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EFFECT OF LOWER CONCENTRATION OF AMMONIUM CHLORIDE ON DEVELOPMENT POTENTIAL OF THE EMBRYOS.

Gynaecology

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ABSTRACT

Background: Addition of amino acids to culture media improves the rate of blastocyst development. But, amino acid metabolism releases ammonia which has deleterious effects on the embryo development. Finding this threshold level above which its deleterious effect starts can help make appropriate changes in media or embryo environment.

Materials and methods: 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 varying concentrations of ammonium chloride (control KSOM, KSOM with 38 µM ammonium chloride and KSOM with 75 µM ammonium chloride). These embryos were cultured individually in embryoscope dishes till 5 days during which morphokinetic, morphometric and morphological parameters were noted and compared within all 3 groups of the embryos. On 5th day apoptosis in blastocysts of each group was studied by staining the blastocysts with TUNEL stain with counter stain of DAPI. Total number of cells and percentage of apoptosis in the blastocysts developed in each group were noted and compared within all 3 groups.

Results : Rate of pronuleus fading and time taken to reach 2 cell stage was significantly less in the embryos cultured in 38 µM and 75 µM ammonium chloride. Significant decrease in percentage of apoptosis and increase in total cell count was observed in the blastocysts obtained from the embryos cultured in 38 µM and 75 µM ammonium chloride in comparison to the blastocysts obtained from the embryos cultured in control group.

Conclusion: Ammonium chloride in lower concentration may have beneficial effect on the embryo development.

KEYWORDS

Ammonium chloride; embryo culture; time-lapse monitoring; embryoscope.

Introduction

In July 1978, there was first live birth from in-vitro fertilisation (IVF) (Steptoe & Edwards, 1978) and since then IVF techniques are helping infertile couples to conceive (Hardy et al, 2002). Out of the live pregnancies achieved one third are multiple gestations. Multiple gestation exposes mother to higher risk of obstetric complications (Dudenhausen& Maier, 2010). To decrease multiple pregnancies to less than 10%, eSet(elective single embryo transfer) is being promoted. For successful implementation of eSET programme, it is important not only to transfer the best quality embryos but also to generate embryos having good implantation potential. This can be achieved by improving culture conditions, optimising gamete quality, and developing new techniques for selecting superior quality embryo for transfer (Hardy et al, 2002). Culture media is one of the important parameters affecting the embryos and one of the important substrates added in culture media is amino acids. Addition of amino acids improves the rate of blastocyst formation. They help in regulating pH, work as anti-oxidant and chelator, synthesize proteins and nucleotides and also act as a source of energy (Houghton1 et al, 2002; Lane et al 2001). Breakdown of amino acids in the culture media and metabolism of embryos leads to accumulation of ammonia (Lane & Gardner, 2003). Ammonia has detrimental effect on the developing embryos which is directly related to its concentration in the culture media, duration of exposure and stage of embryo development at which exposure occurs (Golchin et al, 2016; Lane & Gardner, 2003). Hence the threshold levels at which ammonia starts it deleterious effects should be delineated so that change in culture or substrates can be made accordingly (Gardner et al, 2013). This study was undertaken to see the effect of varying concentration of ammonia chloride on the developmental potential of the embryos. Developmental potential in this project was studied using time-lapse analysis and apoptosis.

Time-lapse analysis is a dynamic assessment of the developmental potential of the embryos (Racowsky et al, 2015). Time-lapse analysis was done with the help of embryoscope as it has an added advantage of grading the embryos using computer-assisted annotations and image analysis software. With the help of time lapse technology, morphokinetic, morphometric and morphological data can be collected and stored simultaneously and thus association between these parameters can be studied (Storr et al, 2015).

Apoptosis was coined by Kerr, Wyllie, and Currie in 1972 to describe morphologically distinct form of cell death (Elmore, 2007). Apoptosis is increased in any stressful condition such as suboptimal culture and presence of reactive oxygen species. These conditions are also responsible for decrease in the developmental potential of the embryos (Kannan & Jain, 2004; Hardy, 1997). Thus, proportion of apoptosis can be considered as important parameter in selection of the embryos having superior embryo development.

