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Fragmentation in embryos: Still an enigma

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Abstract
Fragmentation is commonly seen in human embryos developing in-vitro. Based on severity of fragmentation, development potential of the embryos is estimated. But the proportion of the fragmentation at which developmental potential of the embryos starts to decrease is not known. Recent studies indicate that fragmentation affects developmental potential of the embryos if specific genes are involved in the fragment formation. Thus, considering only percentage of fragmentation can lead to erroneous judgment regarding developmental potential of the embryos. Fragmentation is also considered as parameter of apoptosis happening in the embryos. But this association is also being refuted in newer research. Moreover, time at which fragmentation develops and stage of the embryo at which fragmentation develops and its effect on development of the embryos has not been studied extensively. Thus, extensive research is required to get clear picture of fragmentation and its effect on the developmental potential of the embryos.

Keywords: fragmentation; time-lapse analyses; stage of the embryo; blastocyst

Introduction
After the birth of Louise Brown [1], assisted reproduction technology (ART) has improved significantly. The main objective of ART is to avoid multiple pregnancies and provide single healthy baby to the subfertile couple [2]. For this, it is important to transfer a single embryo having good developmental potential [3]. Developmental potential of the embryo depends upon the stress endured by the embryo [4] and it can be studied using time-lapse imaging. Time-lapse imaging helps in analysing morphokinetic and morphological changes in the embryo [5]. In morphology, blastomere appearance, fragmentation, and multinucleation are the important parameters [6] which are considered for the embryo selection.
Fragmentation is the study of membrane bound anucleate cytoplasmic structures which are of <45 µm diameter in day 2 embryos and <40µm diameter in day 3 embryos [7-9]. These fragments, if are reabsorbed will not have any deleterious effects but if not reabsorbed, then due to loss of cytoplasm will affect the embryo quality [10, 11]. Thus, severity of the fragmentation affects the embryo quality. But at what proportion embryo quality will be affected is not known. Some studies show deleterious effect when fragmentation is >25% [8], Xu et al. [12] observed deleterious effect of fragmentation at >35% while Mateusen et al[7] observed impairment of embryo quality when fragmentation was >5%. Thus, the cut-off limit of fragmentation is still debatable. Fragmentation is also linked to apoptosis [13] but in a study done by Antczak and Blerkom [14], apoptosis occurred only when proportion of genes involved in the developmental process were altered during fragmentation. Due to this uncertainty, many studies are being conducted on fragmentation.

Aims and Objectives
This study was carried out to see the effect of various aspects of fragmentation such as severity, time at which fragmentation started and stage of the embryo at which fragmentation started on the development of the embryos.

Methodology: It is a prospective cohort study carried out on 86 frozen-thawed mouse embryos which were randomly divided into 3 groups and were exposed to the varying concentrations of ammonium chloride*. Ammonium chloride was used to induce fragmentation in the mouse embryos as incidence of fragmentation in mouse embryos is less [15] and ammonium chloride by increasing oxidative stress acts as an eternal stimulus for inducing fragmentation [16, 17]. These embryos were cultured for 5 days in the embryoscope dishes during which grade of

fragmentation, time of appearance of fragmentation and stage of the embryo at which fragmentation started was noted. Grade of fragmentation in the embryos and stage of the embryo development was allotted according to the parameters approved by the Istanbul consensus workshop [8] as mentioned in table 1 and table 2 below.

Table 1: Blastocyst expansion stages according to stages approved by Istanbul consensus [8]

Stage	Description
1	Early blastocyst-blastocoel <50% of total embryo volume.
2	Blastocyst-blastocoel > 50% of embryo volume
3	Full blastocyst-blastocoel occupies almost all the embryo. From this stage, inner cell mass and trophectoderm are assessed.
4	Expanded blastocyst- diameter is now larger than cleavage-stage embryo. Surrounding zona pellucida becomes thinner due to expansion.
5	Hatching blastocyst
6	Fully hatched blastocyst

Table 2: Grading of fragmentation in the embryos according to the grades approved by Istanbul consensus [8]

Grade	Fragmentation
1	0-10% averaging to 5% involvement of the total embryo.
2	10-20% averaging to 15% involvement of the total embryo.
3	20-50% averaging to 35% involvement of the total embryo.
4	50-100% averaging to 75 % involvement of the total embryo.

Following figure (figure 1) shows fragmentation of different severity which was noted in the present study.

