Early cleavage of human embryos is a strong predictor for embryo implantation in ICSI

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Abstract: *Purpose*: To observe whether early cleavage can be a predictor of pregnancy and implantation rates. *Methods*: A total of 193 infertile couples, attending the ART unit, International Islamic Center for Population Studies and Research (IICPSR), -Al- Azhar University in the period from December 2010 to September 2012, were included in this study. The controlled ovarian hyperstimulation protocol was performed according to a long GnRH agonist protocol starting in the midluteal phase (day 21) of the preceding cycle. Embryos were assessed at 25 – 27 hr after ICSI for early cleavage. Embryos which reached the two cell stage at this interval were classified as Early Cleavage (EC) embryos, and the remaining as Non Early Cleavage (NEC) embryos. Embryos were assessed again at 64 - 68 hours post-ICSI for day-three embryo morphology. Day 3 embryo transfer was done. The best two or three embryos, according to day 3 embryo morphology were transferred. The patients were subdivided into two subgroups; one will transfer early cleavage embryos (EC) and the other will transfer non early cleavage embryo (NEC). *Results*: We found that transfer of early cleavage embryos (EC) led to significantly higher pregnancy rates as compared to non early cleavage embryos (NEC) (43.30 % versus 21.88 %; P = 0.005), and also higher implantation rates (25.58 % versus 11.35 %; P = 0.000). Also we found that the EC embryos had significantly higher proportion of good quality embryos when compared to NEC embryos (P = 0.000). *Conclusion*: early cleavage could be an additional factor for selecting embryos with a higher potential of implantation and successful pregnancy
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Introduction

One of the major criticisms of IVF treatment is that it leads to a recognized increase in multiple pregnancies (***Hernandez et al., 2001; Schieve et al., 2002***). Recently, there has been a heightened awareness of the need to control the risk of high order multiple gestations. In several countries these dangers have been reduced by legal restrictions on the number of transferable embryos (***Montag et al., 2001; Zollner et al., 2002***).

In order to select the most viable embryo, embryo scoring systems have been developed based on embryo morphology and blastomere number on the day of transfer ***(Cummins et al., 1986; Steer et al., 1992; Giorgetti et al., 1995; Ziebe et al., 1997).*** Much effort has been devoted to refining existing embryo scoring systems and finding additional simple, non-invasive parameters that could improve the embryo selection procedure ***(Van Royen et al., 1999; Tesarik et al., 2000; Wittemer et al., 2000, Montag et al., 2001; Payne et al., 2005; Senn et al., 2006; Brezinova et al., 2009)***. Early cleavage is one of the most promising new selection parameters as it is easily determined indicator of embryo quality.

Patients and methods

A total of 193 infertile couples, attending the ART unit, International Islamic Center for Population Studies and Research (IICPSR), -Al- Azhar University in the period from December 2010 to September 2012, were included in this study.

Patients were selected according to following criteria: 1) Age ≤ 35 years old; 2) BMI ≥25 - 30 kg/m2; 3) Have two ovaries; 4) Regular cycles; 5) first ICSI cycle; 6) Exclude uterine factor as a cause of female factor infertility; 7) No pelvic masses or diseases (e.g: endometriosis, fibroids, hydrosalpnix, ...); 8) No history of medical disorders (e.g: hypertension, D.M, thyroid dysfunction, liver diseases, renal diseases,...); 9) Exclude azoospermia as a cause of male factor infertility; 10) Long midluteal GnRH protocol.

The study protocol was approved by the Ethics Committee of Obstetrics and Gynecology Department, Faculty of Medicine, Al-Azhar University.

Controlled ovarian hyperstimulation using long GnRH agonist protocol: starting in the midluteal phase (day 21) of the preceding cycle, daily s.c injection with triptoreline-acetate (Decapeptyl, 0.1 mg/day; Ferring, Hoofddorp, The Netherlands). Once adequate pituitary desensitization was achieved as evidenced by oestradiol levels < 50 pg/ml (183 pmol/ml), ovarian stimulation will be started using HMG (Merional 75 I.U, IBSA, Switzerland). Folliculometry were started on day 6 of HMG therapy, then every other day to assess ovarian response. The

dose of HMG was adjusted according to the patient's response either by step up or step down

Triggering of ovulation was done by administration of 10000 IU HCG (Choriomon, IBSA, Switzerland) when at least half of follicles measuring 18 - 22 mm.

