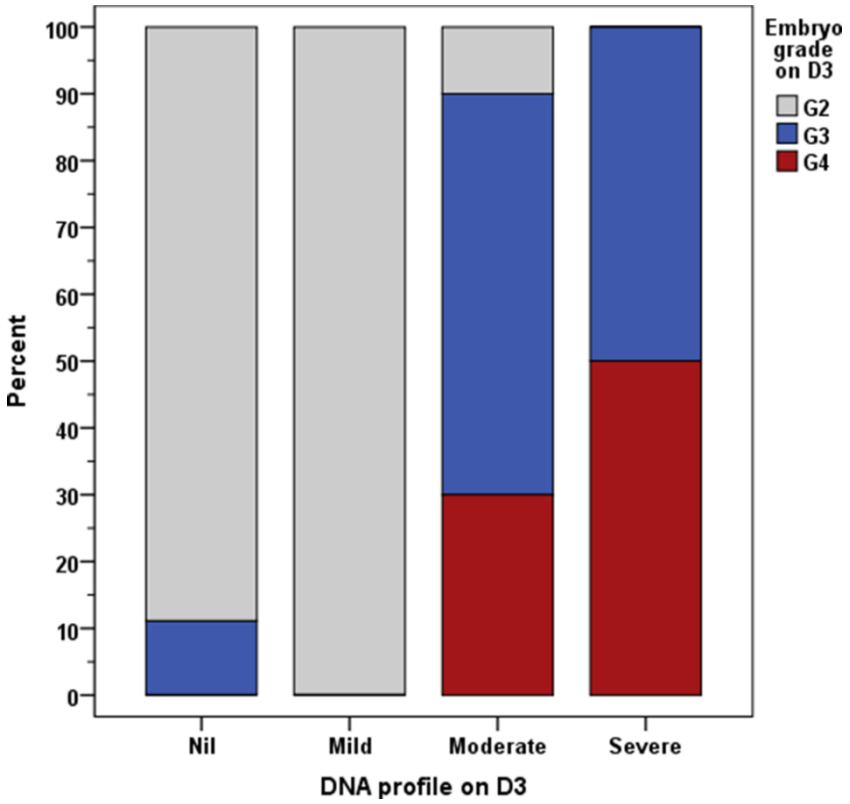

Figure (2):-Relation between the DNA profile and embryo grade on D3.

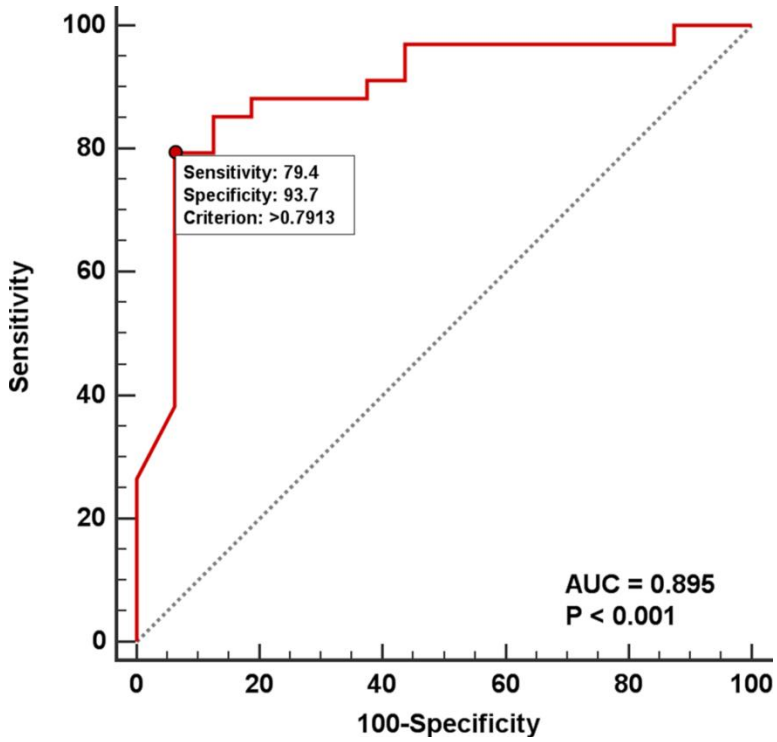

Figure (3):-Receiver operating characteristic (ROC) curve derived from the multivariable binary logistic regression model for prediction of the producing of grade 1 or 2 embryos.

Discussion:-

Fragmentation is a common feature of human embryos after IVF and during culture. Although a number of hypotheses have been proposed to explain the origin of fragmentation in embryos, whether fragments are apoptotic bodies or necrotic blastomeres, or both, remains to be definitively elucidated (Fujimoto et al., 2011).

It is known that replication of mitochondrial genome is strictly regulated during development, passing from primordial germ cells into oocytes and then, through the embryo and the fetus, into mature cells (St John, 2014).

The study idea emerged fromthe hypothesis that fragmentation,whatever the source of it, should reflect the release of mitochondria and/or nuclearDNAinto the embryo culture medium and may establish a positive correlation between mtDNA profiles andembryo fragmentation and grading on Day 3 of early development.

This study demonstrates the presence of cell free mtDNA in human embryo culture media samples of patients undergoing ICSI. It also shows that mtDNA level in human embryo culture media samples is correlated with embryo fragmentation degree and associated with embryo quality. Our data suggest that human embryo culture media cell free mtDNA concentration could be used to predict objectively embryo quality at Day 3 especially in bad quality embryos.

The presence of high mtDNA concentrations in spent culture medium of human embryos could be the consequence but alsoone of the causes of poor embryo quality.

Taken together, our results and previously published data suggest that cell free mtDNAquantification in human embryo culture media could give a better predictive picture of embryo quality and fragmentation, and could be used in future in addition to the subjective morphological criteria.

Further randomized prospective studies, mainly focused on implantation results, will have tobe conducted in a larger number of samples, with the aim at determining usefulness and limitations of the mtDNA quantification in the embryo selection criteria.
Moreover, if further studies will show that high mtDNA levelscontribute to poor embryo quality, it could be possible to investigate the possibility of a therapeutic approach based on DNase treatment to reduce cell free DNA concentration with the final aim of improving ART outcome.

Conclusion:-

This preliminary data suggest thatmtDNA level assessmentin spent media, in combination with morphological grading, mayprovide adjuvant information for selection of Day 3 embryos with good quality and high implantation potential.

In summary, the results of this study demonstratethat mtDNA level assessment in spentculture media in Day 3 embryo secretome, in combination with morphological grading may have the potential to improve proper identification of good quality embryos with highdevelopmental and pregnancy rate potential.

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Conflict of interest:-

Authors declare no conflicts of interest.

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