Although Late; but the First, an Iraqi Success in Human Embryo Cryopreservation Using Vitrification and the Factors Affecting the Pregnancy Rate: Cross-Sectional Study

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Abstract:

Background: it is important to study a history of the science to appreciate the past as a motive for hard work in the present for better future. In the other hand, the factors affecting pregnancy rate of frozen embryos are yet to be clarified at different embryonic developmental stages.
Objectives: to record a brief history of embryo cryopreservation in Iraq and to study many clinical and embryological factors that might affect the pregnancy rate.
Subjects, materials and Method: a cross-sectional study of many clinical and embryological variable, where120 frozen-thawed embryo transfer cycles enrolled in the study at the period from January 2017 till July 2018. The variables were statistically analyzed first by one factor analysis comparing between pregnant and non-pregnant cases; then by multivariate logistic regression analysis to illustrate main factors without a possible confounder effect.
Results: In one factor analysis, the following variables showed a significantly high effect on pregnancy rate (P value <0.001) which are women's age, women's weight, number of retrieved oocytes, mature oocytes and good quality embryos, developmental stage, E2 level, and endometrial thickness. While in multivariate logistic regression analysis the women's weight, the number of retrieved oocytes and good quality embryos were the main factors.
Conclusions: it is judiciously to consider these factors while managing infertile couples with embryo cryopreservation programs especially the modifiable factors.

Keywords: embryo cryopreservation history, vitrification, frozen-thawed embryo transfer.

Introduction:

Due to the past and current situations in Iraq, one of the important medical fields and services delayed significantly until establishing, this is reproductive medicine field. This field itself considered a relatively recent medical specialty worldwide.

Before 2003, there were only two governmental institutions for infertility management which are Kamal Al-Samarraee Hospital and High Institute for infertility Diagnosis and Assisted Reproductive Technologies. Both of them were with very limited resources and achievements.

After 2003, the economic status generally improved, however, it couldn't approach huge needs in medical and health services and, unfortunately, reproductive medicine area comes last after other priorities. Accordingly, infertility cases were facing a big problem which was that no qualified IVF centers were found which necessitating infertile couples to seek help abroad in neighboring countries. This burdens their shoulders with expensive IVF costs in addition to requirements of travel.

It was a big need and priority to upsurge the established IVF centers and build up a new centers to match a growing need for IVF services.

One of the important steps in this way was taken in Al-Najaf Al-Ashraf to establish well recognized specialized IVF center (the Fertility Center) in Al-Sadr Medical City belong to Al-Najaf Al-Ashraf health directorate in collaboration to Faculty of Medicine, University of Kufa, which actually opened in 2008. It, firstly, provided a medical diagnosis for infertility in addition to medical treatments and intrauterine inseminations.

The efforts continued to supply this center with the requirements for intra-cytoplasmic sperm injection (ICSI) procedure in one side, and to recruit qualified personnel with extensive training in different required specialties prominently infertility physicians and embryologists. Both enrolled in abroad and in situ training courses and workshops.

The first recorded pregnancy in this center with conventional IVF was in November 2009, while the first pregnancy by ICSI in January 2010. This center continually works with steadily improving success rates approaching that of well recognized IVF centers worldwide.

However, a very important step was missed due to fluctuations in resources in one side and training opportunities in the other side. This step was a well-organized embryo cryopreservation program.

In 2014, with the help of generous hand of extraordinary professor and one of pioneers in IVF field in Europe, Professor Safaa Al-Hasani a German embryologist originally from Iraq (he was in the team achieving the first IVF baby in Germany 1982) comes to Al-Najaf Al-Ashraf and assisted in establishing a qualified embryo cryopreservation program with his precious instructions and hands on training for the embryologists that they are the core of success as he said. This was a big step that had led eventually to the delivery of the first Iraqi baby born from cryopreserved embryos who was a male baby born in 23th of August 2015, although late; but the first. Meanwhile, Kamal Al-Samarraee Hospital and the High Institute of infertility Diagnosis and Assisted Reproductive Technologies continued their tremendous efforts in developing their work and outcomes regarding ICSI in general and then regarding embryo cryopreservation. The first babies born from frozen embryos were in November 30th 2016 and in June 24th 2018 in Kamal Al-Samarraee Hospital and High Institute for Infertility Treatment and Assisted Reproductive Technologies – Al-Nahrain University respectively.