Oocyte retrieval 34-36 hours later on. The oocytes were placed in culture medium and intracytoplasmic sperm injection **(ICSI)** was done. Fertilization was diagnosed by the presence of two pronuclei in the injected oocyte 16 – 18 hr postinsemination.

Embryo quality assessment:

Embryos were assessed at 25 – 27 hr after ICSI for early cleavage. Embryos which reached the two cell stage at this interval were classified as Early Cleavage (EC) embryos, and the remaining as Non Early Cleavage (NEC) embryos. Embryos were assessed again at 64 - 68 hours post-ICSI for day-three embryo morphology. The proposed embryo score is based on the number of blastomeres or cells observed in relation to number of hours post insemination, the uniformity of cells in terms of size and shape, the clarity of the cytoplasm in terms of presence or absence of granulation, as well as the degree of anuclear fragmentation. The best embryos obtained a score of five ***(Loi et al., 2008).*** We considered embryos obtaining a score of five or four as good quality embryos.

Day 3 embryo morphology score

Features of the embryo	***Yes***	***No***
Is the embryo at a 6–8 cell stage at 46 - 68 hr, post-insemination?	1	0
Are all cells uniform in size?	1	0
Are all cells uniform in shape?	1	0
Is the cytoplasm of cells clear?	1	0
Are the anuclear fragments absent? If present, do they exceed 25%?	1 -1	0 0

Embryo selection and transfer:

Day 3 embryo transfer was done. The best two or three embryos, according to day 3 embryo morphology as prescribed before, were transferred. The patients were subdivided into two subgroups; one will transfer early cleavage embryos (EC) and the other will transfer non early cleavage embryo (NEC). The cumulative embryo score (CES) was calculated by the summation of the individual embryo scores of the embryos transferred.

Luteal phase support was given to the patients in the form of daily 100mg progesterone in oil intramuscular injection for 14 days.

Follow up of the patient after embryo transfer:

A single serum B-HCG measurement will be performed 14 days after embryo transfer. A clinical pregnancy will be determined by identifying the presence of a gestational sac at six weeks gestation on transvaginal ultrasonography.

Different clinical parameters were compared between EC and NEC embryo transfers. The effects of different factors on the clinical pregnancy rate were also estimated. In both of these calculations, the independent-samples Student's t-test and the chi-square x^2 test were used.

Results:

A total of 193 treatment cycles were analyzed. A total of 2023 oocytes were retrieved. Among them, 1232 oocytes were fertilized, 1077 embryos were obtained (overall fertilization rate 87.42%). Of these embryos, 364 (33.80 %) were early cleavage **(EC)** and 713 (66.20 %) were non early cleavage **(NEC)**.

The cycles were divided into two groups, first group (n= 97) was undergone EC embryo transfer and the second group (n= 96) was undergone NEC embryo transfer. A total of 444 embryos were transferred; 215 of them transferred for the first group and 229 transferred for the second group. The overall pregnancy rate was 32.64% (63/193), multiple pregnancy rate was 23.81% and implantation rate was 18.24%.

The transfer of EC embryos resulted in two times higher clinical pregnancy rate (43.30% versus 21.88%; P = 0.005) and more than double implantation rate (25.58% versus 11.35%; P = 0.000) when compared to NEC embryo transfers (table I).

Table I: Comparison of clinical parameters in cycles where early cleavage (EC) and non early cleavage (NEC) embryos were transferred

Parameters	EC	NEC	*P* value
No of cycles	97	96	
Age (ys.)	**28.03± 3.69**	**29.19± 3.39**	**0.024 ***
BMI (kg/m²)	27.52± 1.35	27.50± 1.38	0.953
Duration of infertility (ys.)	5.47± 2.67	5.94± 2.90	0.250
Basal FSH (mIU/ml)	**6.55± 2.16**	**7.39± 1.99**	**0.005 ***
Basal E2 (pg/ml)	63.76± 27.89	62.69± 26.37	0.783
No. of retrieved oocytes	**12.85± 5.88**	**8.09± 3.02**	**0.000 ***
No. of MII oocytes	**7.73± 3.31**	**5.02± 1.88**	**0.000 ***
Fertilization rate	**90.45± 8.86**	**84.95± 12.68**	**0.001 ***
No. of EC embryos (%)	**310/671 (46.20%)**	**54/406 (13.30%)**	**0.000 ***
No. of good quality embryo	**3.55± 1.47211**	**2.10± 1.041**	**0.000 ***
No. of embryo transferred	**2.22± 0.41**	**2.39± 0.49**	**0.010 ***
Cumulative embryo score (CES)	9.14± 1.73	9.32± 2.21	0.532
No. of clinical pregnancy (%)	**42 (43.30%)**	**21 (21.88%)**	**0.005 ***
Multiple pregnancy (%)	**11/42 (26.19%)**	**4/21 (19.05%)**	**0.000 ***
Implantation rate (%)	**55/215 (25.58%)**	**26/229 (11.35%)**	**0.000 ***

