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Hatching: Potential area for research in the embryos

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Abstract
Till now, process of hatching was not studied extensively. But now, this process is being analysed extensively using metabolomics, proteomics and time-lapse imaging. It has been observed that hatching depends on various factors such as presence of lysins produced by trophectoderm and uterus, physical expansion of embryonic mass which leads to zona thinning, contraction and expansion of blastocyst leading to breach of zona, elasticity of zona and site of breach in the zona. was a prospective study carried out on frozen-thawed embryos which were exposed to varying concentrations of ammonium chloride. These embryos were randomly divided into 3 groups and were exposed varying concentrations of ammonium chloride (control KSOM (Pottassium Simplex Optimised Medium), KSOM with 38 µM ammonium chloride and KSOM with 75 µM ammonium chloride). thus difference in ammonium chloride concentration did not have significant effect on the change in the size of blastocyst or zona thinning. Percentage[a] of hatching and fully hatched blastocysts obtained in embryos cultured in different media.

Keywords: hatching, embryos, zona thinning

Introduction
Though knowledge in Assisted Reproduction Technology is growing day by day; take-home pregnancy rate is quite low [1]. It is because the success in Invitro fertilization depends on many factors such as quality of gametes and embryos, receptivity of the endometrium, and the process of hatching [2]. Many aspects of these factors were unknown. But, with the advent of newer technologies knowledge in these fields is improving [3]. One of such factors is the process of hatching. Till now, process of hatching was not studied extensively. But now, this process is being analysed extensively using metabolomics, proteomics and time-lapse imaging. It has been observed that hatching depends on various factors such as presence of lysins produced by trophectoderm and uterus, physical expansion of embryonic mass which leads to zona thinning, contraction and expansion of blastocyst leading to breach of zona, elasticity of zona and site of breach in the zona [4].

Here a study is being presented where hatching process in the embryos has been analysed. These embryos were exposed to varying concentration of ammonium chloride and morphokinectic and morphological changes occurring in the embryos were studied over a period of 5 days using time-lapse imaging i.e via embryoscope. By time-lapse imaging, morphokinetic, mechanical, morphometric and morphological changes occurring during development of the embryos can be analysed easily without damaging the embryos [5]. During this study, changes in relation to hatching of the blastocyst such as expansion of blastocyst, zona pellucida thinning and development of slits in the zona were analyzed.

Methodology
It was a prospective study carried out on frozen-thawed embryos which were exposed to varying concentrations of ammonium chloride. These embryos were randomly divided into 3 groups and were exposed varying concentrations of ammonium chloride (control KSOM (Pottassium Simplex Optimised Medium), KSOM with 38 µM ammonium chloride and KSOM with 75 µM ammonium chloride). These embryos were analysed for 5 days via time-lapse imaging i.e embryoscope. During this time; change in thickness of zona pellucida, increase in volume of the embryos, site and size of slit formed at the time of hatchinng were analysed.

Statistical analysis: Morphometric, morphokinetic, and morphological data was tested for its normalcy using Shapiro-Wilk test and then compared in all three groups.

If data was normally distributed, significant difference was calculated using one-way ANOVA and if not normally distributed then Kruskal-Wallis test was used. Statistics were performed using SPSS software (IBM SPSS statistics 23) and p-value of <0.05 was considered significant.

Results

Ideally, there should be increase in volume and decrease in width of zona at start of hatching[6]. This change in morhometrics helps in the hatching process. Keeping this in consideration, internal perimeter of the embryo and width of zona were measured before and at time of hatching, and the difference was compared using paired t-test.

Values of internal perimeter and zona at starting of embryo development and at time of hatching. 38 µM means 38 µM ammonium chloride in KSOM and 75 µM means 75 µM ammonium chloride in KSOM.