In addition, other two governmental centers had been established; one in Basra (2012) and the other in Baghdad (Um Al-Baneen fertility center-2015); both belong to the Ministry of Health. Moreover, due to relative huge needs, many private centers established in Iraq, especially in Baghdad, before and after these dates; however, mainly depended on foreign teams due to a vast shortage in adequate well trained staffs.
All these centers, especially governmental ones, had different levels of collaborations and communications to improve their results.
Even globally, vitrification procedure in human embryo cryopreservation considered relatively new due to the slow shifting from traditional slow-freezing procedure to it, but, eventually, the irresistible comparative evidence made clear to almost everybody that in all developmental stages, vitrification produces better survival and more competent embryos than traditional freezing (1).
However, there is limited understanding of the factors affecting success rates after frozen-thawed embryo transfer using vitrification procedure (2).
Few innovations have had such a great transformative impact on the assisted reproductive technologies (ART's) field as vitrification had; and this impact will only raise more if the work on development and refinement of this novel procedure continue regarding its technology and methodology (3).
In this study, we will summarize a cryopreservation results and analyses the factors that could affects the pregnancy rate in embryo cryopreservation program using vitrification in Iraqi patients attending the fertility center in Al-Najaf Al-Ashraf and High Institute for infertility Diagnosis and Assisted Reproductive Technologies.

Subjects, Materials and Methods:

One hundred twenty infertile couples entering frozen-thawed embryo transfer (FTT) cycle in the period from January 2017 till June 2018 were enrolled in this cross-sectional study. From each couple, detailed history was taken with comprehensive physical examination and related investigations.
The female partners' mean age was (30.48 ± 4.99 SD) years, mean duration of infertility period was (6.14±1.59 SD) years.
The couples were of either male factor (less than reference value of sperm concentration or progressive motility or normal morphology or any combination) (46%) or of female factor (54%) (Polycystic ovarian syndrome (PCOS); Tubal factor; and hypogonadotrophic hypogonadism (HH)).
Couples involved in this study were either of primary infertility 82.5% (99 in number) or secondary infertility 17.5% (21 in number).
Regarding vitrification solutions, they were ready to use directly (Kitazato, Japan). For vitrification; two-steps solutions were used which are:

1. Equilibration solution (ES): consist of 7.5% Ethylene Glycol (EG) and 7.5% Dimethyl Sulfoxide (DMSO) in culture medium supplemented with 20% Human Serum Albumin (HSA). The embryos were placed in this solution for 5-15 minutes according to shrinkage and recovery process.
2. Vitrification solution (VS): consist of 15% EG, 15% DMSO and sucrose (0.5 M) in culture medium supplemented with 20% HSA. The embryos were placed in this solution for about 1 minute; then loaded on ivitri straw (Sunlight Medical, USA) with minimum volume and plunged in liquid nitrogen (LN) for storage.

For Thawing (Kitazato, Japan); the following solutions were used:

1. Thawing solution (TS): consist of medium with 1 M sucrose. After remove the straw from LN, rapidly placed in about 1 mL of this solution for 1 minute.
2. Dilution solution (DS): consist of medium with 0.5 M sucrose. Place the embryos in this solution for 3 minutes.
3. Washing solution (WS): only medium supplemented with 20% HSA. Wash the embryos in this medium for several times and transfer them to prepared culture dish in CO2 incubator for about two hours till embryos assessment and transfer.