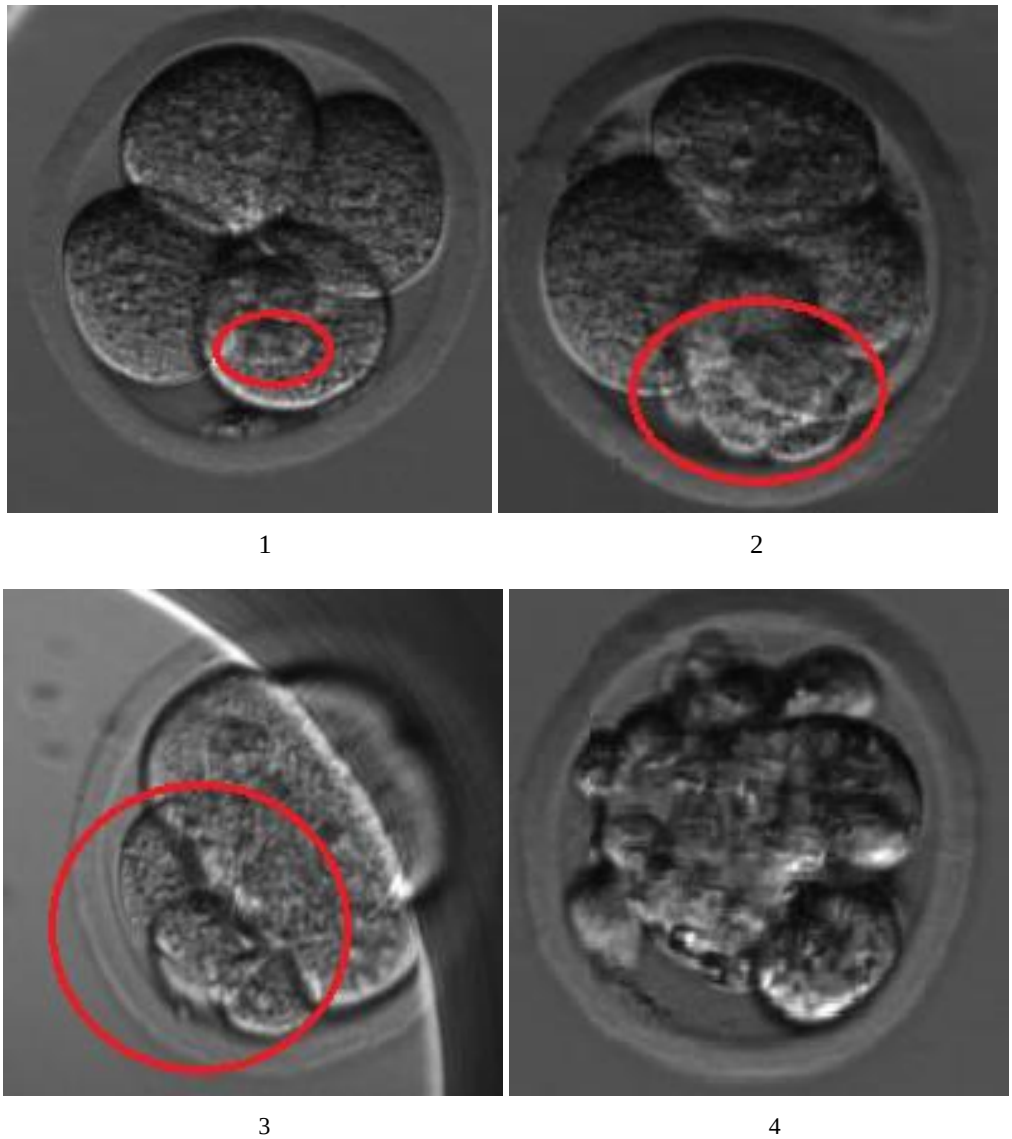

Fig 1: Image 1 shows 0-10% fragmentation at cleavage stage, Image 2 shows 10-20% fragmentation at cleavage stage, image 3 shows 20-50% fragmentation at morula stage and image 4 shows 50-100% fragmentation at morula stage. Areas showing fragmentation are circled in red.

Comparison of grade of fragmentation, time of appearance of fragmentation and stage of the embryo at which fragmentation appeared was done between all three groups. Association of above mentioned parameters with development of blastocysts was analysed.