Table 1: Values of internal perimeter and width of zona at start of embryo development and at time hatching

	Overall	Control	38 µM	75µM
Internal perimeter of embryo at start of the culture	5351.16 ± 442.82	5443.29 ± 497.78	5245.72 ± 477.32	5455.55 ±344.96
Internal perimeter of embryo at time of hatching	8081.16 ± 1656.78	8186.60 ± 1935.75	7809.67 ±1614.37	8417.92 ±1602.55
Width of zona at start of culture	7.659 ± 0.813	7.662 ±0.253	7.983 ±0.467	7.171 ±1.189
Width of zona at time of hatching	3.847 ± 1.580	3.928 ± 1.693	3.944 ±1.349	3.636 ±1.963

Table 2: P-values of overall and group-wise comparison of internal diameter and width of zona before and at time of hatching

	Overall p-value	Control p-value	38µM p-value	75µM p-value
Internal perimeter before and after hatching	0.000	0.011	0.000	0.000
Width of zona before and after hatching	0.000	0.001	0.000	0.001

P-values in all groups was <0.05 indicating that in all groups there was significant change in thickness of zona pellucida and blastocyst volume. Thus, irrespective of ammonium chloride concentration all blastocyst underwent thinning of zona pellucida and increase in blastocyst volume.

To see whether the proportion of change in the area/internal perimeter of the embryo and the width of the zona differed from one group to another group, comparison of means of difference between the internal perimeter and width of zona before hatching and at time of hatching was done.

Group wise values (mean and standard deviation) of internal perimeter, difference in value of internal perimeter, width of zona and difference in width of zona at the time of hatching. Area was measured in μm^2 and width was measured in µm.

Table 3: The mean values of the parameters compared and the p-value of difference between the means

	Control Mean ± SD	38µM Mean ± SD	75µM Mean ± SD	p-value
Area of embryo at hatching (μm^2)	8186.88 ± 1935.75	7781.26 ± 1573.76	8417.92 ± 1602.55	0.780
Difference in area (area at hatching-area at start of culture) (μm^2)	2778.71 ± 2008.21	2736.67 ± 1294.46	2962.33 ± 1719.02	0.188
Width of zona (µm)	3.5625 ± 1.8791	3.9474 ± 1.3122	3.6364 ± 1.96330	0.591
Difference in width (width at the start of culture-width at time of hatching) (µm)	3.9693 ± 2.19683	4.3217 ± 1.9189	3.4600 ± 2.34292	0.794

From the above table it is seen that P-value is >0.05, thus difference in ammonium chloride concentration did not have significant effect on the change in the size of blastocyst or zona thinning.

Percentage[a] of hatching and fully hatched blastocysts obtained in embryos cultured in different media.

Table 4: Percentage of blastocysts undergoing hatching process and percentage of blastocysts which were fully hatched

	Control (%)	38 µM (%)	75 µM (%)
Hatching blastocyst	44.4	55.6	41.7
Fully hatched blastocyst	0	7.4	8.3

Different patterns were observed in relation to the process of hatching and parameters affecting the hatching process such as presence of thick or thin zona, presence of big or small slits and occurrence of secondary breach near or away from the primary hatching site.

Presence of thick zona impeded the process of hatching. Similarly, big slits in the zona favoured the process of hatching while small slits hampered the process. It was also seen that hatching process was not affected when secondary breach was formed near the primary hatching while it was impaired when secondary breach in zona occurred away from the primary breach site. Table 5 summarizes the events seen in various embryos.

Table 5: Percentage of embryos showing trends in the process of hatching

	Control (%)	38 µM(%)	75 µM (%)
Multiple breach	0	6.25	3.125
Small slits in zona	18.18	15.62	12.5
Thick zona	18.18	6.25	3.125

Figures showing different trends (mentioned in table 5) which have been observed during hatching process are being presented now.

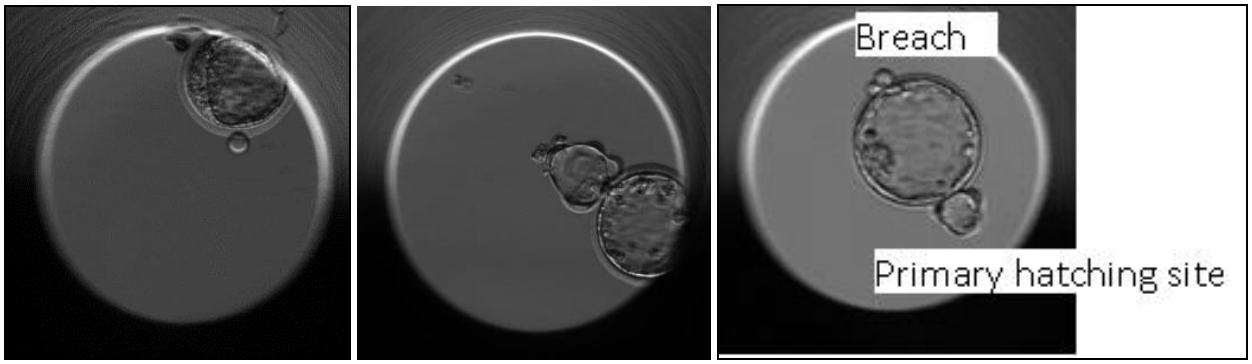

Fig 1: Figure on left hand side shows small slit causing inadequate escape of trophectoderm. Middle figure shows in-adequate expansion and thick zona at the time of hatching. Figure on right hand side shows breach at a site away from the primary hatching site. These trends prevented complete hatching of the blastocyst.