According to literatures, work experience and availability of data or procedures, the following variables were included in our study: Age; Body mass index (BMI) kg/m2; Cause of infertility; Duration of infertility; Type of infertility (primary or secondary); Cycle day 2 hormonal profile (FSH, LH and PRL) and E2 at the day of hCG injection; Induction protocol for controlled ovarian hyperstimulation COH; Number of retrieved oocytes; Number of mature oocytes; Fertilization rate; Good quality embryos; Number of transferred embryos post-thawing; Pregnancy rate; and Implantation rate.

Statistical analysis was done by using SPSS (statistical package for social sciences) version 20. Frequency, percentage, mean and standard deviation were used as descriptive statistics. For analysis, chi square test for categorical data and independent sample t-test and analysis of variance with LSD for numerical variables were used in addition to multivariate regression analysis. P value ≤ 0.05 regarded significant.

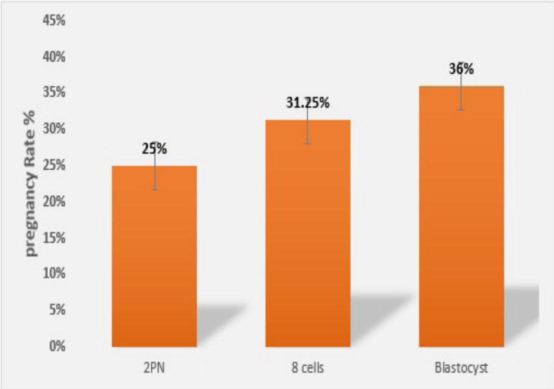
Results:

Pregnancy rate in different embryo stages was presented in Figure (1). The cases ended with pregnancy were 42 cases with an overall 35% pregnancy rate. However, within each separated group (2PN, 8-cells stage and blastocyst) the pregnancy rate was 25%, 30% and 35% respectively.

The comparison of different variables between pregnant and non-pregnant women after frozen-thawed embryo transfer cycle by one factor analysis statistics was displayed in Table (1). Age and BMI of women significantly higher in non-pregnant women; while the number of oocyte, mature oocyte, good quality oocyte, endometrial thickness and E2 significantly higher in pregnant women.

To know the independent factors among them, in this study, multivariate logistic regression analysis of factors was applied as shown in table (2) which revealed that the most important factors for success of pregnancy after frozen-thawed embryo transfer cycle of this study were BMI, number of oocyte and good quality.

There was no significant association between type of protocol and pregnancy rate (table 3). Also, there was no significant association between pregnancy occurrence and cause of infertility (table 4). Table (5) shows significant positive association between OHSS, as a cause of cryopreservation, and pregnancy rate. Figure (2) shows no significant association between type of infertility and pregnancy and in all women the primary infertility is the common.



Figure(1): Pregnancy rate in different embryo stages (within the same developmental stage) (P value 0.853). 2PN: 2 pronuclear stage (zygote).

Table (1): Comparison between pregnant and non-pregnant women in different variables.

Variable	Pregnant (42) Mean+SD	Non-Pregnant (78) Mean+SD	P value
Age/ years	27.2±3.7	32.2±4.7	<0.001
BMI Kg/m²	23.4±2.4	26.9±3.3	<0.001
Duration/years	6.3±1.2	6.03±1.7	0.336
No. of oocyte	16.7±5.5	11.7±4.7	<0.001
No. of mature oocyte	12.3±4.8	8.2±3.6	<0.001
Fertilization rate	76.9±14.1	81.2±16.6	0.158
Good quality	6.6±2.8	3.5±1.7	<0.001
No. of transfered embryo	1.8±0.56	1.8±0.51	0.914
Endometrial thickness	12.83±0.88	9.93±0.79	<0.001
E2	2635.2±683.7	1692.6±791.09	<0.001
Prolactin	15.24±7.19	18.28±10.37	0.093
LH	2.95±1.04	3.24±1.46	0.276
FSH	5.02±2.04	4.83±2.16	0.650