Methodology

It is a prospective cohort study of commercially purchased 86 frozen-thawed mouse embryos (Embryotech laboratories, Inc; Wilmington, MA). Study was done in two phases and there were three runs of the experiment. Number of embryos per group in all three runs has been mentioned in table 1.

Table 1 Number of embryos cultured in different culture media during 3 runs of the experiment.38 µM indicates 38 µM of ammonium chloride in KSOM and 75 µM indicates 75 µM ammonium chloride in KSOM.

Run	Control	38µM	75µM
1st	11	11	11
2nd	11	10	10
3rd	-	11	11

First phase consisted of time-lapse analysis of the embryos and second phase consisted of analysis of apoptosis using fluorescent confocal microscopy. Base culture media used was 'Potassium simplex optimised medium' (KSOM) from Millipore. KSOM was chosen as it is preferred media for the mouse embryos (Yao & Asayama, 2016) and it a single step media (Gruber & Klein, 2011) and in this study single step media was required.

Mouse embryos were thawed according to the instructions given by

Embryotech laboratories and code was saved for further referencing. These embryos were divided in 3 groups and were cultured in KSOM, KSOM with 38μM ammonium chloride and KSOM with 75μM ammonium chloride. Culture was carried out in pre-incubated EmbryoscopeTM slides (FertiliTech) loaded with culture media.

Petridishes containing microdroplets of different aliquots of culture media overlaid with oil were also prepared to analyse pH. Average of the time at which the embryos were thawed was taken as the starting time of the time-lapse analysis (t0). Embryos were cultured till day 5. During these days morphokinetic, morphometric and morphological data was collected.

Morphokinetic data collected was as follows; time of pronuleus fading (tPNF); appearance of 2 cells (t2), 3 cells (t3), 4 cells (t4), 5 cells (t5), and 8 cells (t8); time interval of development of 3 cells from 2 cells (t3-t2), 4 cells from 3 cells (t4-t3), 8 cells from 5 cells (t8-t5). Other data used for morphokinectic analysis was time of formation of morula (tM), early blastocyst (tSB), blastocyst (tB), expanded blastocyst (tEB) and hatched blastocyst (tHB). Morphological analysis consisted of staging of the blastocysts according to the stage approved by the Istanbul consensus workshop (Balaban et al, 2011), which is mentioned in table 2. In morphometric analysis, change in the value of internal perimeter of the embryo and thickness of zona during hatching of the blastocyst were analysed.

Table 2 Blastocyst expansion stages according to the stages approved by Istanbul consensus.

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 zonapellucida becomes thinner due to expansion.
5	Hatching blastocyst
6	Fully hatched blastocyst

On day 5, embryos which had developed to the stage of blastocyst were analysed for apoptosis by double staining protocol i.e. FITC (fluorescein isothiocyanate) conjugated TUNEL (terminal deoxyribonucleotidyl transferase mediated digoxigenin nick end labelling) stain with counterstain of DAPI (4'-6-diamidino-2-phenylindole) according to the instructions given by sigma laboratories (www.sigmaaldrich.com, n.d). Throughout the procedure, blastocysts cultured in 3 different media were kept separate. Stained embryos were examined in multiple focal planes under fluorescent microscope (Nikon eclipse 90i) (Collazo et al, 2005). In Nikon microscope there is presence of dual band DAPI-FITC filter which can detect both fluorophores simultaneously i.e blue fluorescence emitted by normal cells stained with DAPI and green fluorescence emitted by apoptotic cells stained with TUNEL stain. Figure 1 shows the images produced by different stains. Percentage of apoptotic cells in the total cells was calculated and used as apoptotic index (AI). Comparison of the apoptotic index was done in all 3 groups.

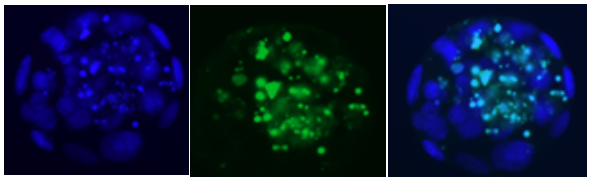

Figure 1 All images are of the same embryo which was subjected to the double stain. Photograph on the left-hand side shows the image produced by DAPI. The middle photograph shows apoptotic cells stained with TUNEL stain, while the photograph on right-hand side shows the merged picture of both stains.