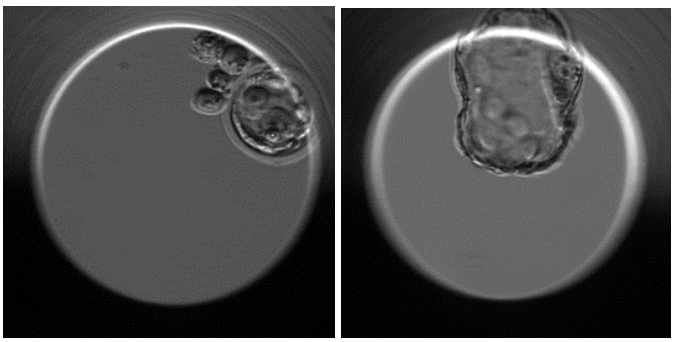

Fig 2: Comparison of small slit to wide slit in zona at the time of hatching. Image on left side shows inadequate expulsion of trophectoderm from small slit which resulted in damage of the trophectoderm and blastocyst. Image on right side shows big slit which favours smooth process of hatching.

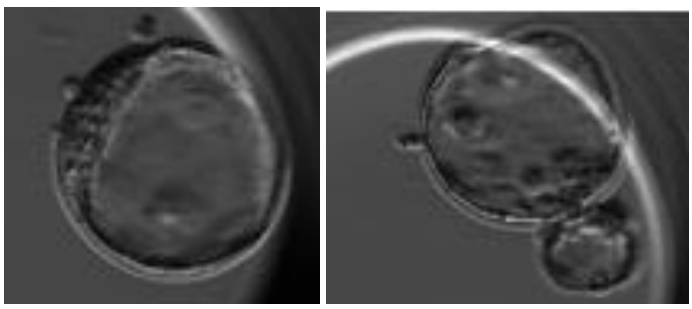

Fig 3: comparison of different sites of multiple breach. Image on left shows multiple breach in approximation of each other occurring simultaneously which favours hatching. Image on right shows breach at a place away from primary hatching site, occurring after hatching process which impeded the hatching process.

Discussion

It was observed that though hatching blastocyst were 44.4%, 55.6% and 41.7% in three groups i.e control, 38 µM ammonium chloride in KSOM and 75 µM ammonium chloride in KSOM group respectively; percentage of fully hatched blastocysts was very less i.e 7.4% and 8.3% in 38µM and 75µM ammonium chloride group respectively, while in control group there were no fully hatched blastocysts seen. It was also observed that parameters important for hatching such as thinning of zona pellucida and increase in the blastocyst area had occurring in all embryos irrespective of the concentration of ammonium chloride. So, there might be some other hindrances in path of hatching embryos due to which completely hatched embryos were less in proportion.

So, it can be considered that other parameters such as size of the slits formed in zona and site of secondary breach in zona at time of hatching might also affect the end result i.e completely hatched blastocyst. This fact has been supported in studies done in mouse and rat embryos by Niimura and Fuji [7], where it was seen that formation of big slit is required for completion of hatching process. They also observed that if breach in zona occurred near the primary hatching site fully hatched embryos will be formed but if they appear away from the primary hatching site then process of hatching is not completed. Similar patterns in formation of slits and the association of the size of slit to the hatching of blastocyst were observed the studies carried out in the cattle [8].

Conclusion

To improve the rate of take-home baby rates in In-Vitro fertilization, it is very important that more number of embryos should undergo complete hatching [9]. Thus, many studies are being carried out to study the processes favoring hatching process. Assisted hatching is also being studied and implemented recently to improve the process of implantation after Invitro fertilization [10]. To summarize in nutshell; embryo development is very complex process. Still many aspects of this process are unknown. In future, with help of technology these aspects can be explored and modified so that success of In-Vitro fertilization can improved.

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