Table (2): Multivariate logistic regression analysis of factors.*

	B	S.E.	Wald	df	P value	OR	95% C.I.for OR	
							Lower	Upper
Age	0.167	0.102	2.673	1	0.102	1.181	0.967	1.443
Good quality	0.357	0.121	8.683	1	0.003	1.430	1.127	1.813
Oocyte no.	0.366	0.139	6.887	1	0.009	1.442	1.097	1.895
BMI	-0.666-	0.218	9.307	1	0.002	0.514	0.335	0.788
Constant	-8.197-	3.502	5.477	1	0.019	0.000		

* B regression coefficient, SE of regression coefficient, the modified Homser-Lemshow goodness of fit X2 test statistics was 17.11 (p =0.029)

Table (3): Association between type of protocol and pregnancy rate.

		Pregnancy Test		Total	P value
		Positive	Negative		
Protocol	Agonist	18	29	47	0.543
		38.3%	61.7%	100.0%	
	Antagonist	24	49	73	
		32.9%	67.1%	100.0%	
Total		42	78	120	
		35.0%	65.0%	100.0%	

Table (4): Association between cause of infertility and pregnancy rate

		Pregnancy Test		Total	P value
		Positive	Negative		
Cause	HH	2	3	5	0.689
		40.0%	60.0%	100.0%	
	M	17	38	55	
		30.9%	69.1%	100.0%	
	PCOS	18	32	50	
		36.0%	64.0%	100.0%	
	Tubal	5	5	10	
		50.0%	50.0%	100.0%	
Total		42	78	120	
		35.0%	65.0%	100.0%	

HH: hypogonadotrophic hypogonadism; M: male factor; PCOS: Polycystic ovarian syndrome; Tubal: uterine tube obstruction

Table (5): Association between causes of cryopreservation and pregnancy rate.

		Pregnancy Test		Total	P value
		Positive	Negative		
Causes of cryopreservation	All (other cause)	1	10	11	<0.001
		9.1%	90.9%	100.0%	
	Leftover	24	60	84	
		28.6%	71.4%	100.0%	
	OHSS	17	8	25	
		68.0%	32.0%	100.0%	
Total		42	78	120	
		35.0%	65.0%	100.0%	

OHSS:ovarian hyperstimulation syndrome

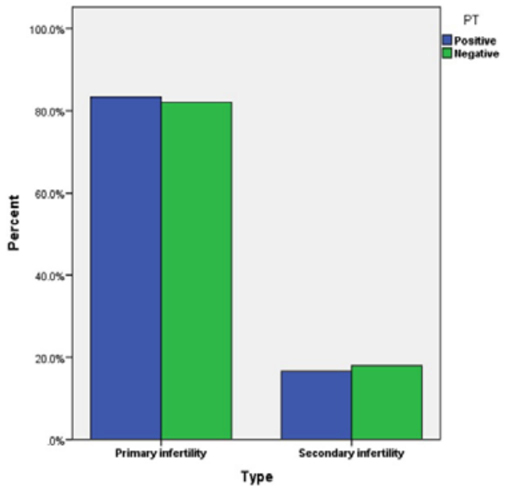


Figure (2): Association between type of infertility and pregnancy outcome.
PT: Pregnancy Test

Discussion:

In the present days, embryo cryopreservation with vitrification is a usual daily work in IVF centers worldwide.

Actually, without qualified cryopreservation program, it is hardly to consider such place an IVF center because it loses a precious materials for cryopreservation and greatly minimizes an overall chance of getting pregnancy in many cases; and, moreover, even abolishes such a chance in other cases as in some cases with OHSS when fresh embryo transfer is highly not recommended (4, 5, 6).

In spite of great importance of embryo cryopreservation, there is a limited understanding of laboratory and clinical factors that could affect the success rate of frozen embryo transfer (2, 3). In view of that, this Iraqi pioneer study comes to participate in directing a spot of light to this vital area of reproductive medicine.