*** Significant**

Table II: The analysis of factors predicting the clinical pregnancy rate of ICSI cycles

Parameters	Clinical Pregnancy		*P* value
	Yes	No	
No of cycles	63	130	
Age (ys.)	28.52± 3.49	28.65± 3.63	0.824
BMI (kg/m²)	27.69± 1.44	27.43± 1.31	0.214
Duration of infertility (ys.)	5.612± 2.53	5.75± 2.92	0.767
Basal FSH (mIU/ml)	6.97± 2.14	6.97± 2.11	0.997
Basal E2 (pg/ml)	65.56± 25.21	62.10± 27.96	0.407
No of retrieved oocytes	**12.33± 5.51**	**9.58± 4.88**	**0.001 ***
No of embryo	**6.65± 2.84**	**5.06± 2.50**	**0.000 ***
Fertilization rate (%)	419/473 (88.58%)	658/759 (86.69%)	0.071
No of EC embryos (%)	**164/419 (39.14%)**	**200/658 (30.40%)**	**0.000 ***
No of good quality embryo	**4.08± 1.38310**	**2.22± 1.07**	**0.000 ***
No of EC embryo transfers (%)	**42/63 (66.67%)**	**55/130 (42.30%)**	**0.008 ***
No of embryos transferred	2.38± 0.49	2.26± 0.44	0.091
Cumulative embryo score (CES)	**10.35± 2.126**	**8.69± 1.66**	**0.000 ***

*** Significant**

The analysis of all factors predicting the clinical pregnancy (table II) demonstrated a clear correlation between the early cleavage of transferred embryos and the establishment of the pregnancy, as more EC embryos were transferred to the pregnant than to non-pregnant women (66.67 versus 42.30%, P = 0.008). Also the proportion of number of the EC embryos was higher in the pregnant group (P = 0.000). It was demonstrated a clear correlation between the proportion of good quality embryo, CES and the occurrence of the pregnancy (P = 0.000 *for both parameters*). Other clinical parameters, as the mean age of patients, the BMI, the duration of infertility, the basal FSH and basal E2, fertilization rate and the number of embryos transferred did not differ between pregnant and non-pregnant women.

One hundred thirty six couples (70.47% of patients) produced at least one early cleavage embryo (EC) while fifty seven couples (29.53% of patients) produced. Only non early cleavage embryos (NEC) (table III). Patients with at least one early cleavage embryo showed about four times higher pregnancy

rate when compared to patients had only non early cleavage embryos (41.91 % versus 10.53 %; P = 0.000). Also they had higher implantation rate and more good quality embryos. There was no difference between the two patients groups in the female age, BMI, duration of infertility, basal E2, number of transferred embryos and Cumulative embryo score (CES) of the transferred embryos.

Table III: characteristics of patients with at least one early cleavage embryo and those without early cleavage embryo.

Parameters	At least one early cleavage embryo	Only Non Early cleavage embryos	P value
No of cycles	136	57	
Age (ys.)	28.29±3.63	29.35±3.37	0.061
BMI (kg/m²)	27.57±1.31	27.38±1.48	0.377
Duration of infertility (ys.)	5.58±2.76	6.00±2.86	0.342
Basal FSH (mIU/ml)	**6.72±2.062**	**7.56±2.15**	**0.012 ***
Basal E2 (pg/ml)	62.81±26.69	64.23±28.21	0.741
No. of retrieved oocytes	**11.99±5.32**	**6.89±2.71**	**0.000 ***
No of embryo	**6.42±2.70**	**3.58±1.38**	**0.000 ***
Fertilization rate %	**88.85±10.20**	**85.00±13.12**	**0.030 ***
No. of good quality embryo	**3.32±1.40**	**1.65±0.77**	**0.000 ***
No. of transferred embryos	2.28±0.45	2.35±0.48	0.326
Cumulative embryo score (CES)	9.33±1.94	9.00±2.06	0.291
No. of clinical pregnancy (%)	**57/136 (41.91%)**	**6/57 (10.53%)**	**0.000 ***
Implantation rate (%)	**73/310 (23.55%)**	**8/134 (5.97%)**	**0.000 ***
Multiple pregnancy (%)	**14/57 (24.56%)**	**1/6 (16.67%)**	**0.000 ***