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. In normally distributed data where difference was significant, Bonferroni post hoc test was used to do pairwise comparisons while in Kruskal-Wallis test pairwise comparison was done when there was significant difference. Statistics were performed using SPSS software (IBM SPSS statistics 23) and p-value of <0.05 was considered significant.

Results

Measurement of pH of control KSOM, 38 μM ammonium chloride in KSOM and 75 μM ammonium chloride in KSOM was done using Mettler Delta 320 and Inlab ultra-micro electrode. The pH values did not undergo significant change on addition of ammonium chloride (range of 7.50 to 7.64).

Rate of blastocyst formation was assessed in all three groups of the embryos. Figure 2 shows the pictorial presentation of percentage of blastocysts formed in each group. On comparison of means, there was no significant difference in the rate of blastocyst formation with regards to change in culture media (p-value=0.256).

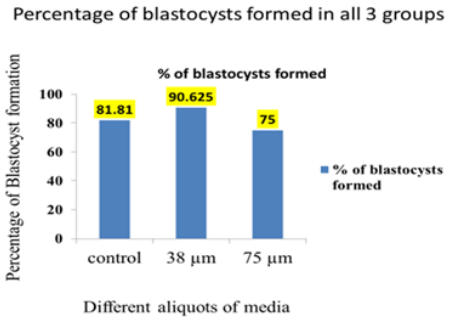

Figure 2 Percentage of blastocysts formed in all groups. 38 μM indicates 38 μM of ammonium chloride in KSOM and 75 μM indicates 75 μM ammonium chloride in KSOM.

Morphokinetic analysis:-To analyse whether the quality of the blastocysts formed was affected by the change in the culture media, stage of blastocyst formed in each group were studied. The stage was allocated according to the stages approved by the Istanbul consensus. Table 3 shows the values of different blastocyst stages observed in all three groups. The p-value was not significant indicating that stage of blastocyst formed in the embryos was not influenced by the change in ammonium chloride concentration.

Table 3 Percentagea of different stage of blastocysts obtained in embryos cultured in different media. Highest values are marked bold. 38 μM indicates 38 μM of ammonium chloride in KSOM and 75 μM indicates 75 μM ammonium chloride in KSOM.

	control	38 μM	75 μM
Early blastocyst	16.7	3.7	8.3
Blastocyst	11.1	7.4	0
Expanded Blastocyst	**27.8**	**25.9**	**41.7**
Hatching blastocyst	**44.4**	**55.6**	**41.7**
Fully hatched blastocyst	0	7.4	8.3

[a]Percentage of blastocyst$=\frac{number\ of\ blastocyst\ of\ specific\ grade\ in\ the\ group}{total\ number\ of\ blastocysts\ developed\ in\ that\ group}\ X\ 100$

To analyse change in the morphokinetic milestones in association with the change in culture media, comparison of morphokinetic data was done in all 3 groups. It was observed that difference of means of tPNF and t2 was significant. Bonferroni post hoc test was done to see between which groups the value was significant. Data showed p-value of difference in means of tPNF was significant in control group vs the group cultured in 38 μM ammonium chloride while p-value of difference in the means of t2 was significant in control group vs group cultured in 38 μM ammonium chloride as well as control group vs group cultured in 75 μM ammonium chloride in KSOM. The value of mean tPNF was more in the control group (6.585 h) compared to the group developing in 38 μM ammonium chloride in KSOM (5.139 h) indicating that embryos developing in control KSOM took more time for pronucleus fading in comparison to the embryos developing in 38 μM ammonium chloride. Similarly, mean t2 was 10.314 h in control group in comparison to 8.380 h and 8.209 h in the group cultured in 38

μM ammonium chloride and 75 μM ammonium chloride respectively indicating that time taken by embryos to reach 2 cell stage was more in control group when compared to other groups. Results are summarised in table 4.

