Introduction

**Fertilization** - the process of union of two haploid gametes to form a single diploid cell. The zygote proves potential of gametes for reproduction. Formation of pronuclei is the first sign of successful fertilization. The Pro-Nuclear (PN) morphology reflects gamete quality as well has prediction potential for subsequent development of embryo. The pronuclear stage evaluation is descriptive, time requiring and demanding for expertise observation. However being a complex, multistep process error or asynchrony at one or other step is most likely to occur and get reflected in zygote morphology.

Intra cytoplasmic sperm Injection ICSI activates the oocyte and triggers meiosis resumption. The appearance of male and female pronuclei approximately 6 hrs after ICSI is the first easily observable sign of fertilization. Approximately 16-18 h post injection the pronuclei migrate to center of oocyte cytoplasm, increases in size and nuclear precursor bodies get aligned with each other (Payne et. al. 1997, Van Blerkorn et. al. 1995). This is the ideal time for grading of zygote for its selection in IVF treatment because the arrangement and morphology of pronuclei and nuclear precursor bodies changes over a time.

Assessment of zygote morphology needs careful observations and demand for expertise. Hence we thought of designing a Numerical Scoring System (NScS) to assign a numerical score to the zygote. The numerical scoring system is also based on observations of morphological characteristics of zygote and is simple, reproducible, highly economical and fast. It makes the analysis of images of the zygote capture during IVF programs to ascertain a numerical score for its every ideal character and deviations.

This study has been carried out by retrospective analysis of data of zygotes accumulated at Niramaya IVF Centre from June 2010 to March 2016.

**Materials and methods**

I) **Materials**

A. **Biological material**

i. Fertilized oocytes obtained during in-vitro fertilization treatment.
B. Instruments
i. Stereo zoom microscope (Olympus SZ40)
ii. Microfilt laminar flow (Life Science Technology)
iii. Hera cell CO₂ incubator (HereusHeracel)
iv. Inverted microscope with micromanipulator (Olympus IX70 microscope)
v. Micropipettes (Tarson 200-1000µl)
vi. Flexipetter (Tarson)
vii. Nikon camera attached to microscope
viii. Image capturing software
ix. Camera attached to microscope
x. Laptop

C. Disposables
i. Cook’s Flexipet 175 µm

II) Method:

A) Evaluation of Zygote morphology:
i. Zygotes were observed under Olympus IX70, inverted microscope at 400x magnification by a single observer to avoid biased evaluation as described in chapter 3 materials and method.
ii. The zygote morphology is assessed for number, size and symmetry of pronuclei, appearance of cytoplasm as well as size, number and distribution of the nucleoli.
iii. Images were captured with the camera attached to microscope.

B) Zygote scoring
Zygote morphology is assessed for all parameters which include 1) number, 2) size 3) position of nucleoli 4) number and distribution of NPBs and 5) appearance of cytoplasm. Zygotes were assigned on the scale of 0 – 9. Zygote exhibiting all parameters absolutely ideal is assigned the score of 9. Deviations in the number of PN are denoted by number D as described in detailed in Chapter 3 material and methods.
Numerical score is assigned to the zygote obtained after ICSI procedure by taking in to consideration the characteristics of all its different regions. Each of the morphological deviation from the character of an ideal zygote has been given a deviation score of (2). Score was assigned to the zygote by subtracting the total deviation score from the score of the ideal zygote.

II) Results
Zygotes obtained after ICSI technique were observed under inverted microscope (Olympus IX 70) at 400x magnification by moving microscope stage in x, y and z direction. Zygote morphology was assessed with respect to pronuclear number, size and their relative position, number and alignment of NPBs in both pronuclei and appearance of cytoplasm.
Morphological variations considered in the present study include size and shape of PN, number and distribution of NPBs in both PN and appearance of cytoplasm. This retrospective study is based on the data of 119 zygotes accumulated at Niramaya IVF Center from June 2010 to March 2016. Table no. 5.1 shows the morphological parameters and their deviations related to every region of the zygote as observed under microscope at 17 -19hr after ICSI.
Table 5.1: Zygotes morphology at 17 -19hr after ICSI.

<table>
<thead>
<tr>
<th>SrNo</th>
<th>Zygote morphology</th>
<th>Ideal character</th>
<th>Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pronuclear number</td>
<td>2PN</td>
<td>1.1 one PN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.2 three PN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.3 ≥ three PN</td>
</tr>
<tr>
<td>2</td>
<td>Relative size of two Pronuclei</td>
<td>Approximately same sized</td>
<td>2.1 Relatively small</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pronuclei</td>
<td>2.2 large size</td>
</tr>
<tr>
<td>3</td>
<td>Relative Position of Pronuclei</td>
<td>Centrally juxtaposed</td>
<td>3.1 peripherally positioned PNs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.2 Separation between PNs</td>
</tr>
<tr>
<td>4</td>
<td>Nuclear precursor bodied</td>
<td>Numbers similar</td>
<td>4.1 differ in number</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Similar size of NPBs</td>
<td>4.2 differ in sizes (appearance)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aligned</td>
<td>4.3 scattered/differential in appearance between PNs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.4 Ghost PNs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.5 Single NPB in one or more PNs (bull’s eye)</td>
</tr>
<tr>
<td>5</td>
<td>Cytoplasm</td>
<td>without inclusions</td>
<td>5.1 Small vacuoles</td>
</tr>
</tbody>
</table>

Variations of zygote morphology are enlisted in the table 5.1 and denoted by D = 1.1, 1.2, ------- 5.1 according to region of zygote.