*** Significant**

Discussion

In the current study we find that transfer of early cleavage embryos (EC) led to significantly higher pregnancy rates as compared to non early cleavage embryos (NEC) (43.30 % versus 21.88 %; P = 0.005), and also higher implantation rates (25.58 % versus 11.35 %; P = 0.000). This is in agreement with the results obtained in previous studies ***(Tesarik et al., 2000; Ludin et al., 2001; Salumets et al., 2003; Fancsovits et al., 2005; Hammoud et al., 2008; Brezinova et al., 2009)***. The possibility that early embryo cleavage, a highly significant biological indicator of embryo growth potential, may predict IVF outcome was first proposed by ***Shoukir et al., 1997 and Sakkas et al., 1998***. However, most studies examined transfers in which two or more embryos were transferred of which at least one embryo had shown early cleavage. This makes it difficult to conclude to which embryo the pregnancy can be attributed. In our study, data were analysed from cycles where two or three embryos were transferred eithr all EC or NEC embryos, which makes it possible to determine the relationship between early cleavage and pregnancy arising from one specific embryo category.

Recently, ***Lee et al. (2012)*** found that the EC group had statistically higher implantation rates than the non-EC group in both the IVF (42.9 versus 19.7 %, P < 0.05) and ICSI cycles (48.1 versus 24 %, P < 0.05). Interestingly, the clinical pregnancy rate was statistically significantly higher for the EC group than the non-EC group for the IVF cycles (75 versus 37.5 %, P < 0.05) but, in contrast to our results, it was not statistically higher for the ICSI cycles (68.2 versus 51.4 %, P = 0.27).

It has been demonstrated that EC embryos have better morphology ***(Lundin et al., 2001; Sakkas et al., 2001; Fenwick et al., 2002; Ciray et al., 2004; Hammoud et al., 2008)***. Consistent with this, we found that the EC embryos had significantly higher proportion of good quality embryos when compared to NEC embryos (P = 0.000). ***Van Montfoort et al. (2004)*** concluded that early-cleaving embryos had a significantly higher embryo score on the day of transfer. ***Terriou et al. (2007)*** claimed that even EC embryos are strongly associated with good embryo morphology (53 % in IVF, 69 % in ICSI). ***Lemmen et al. (2008)*** concluded, according to information obtained from time-lapse study, that first cleavage has been shown to be associated with a higher number of blastomeres on day 2 after oocyte retrieval.

Female age was suggestive to be one of the most important prognostic factors of an ongoing pregnancy after ART. However, in our study we find no differences were found between the pregnant and non pregnant groups with regard to maternal age (28.52 years versus 28.65 years; P = 0.824). This is in agreement with studies (***Salumets et al., 2003a; Fu et***

al., 2009). A possible reason why we did not observe the effect of age of patients on the pregnancy rate was that all patients were ≤ 35 years old. In contrast to this, ***Lundin and his colleagues*** confirmed that the early cleavage and female age were shown to be positively correlated with pregnancy (***Lundin et al., 2001***).

Our observation that significantly more EC embryos occurred in younger patients (< 30 years) contrasts with previous reports were this difference was not detected (***Shoukir et al., 1997; Sakkas et al., 1998***). However our results are in consistent with previous studies (***Bos-Mikich et al., 2001***).

One advantage of embryo selection based on the timing of first cleavage is that this is a clearly visible event, whereas pronuclear morphology and cleavage embryo morphology may vary during the dynamic process of syngamy. The reason why early cleavage yields better quality embryos and higher pregnancy rates is unknown, but it may be speculated that such zygotes derive from oocytes with adequately synchronized cytoplasmic and nuclear maturation ***(Ebner et al., 2003)***. Paternal factors have been observed to play some role but they are not well documented. There is still a shortage of information concerning the importance and functions of transcripts trapped in spermatozoa. Therefore, a possible involvement of these factors in the regulation of early zygotic cleavage cannot be ruled out. Moreover, it would be important to know if spermatozoa produced by males of high and low in vivo fertility show different pools of transcripts that could be associated with male fertility ***(Lechniak et al., 2008)***.

Conclusion:

The current study suggests that early cleavage could be an additional factor for selecting embryos with a higher potential of implantation and successful pregnancy while avoiding multiple pregnancies. However, larger prospective studies are suggested to be done on a larger patient cohort of wider age limits are needed to confirm the usefulness of early cleavage in embryo selection.

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