Statistical analysis

Data was tested for normalcy using Shapiro-Wilk test and then compared in all three groups. In normally distributed data, significant difference was calculated using one-way ANOVA and if not normally distributed then Kruskal-Wallis test was used. If p-value in one-way ANOVA was significant then Bonferroni post hoc test was carried out while in Kruskal-Wallis test, pairwise comparison was done when p-value was significant. Formation of the blastocyst was studied in association with severity of fragmentation, time at which fragmentation started and stage of the embryo at which fragmentation started. It was compared within all groups using Chi-square test. In case of significant difference, z-score was calculated, and Bonferroni correction was carried out to obtain significant p-value. Statistics were performed using SPSS software (IBM SPSS statistics 23) and p-value of <0.05 was considered significant

Results

From figure 2, it is seen that all embryos mainly exhibited grade1, grade2 and grade 3 fragmentation, and no significant difference was noted in grade of fragmentation across all 3 groups* of the embryos.

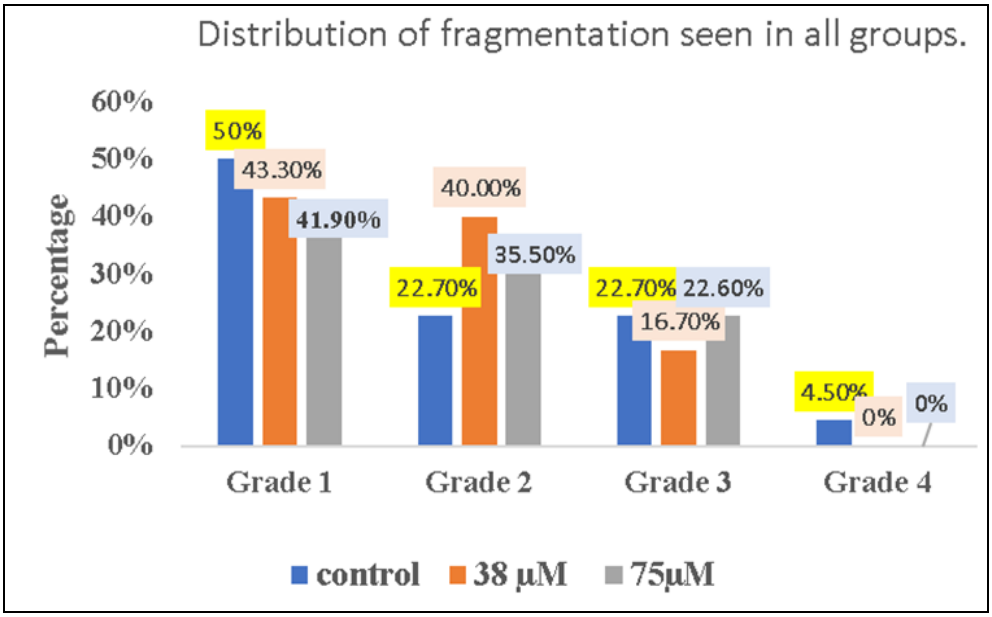

Fig 2: This figure shows comparison of the grade of fragmentation across all groups*. Grade was calculated according to the grades approved by Istanbul consensus[8]. Grades of fragmentation were taken at the starting of fragmentation.

Figure 3 shows the rate of blastocyst development in all three groups. On comparison, p value was not significant, which indicates that blastocyst development was same in all three groups* of the embryos.