First of all, it is worthy to proud by overall pregnancy rate in the current study (35%) which is comparable to that of Europe (7). Europe could be considered a good standard to compare with, because it actually leads the world in ART's where it contributes in approximately 50% of all reported ART's cycles globally (7). In more details, the pregnancy rates according to the developmental stage at the time of transfer in this study were 25%, 31.25% and 36% for 2PN stage, cleavage stage (day 3) and blastocyst stage respectively (Figure 1) with no significant difference.

As in table (1) and as it expected and well known, as a fact, that female age is a single most important factor and has a significant detrimental effect on both: the fertility potential where studies demonstrated that fertility declines with increasing age and started to decline at age 25 years on (8, 9, 10); and on ART's success rates (11, 12, 13). It has been found that there was an increment in the prevalence of infertility from 1% to 55% at age 25 years and 45 years respectively (9). This effect of age on fertility potential and ART's outcomes could be explained by several aspects including increasing aneuploidy, decreasing ovarian reserve, decreasing oocyte quantity and quality and decreasing availability of competent embryos for transfer in IVF cycles (13, 14).

On the other hand, no such effect is observed regarding duration of infertility (Table 1); where there was no significant deference in duration of infertility between pregnant and non-pregnant cases. This might be clarified by that the age itself is the main factor regardless of duration of infertility where, in addition, the infertile patient commonly delay the decision of ART's.

Although the age impact was significant in single factor analysis; it has no such an effect with multivariate logistic analysis (Table 2). This could be explained by the fact that the majority of cases in our study (pregnant and non-pregnant) were under the age of 30 years and much less number of cases above 35 years.

Obesity was considered to have a drawback effect on different outcomes of many ART's procedures including ICSI outcome and frozen embryo transfer according to Rahim AI and Chen R *et al.* respectively (15, 16). However, it is still unclear if these detrimental effects of obesity are due to the effect of obesity on oocytes or embryos or endometrial milieu or all of these (17). Many explanations for such an effect of high BMI were hypothesized. Some researches blame its effect on oocytes quality and subsequent embryonic development (16, 18). Others blame the uterine milieu itself even when there were euploid embryos for transfer by increasing miscarriage rate in frozen embryo transfer cycles (17). Also, it was obviously that obesity detrimental effects are still even with donor programs with young, lean donors if the recipients were with high BMI (19). Some researchers found that obesity associated with low progesterone levels produced by corpus luteum (20, 21). Others found that obesity impairs decidualization (22) and alters a mid-luteal endometrial protein profile (23). Some researchers postulated a possible epigenetic effect of high BMI on the oocytes or embryos, even though when the pre-implantation genetic screening was normal (17); the possibility of this idea raised by others where they did reported an alteration of placental gene expression (24) and alteration of metabolomics signature of early embryos (25) in obese women.

Normal body-mass index (BMI) has a highly significant effect on the success rate in this study (Table 1). Actually there was no single pregnancy in obese women in the studied cases. Moreover, not merely a single factor analysis shows such an effect of high BMI; but also with multivariate logistic analysis (Table 2) indicating that high BMI and obesity is an independent negative impact factor in the prediction of success rate with frozen embryo transfer cycles.

Although using the antagonist protocol was more than the use of agonist protocol in all studied cases and in pregnant cases, there was no significant impact of the used protocol on the pregnancy rate (Table 3).

This was in agreement with Tulic L (26) and Singh N *et al.* (27) that they found no difference between these two protocols in general milieu and in outcomes; so it is clearer to find this neutral effect in FTT cycles too. However; the explanation of the use of antagonist protocol more popularly could be returned to its assumed decreasing OHSS likelihood and its simple use and follow up with more patients' compliance (27, 28, 29).

More pregnancy cases were found in cases where the causes of infertility were PCOS and male factor (Table 4). This could be refereed to that more cases with these two infertility causes were recruited in this study reflecting the usual cases seeking ART's. Though, there was no significant effect of the cause of infertility on the pregnancy rate (Table 4) indicating a high ability of ART's, including frozen embryo transfer, to overcome a variety of infertility obstacles.