Table 4 Comparison of mean, standard deviation (SD) and p-value of morphokinetic milestones. Parameters compared are tPNF (time of pronucleus fading), t2 (time of appearance of 2 cells), t3 (time of appearance of 3 cells), t4 (time of appearance of 4 cells), t5 (time of appearance of 5 cells), t8 (time of appearance of 8 cells), time taken to form morula (tM), early blastocyst (tSB), blastocyst (tB), expanded blastocyst (tEB), and hatching blastocyst (tH). Significant values have been marked bold. 38 μM indicates 38 μM of ammonium chloride in KSOM and 75 μM indicates 75 μM ammonium chloride in KSOM. 38 μM indicates 38 μM of ammonium chloride in KSOM and 75 μM indicates 75 μM ammonium chloride in KSOM.

	Mean ± SD (control)	Mean ± SD (38 μM)	Mean ± SD (75 μm)	p-value
tPNF	**6.585 ± 1.838**	**5.139 ± 1.200**[a]	**5.941 ± 1.640**	**0.004**
t2	**10.314 ± 2.976**	**8.380 ± 3.003**[b]	**8.209 ± 2.361**[b]	**0.016**
t3	29.266 ± 2.455	27.829 ± 1,614	28.290 ± 3.428	0.177
t4	29.978 ± 2.353	28.987 ± 2.392	30.164 ± 4.671	0.354
t5	40.785 ± 4.430	39.044 ± 2.440	39.611 ± 3.784	0.280
t8	41.708 ±2.583	41.021 ± 2.777	41.764 ± 3.535	0.664
tM	53.157 ± 8.875	49.742 ± 4.472	51.116 ± 5.477	0.164
tSB	72.039 ± 7.894	69.363 ± 5.746	70.986 ± 7.615	0.430
tB	72.919 ± 6.149	73.093 ± 6.330	73.328 ± 6.197	0.980
tEB	78.184 ± 6.279	79.120 ± 7.842	79.057 ± 6.170	0.922
tHB	79.263 ± 10.028	79.765 ± 8.867	82.096 ± 6.770	0.700

[a]positiveBonferroni post-hoc for tPNF, p-value (0.003)

[b]positiveBonferroni post-hoc test for t2, p-value (0.041) between control and 38 μM group and 0.023 between control and 75 μM group.

There was chance of bias in the experiment as exact time of insemination is not known in the embryos which are frozen-thawed and thawing time is not precise as process of embryo freezing can vary by several hours. Thus, to remove the bias, t2 was considered as reference point and all parameters were examined again in accordance to t2. No significant difference was seen in p-value of various parameters.

Morphometric analysis:-Ideally, there should be increase in volume and decrease in width of zona at start of hatching (Kirkegaard et al, 2012). Keeping this in consideration, internal perimeter of the embryo and width of zona were measured before and after hatching, and the difference was compared using paired t-test. Overall comparison and group-wise comparison of change in internal perimeter of the embryo and width of zona was done to analyse whether this function was affected by the change in ammonium chloride concentration. P-value in all the groups was significant indicating that volume expansion and zona thinning was not affected by change in the culture media. Table 5 shows the values of internal perimeter and width of zona at start of embryo development and at time hatching. Table 6 shows the p-values of overall and group wise comparison of area of the embryos and width of zona before and after hatching.

Table 5 Values of internal perimeter and zona at starting of embryo development and at time of hatching. 38 μM indicates 38 μM of ammonium chloride in KSOM and 75 μM indicates 75 μM ammonium chloride in KSOM.

	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 6 P-values of overall and group-wise comparison of internal diameter and width of zona before and after hatching.

	Overall p-value	Control p-value	38 μM 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

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 after hatching was done. P-value was not found to be significant indicating that change in the culture media did not affect the proportion of expansion and zona thinning occurring at hatching. Taking into consideration the findings it was interpreted that all embryos underwent volume expansion and thinning of zona but there was no significant difference with regards to proportion of the change in the area/internal diameter or width of the zona.