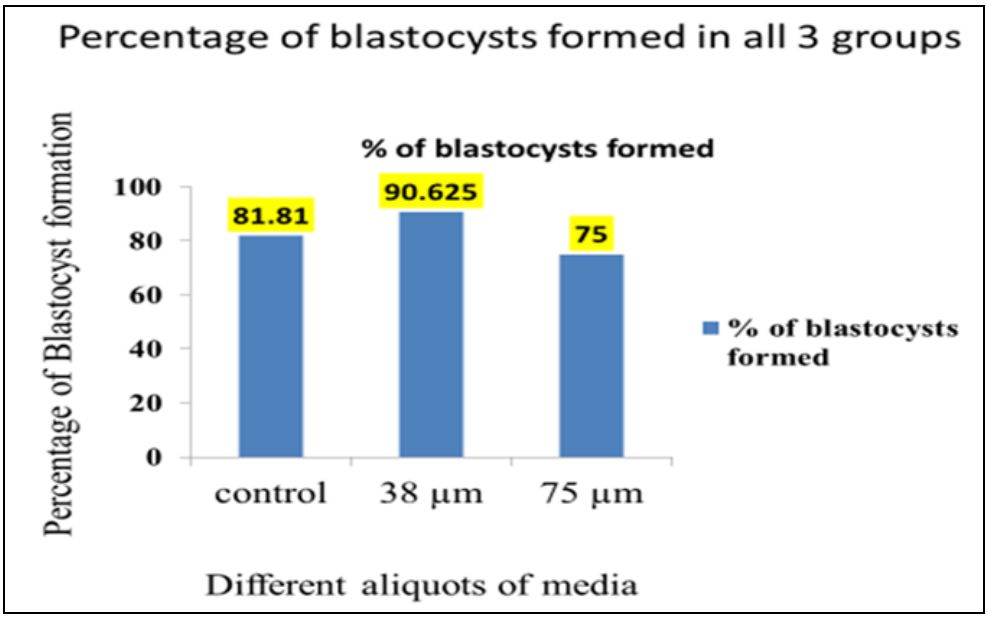

Fig 3: Percentage of blastocysts formed in all groups*.

Table 3 compares the starting time of fragmentation in all 3 groups* of the embryos. P-value was >0.05 indicating that there was no change in commencement of fragmentation in all three groups* of the embryos.

Table 3: Values of mean, standard deviation, and p-values of starting time of the fragmentation in all three groups*.

	control	38µM	75µM	p-value
Time at starting of fragmentation(h)	57.792 ± 15.818	62.155 ± 12.55	59.137 ± 18.16	0.583

It was seen that fragmentation started at different stages in different embryos. Thus, stage of the embryo (cleavage, morula, or blastocyst) at which fragmentation started was analysed and compared. Figure 4 shows the distribution of fragmentation according to the stage at which it appeared. It was seen that maximum number of embryos in each group* had fragmentation at morula stage/stage of compaction and there was no statistically significant difference when compared in all three groups*.

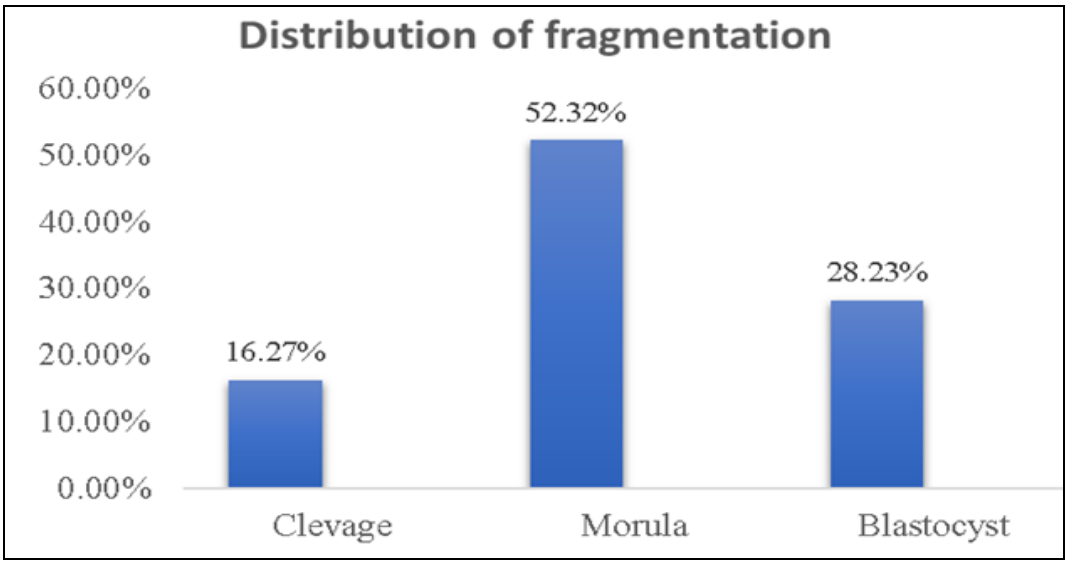

Fig 4: Overall percentage of the stage of the embryos at which fragmentation started.

Table 4 shows the outcome of the embryos in terms of blastocyst development in relation to the stage of the embryo at which fragmentation appeared. Significant difference in the development of blastocysts was noted when fragmentation occurred at morula stage/stage of compaction. More number of blastocysts developed when fragmentation occurred at morula stage.