Considering the characteristics of all different regions of the zygote a score is assigned for its selection in IVF program. While assigning score to the zygote total deviation score is subtracted from the score 9 of the ideal zygote. Table 5.2 enumerates the observations of zygote morphology and the numerical score assigned to it as per the scoring scheme designed in this study.

C) Assigning selection score to the zygote:

Zygotes obtained after ICSI technique during IVF program are observed microscopically for morphological analysis and incubated further under optimum culture conditions.
Growth of the developing zygote is monitored and subsequently developed embryo is transferred at suitable time. In the present scenario it is difficult to trace out the quality of zygote resulting into embryo. With the numerical scoring system for zygote designed in this study the quality of zygote resulting in embryo formation can be traced back.

With this scoring system for zygotes a method of its selection for its use in IVF treatment has been formulated.

This method is based on the following assumptions

1. Score for zygote with ideal characteristic = 9.
2. Each morphological deviation from the ideal characteristic is assigned a deviation score (2).
3. Score is assigned to the zygote by subtracting the total deviation score from the score of the ideal zygote.
4. Successful zygote formation i.e. the completion of first developmental stage during IVF is merited by applying Stage specific multiplication factor (St MF) of $10^1 = 10$.

Selection Score of the zygote (SScZ) is calculated by multiplying ScZ with the multiplication factor of $10^1$.

Thus

**A) For ideal zygote**

a) Score of ideal zygote = 9

b) Total deviation Score (TDS) zygote = 0

c) Score of ideal zygote = 9 - 0 = 9

d) Selection Score of the zygote (SScZ)

$$SScZ = ScZ \times St \, MF$$

$$SScZ = ScZ \times 10^1$$

$$= 9 \times 10$$

$$= 90$$

**B) For zygote with D =2.2 and 3.1,**

a) Score of ideal Zygote = 9

b) Morphological deviation of zygote = (4)

c) Score of zygote (Sc Z) = 9 - 4 = 5
d) Selection Score of the Zygote (SSc Z) = TGS x 10^1
   = 5 x 10^1
   = 50

e) Cumulative Selection Score at zygote stage (CSScZ) = SScG + SScZ
   = 9 + 90
   = 99

Table no 5.2 shows the selection scores assigned to the zygote employing this method. Total score of the ideal zygote is shown for the reference.
### Table 5.2: Selection Score of Zygote

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Zygote</th>
<th>Morphology of zygote</th>
<th>Deviation score (D)</th>
<th>Zygote score (ZSC)</th>
<th>Stage multiplication factor (St MF)</th>
<th>Selection score of zygote (SSC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal cytoplasm 2 Equal sized PN Equal, Symmetrical NPB</td>
<td>0</td>
<td>9</td>
<td>$10^1=10$</td>
<td>$9 \times 10=90$</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>un aligned NPB (D = 4.3) normal cytoplasm Equal sized PN, Homogenous cytoplasm</td>
<td>-2</td>
<td>7</td>
<td>$10^1=10$</td>
<td>$7 \times 10=70$</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Un equal sized PN (D=2.1), un aligned (D=4.3) equal NPBs,</td>
<td>-4</td>
<td>5</td>
<td>$10^1=10$</td>
<td>$5 \times 10=50$</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Un equal sized PN (D=2.1), un aligned PN, unequal NPBs (D=4.1), un aligned (D=4.3)</td>
<td>-6</td>
<td>3</td>
<td>$10^1=10$</td>
<td>$3 \times 10=30$</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3 PN (D = 1.1) Dark Cytoplasmic</td>
<td>9</td>
<td>0</td>
<td>$10^1=10$</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Morphological studies of zygotes were carried out by single observer to avoid biased selection. Observed under Olympus IX 70. Sc D= Score for deviation was found out for zygote and score of the zygote was calculated (ScZ). Selection score of the zygote (SScZ) was calculated by multiplying with St MF = $10^1$ Cumulative Selection Score of zygote (CSScZ) = SScG + SSc Z.
III) Discussion

Assessment of zygote morphology and its correlation with the subsequent development is routinely performed in the IVF program. Predictive value of zygote quality for embryo development has been documented in the literature. Zygote exhibiting deviation in number of pronuclei, relative sizes of PN considered as abnormal. Unequal number, size and alignment of NPBs reflect delay or fast condensation in one nucleus. This represents abnormal or asynchronous cytokinesis and karyokinesis reducing the developmental potential of embryo. Since the chromosomes bearing NPBs are most likely to be abnormal in any aneuploidy screen, its evaluation plays important part in zygote assessment.

In the present work microscopic observation of zygote to assess the characteristics of each of its regions was performed in alliance with the existing zygote grading system. Every parameter and the character of every region of zygote when assigned a numerical number reflected the quality of the developing embryo. Deviation in any morphological characteristic of the zygote from ideal characters has been assigned a deviation score. This has made the scheme highly accommodated and realistic. Selection Score of the zygote (SScZ) obtained by this scheme exactly reflects the scenario during the formation of this zygote. Since the cumulative score of the zygote reflects gamete quality the system of numerical scoring give an opportunity for better understanding of the possibility of IR.