Apoptosis:-Apoptosis was analysed considering 3 parameters; number of apoptotic cells, number of total cells and percentage of apoptotic cells. Distribution of these parameters within the groups was studied by taking out mean, standard deviation (SD) and standard error of mean (SE) for each parameter and comparing them in all three groups. P-value was significant on comparing total number of cells and percentage of apoptosis. To find out between which two groups the difference was significant, data of pair-wise comparison of Kruskal-Wallis test was taken. It was seen that p-value was significant between control group and group cultured in 75 μM ammonium chloride in KSOM. Table 7 shows the values of mean, SD of each parameter and p-value of difference between the means of each parameter within all groups. The mean value of total cells in control group and group cultured in 75 μM ammonium chloride was 38.64 and 59.06 respectively. Similarly, percentage of apoptosis in the control group and group cultured in 75 μM ammonium chloride was 28.31% and 19.43% respectively. This indicated that embryos cultured in 75 μM ammonium chloride had more number of cells and less percentage of apoptosis.

Table 7 Group-wise comparison of mean, standard deviation (SD) and p-value of difference in means of apoptotic cells, total cells, and percentage of apoptosis. Significant values have been marked in bold.

	Control	**38 μM**	**75 μM**	**p-value**
Apoptotic cells	9.18 ± 3.573	10.24 ± 5.495	7.00 ± 2.351	0.063
Total cells	**38.64 ± 20.393**	**46.90 ±15.522**	**59.06 ±19.395**[a]	**0.013**
Percentage of apoptosis	**28.316 ± 13.837**	**26.552 ±22.281**	**19.438 ±19.819**[a]	**0.032**

Discussion

There was no change in the proportion of blastocyst formed and stage of blastocyst formed in the embryos in relation to the addition of ammonium chloride. These results are comparable to the study done by Golchin et al (Golchin et al, 2016); which reported that rate of cleavage and blastocyst development in the sheep oocytes obtained by in-vitro maturation was not statistically different when they were exposed to 28 μM and 88 μM ammonium chloride but decreased when exposed to 132 μM and 176 μM ammonium chloride. Similar findings were observed by Tareq et al., where developmental arrest in porcine embryos were observed at concentration of ≥ 300 μM (Tareq et al, 2007).

Results of this study also showed that time taken by embryos in control group to achieve pronucleus fading(tPNF) and 2 cell (t2) was more in comparison to other groups. This finding points out towards the beneficial effect of the ammonium chloride. Studies supporting this statement are ofShoukiret al (Shoukir et al, 1997) and Tsai et al (Tsai et al, 2002); who stated that early tPNF and t2 are indicators of human embryos having good implantation. Lemmen et al (Lemmen et al, 2008) also suggested that early tPNF and t2 resulted in embryos having more number of blastomeres.

In this study increase in total number of cells and decrease in

percentage of apoptosis was observed in blastocysts obtained from the embryos cultured in media supplemented with ammonium chloride. More number of cells in pre-implantation embryo is an indicator of superior embryo quality (Newmark et al, 2007). Thus, it can be inferred that embryos which were cultured in 38 μM and 75μM ammonium chloride might have improved development in comparison to those cultured in control media. Beneficial effects of ammonium chloride at lower concentration was also observed by Hammon et al (Hammon et al, 2000). Their study showed increased percentage of hatched/expanded blastocysts in group of bovine embryos cultured in 38 μM (16.1%) and 88 μM (16.9%) ammonium chloride in comparison to those developing in control media (10%).

Conclusion

Addition of amino acids is helpful if concentration of ammonia which is by-product of amino acid metabolism is kept low. Glutamine is the most labile of all amino acids and its breakdown results in ammonium and pyrolidine carboxylic acid (Gardner et al, 2013).Production of ammonium in culture media can be reduced by replacing glutamine with glycyl-L-glutamine (Summers et al, 2005).

This study also emphasised the role of non-invasive technologies in Assisted Reproduction Technology (ART). Time-lapse imaging is already being used for observing embryo development but in areas such as detection of apoptosis, non-invasive techniques may provide better outcome. New studies are being conducted with the use of non-invasive technology like Raman microspectroscopy for detecting apoptosis/necrosis in the embryo (Brauchle et al, 2014; Pascut et al, 2011) which can help to distinguish apoptosis and necrosis in the embryos and thus help in selection of the embryos with good implantation potential.

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