Table 4: Outcome of the embryos in relation to the stage of embryo at the time of appearance of fragmentation. Significance was calculated using Chi-square. As Chi-square test indicated significant difference, post-hoc test was done to find out z-scores. These z-scores were then subjected to Bonferroni correction.

Stage of embryo at which fragments appeared	Blastocyst formed (%)	Blastocyst not formed (%)
Cleavage	42.9	57.1
Morula	86.7	13.3
Blastocyst	95.8	4.2[a]

[a]there was one embryo which had not reached blastocyst stage and fragmentation started at start of cavitation.

Table 5 compares the group-wise development of the embryos to blastocyst stage in relation to the stage of the embryo at which fragmentation occurs. Here also positive trend was seen in the embryos having fragmentation in morula stage.

Table 5: Outcome in all groups*, in accordance to the stage of embryo at appearance of fragmentation. Comparison between groups was done using Chi-square. Values in stage of morula are marked bold. Chi-square test was carried out for comparison and as it was significant z-scores were taken out and Bonferroni correction was applied.

Groups	Stage of embryo at which fragments appeared	Blastocyst not formed (%)	Blastocyst formed (%)
Control	Cleavage	40	60
	Morula	16.7	83.3
	Blastocyst	0	100
38µM	Cleavage	50	50
	Morula	15.8	84.2
	Blastocyst	0	100
75µM	Cleavage	71.4	28.6
	Morula	7.1	92.9
	Blastocyst	10[a]	90

[a]indicates, there was one embryo in which fragmentation had occurred at starting of cavitation.

Overall, the results inferred that blastocyst development was not affected in any of the groups*. It was also seen that when fragmentation occurred at morula stage instead of cleavage chance of blastocyst development was more.

Discussion

Fragmentation is an important morphological parameter for assessing the embryo quality [8, 18]. In this study it was seen that there was no significant difference observed in starting time of fragmentation in all groups* of the embryos. It was also observed that development of the embryos to blastocyst stage was good in all the groups*. Several reasons might be responsible for this phenomenon.

Firstly, the severity of fragmentation observed in all groups* of the embryos was mainly grade 1, grade2 and grade3 i.e <50% which might not have affected the development of blastocysts. There are many studies citing this trend. Xu et al. [12] indicated that developmental potential of the embryos is affected only when fragmentation is >35%; Hardy et al. [19] observed that <25% fragmentation did not affect the development of inner cell mass and rate of blastocyst development; Stone et al. [20] also observed that blastocyst development was affected only when the grade of fragmentation was grade 4 i.e>50%.

Other reason of fragmentation not affecting the blastocyst development might be that fragments might not have incorporated the genes responsible for the development. This was observed by Antczak and Blerkom [14] in their study. Third reason behind good development of blastocysts might be that fragmentation in the present study was mainly observed at the morula stage/stage of compaction in all groups* of the embryos. Similar finding was observed by Ebner et al[21] in their study.

Limitation of this study

This study was carried out on mouse embryos which have high development rate i.e 80% [15] and thus the results obtained may not be applied to the human embryos. Moreover, this study did not incorporate the study of genes which might have helped in understanding the significance of specific genes in determining the effect of fragmentation on blastocyst formation.

Future implications

After the advent of time-lapse imaging, fragmentation and its effect on the blastocyst development can be carried out in the human embryos also as several phenomenon can now be studied in the embryos without harming them [22]. New research is also focussing on studying apoptosis and necrosis in the embryos using non-invasive techniques such as Raman spectroscopy [23, 24]. This can further clear the picture of association of fragmentation with apoptosis or necrosis. More research should be undertaken to study the association of genes such as Bax, Bcl-x, transforming growth factor β2 (TGFβ2), vascular endothelial growth factor (VEGF), c-kit and epidermal growth factor R (EGF-R) in starting apoptosis in the fragmented embryos [14, 25].

Conclusion

Role of fragmentation in the embryo development is not clear. It related to apoptosis by some [7] and refuted by others [14] while some relate it to necrosis also [26]. Newer studies also indicate that fragmentation occurring at morula stage may not hinder the developmental potential of the embryos [21]. Recent studies indicate positive effects of the fragment removal on the blastocyst development [9]. If more studies are carried out on fragmentation, then new solutions will emerge on how to choose the embryo having good developmental potential and also how to improve the developmental potential of the embryos.

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