Regarding the causes of cryopreservation, leftover embryos was the first cause followed by OHSS cases and then freeze-all embryos due to non-medical causes like difficulty reaching to the center in appropriate time for embryo transfer (Table 5). This was, also, the order in pregnant cases. Though, pregnant cases were significantly higher in cases where OHSS was the cause of cryopreservation (Table 5). This result is in agreement with Shi W *et al*. study (30). It had been found that women failed to conceive in fresh cycle embryo transfer have less chance to conceive in frozen cycle (31, 32) and, in opposite, women have higher chance to conceive if they had pregnancy in their fresh embryo transfer cycle and women with OHSS in frozen transfer cycle (33, 34, 35). Also, it is logic to transfer the most component embryos in fresh cycle to maximize the pregnancy rate which might lower the availability of adequate component embryos for cryopreservation. Whereas, in OHSS, all competent embryos, which usually adequate in number, were subjected to the cryopreservation increasing the chance of having more competent embryos post thawing. Regarding third group of this study, as a cause of cryopreservation, it could be considered an emergency plan to rescue the available embryos by cryopreservation, and not a matter of embryo selection; in addition to their lower number in comparison with OHSS cases and left-over cases (Table 5).

Also there was no difference in outcomes between primary infertility and secondary infertility cases regarding frozen embryo transfer cycles (Figure 2); reflecting the power of ART's to overcome a variety of infertility causes and situations; however, as usual, in both pregnant and non-pregnant cases, the women with primary infertility were more than the women with secondary infertility.

Regarding to the studied embryological variables in our study, with one factor analysis, the number of retrieved oocytes, the number of mature oocytes, and the number of good quality embryos, all these were significantly higher in pregnant cases (Table 1). The same highly significant impact was for high E2 levels (Table 1) reflecting the higher possibility of getting more oocytes.

This higher significant effect of these factors comes with logic and sense where more available competent materials provide wider spectrum of selection and could anticipate higher results. This is comes in agreement of other studies which are Zdravka V *et al*. (2), Lin YP *et al*. (31) and Darkopoulos P *et al*. (36).

Although, high E2 level could have detrimental impact on the endometrium receptivity (37, 38, 39); no such effect was found on the oocyte and embryo quality as stated in some studies (38, 40).

In addition, such a negative impact of high E2 levels on the endometrium and its receptivity was eliminated by cryopreservation and postponed embryo transfer to controlled preparation of endometrium.

In the same conscious, endometrial thickness had highly significant effect on the pregnancy rate (Table 1). Endometrial receptivity might be considered, till now, a mysterious factor of success rate where although with transfer of high quality embryos, the success rate still below the desired goal indicating a crucial rule of endometrium (41, 42, 43).

Neither fertilization rate nor other hormonal levels (FSH, LH and PRL) (Table 1) had significant effect on the outcome. Although fertilization rate didn't significantly differ in both groups (pregnant and non-pregnant cases), the more fertilized oocytes that resulted in more good embryos were higher in pregnant cases. Also, the hormonal levels, other than E2, were within accepted levels in all women especially regarding FSH and LH hormones (Table 1).

The multivariate logistic analysis approves the effect of the number of retrieved oocytes and the number of good quality embryos (Table 2). No such impact was seen regarding ET in spite of its significant effect in one factor analysis (Table 1 and 2). this could be referred to the fact that we didn't enrolled the women in the FTT cycle unless they approach ET of 9 mm and more which is within the range of accepted ET for embryo transfer in other studies like El-Toukhy T *et al.* study (44).

Regarding the number of embryo transfer, there was no significant difference between pregnant and non-pregnant cases (Table 1). This result comes in the consistent of the present attitude of lowering the number of transferred embryo, more precisely elective single embryo transfer, to lower the likelihood of multiple pregnancy and its complication.

However, it is wisely to state that to decide the number of embryos for transfer, it depends on many factors including the age of the women, the embryos stage and quality, the rank of the transfer trial and even the success rate of the center where the procedures done in it (45).

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