

Original Article

Lower limit of antioxidant activity in follicular fluid: relationship to embryo quality in IVF cycle

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Abstract: Human follicular fluid (FF) forms the microenvironment of the developing oocyte and has an important influence on embryo development. Although some published report showed their results of associations between antioxidant activity of FF and embryo quality, there was no information on the lower limit of antioxidant activity in FF which had negative impact on embryo quality. The antioxidant activity of 407 FF samples from 52 infertile patients undergoing IVF were collected and determined by α -diphenyl- β -picrylhydrazyl (DPPH) radical scavenging and reducing power assay. The lower limit of DPPH radical scavenging percentage and reducing power were determined to be 18.3% and 0.257, respectively. It means FF may adversely affect the embryo development when the DPPH radical scavenging ability or reducing power below the lower limit. This estimated lower limit was further validated in FF samples of embryo grading I-IV. This cut off value of antioxidant activity of FF is expected to assist embryologists and clinicians in predicting development of embryo from infertile patients undergoing in vitro fertilization (IVF).

Keywords: In vitro fertilization, antioxidant activity, lower limit, embryo quality

Introduction

Nowadays, assisted reproductive techniques (ART) account for the birth of more than 3 million babies worldwide, and the number of in vitro fertilization (IVF) cycles performed increases every year [1, 2]. However, an overall pregnancy rate of 30-40% per started cycle is obtained in many centers [3, 4]. Amongst the various factors that adversely affect IVF outcome, oxidative status has proved as one of the important factors that affect embryonic development [5].

Human follicular fluid (FF) forms the microenvironment of the developing oocyte and has an important influence on embryo development [6]. FF is rich in low-molecular weight metabolites, which are direct or indirect regulators of oxidative stress and antioxidant activity [7]. Oxidative status is involved in the etiology of defective embryo development [8]. Various published report has shown that significantly increased reactive oxygen species, high lipid peroxidation and decreased antioxidant capacity in follicular fluid (FF) correlate with embryo

quality [9-12]. At the same time, FF has its antioxidant system, which include superoxide dismutase, catalase, thioredoxin and glutathione peroxidase [13-15], vitamins [16, 17]. The free radical activity is also associated with parameters of ovarian responsiveness and in vitro fertilization (IVF) outcome [18]. This suggests clear associations between antioxidant activity of FF and embryo quality [19]. However, some researchers showed embryo quality does not vary with the antioxidant status of FF [20]. Furthermore, some studies found that patients who become pregnant by IVF had lower antioxidant activity than non-pregnant women [21]. Therefore, the lower limit of antioxidant activity in FF may be required for consideration in IVF.

The goal of the current study was to describe the lower limit of FF's antioxidant activity below which embryo development will be adversely affected. The control chart is applied to investigate the antioxidant cut-off level in FF of patients undergoing IVF. The control chart could obtain the threshold value at which the process output was advised statistically unlikely while a central line shows the process mean [9]. To the

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best of our knowledge, few studies have examined the lower limit of antioxidant activity in FF by free radical scavenging and reducing power assays. For this purpose, 407 FF samples from 52 infertile patients undergoing IVF were collected for this study.

Materials and methods

Chemicals

α,α -diphenyl- β -picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used for analysis were AnalaR grade and obtained from China Medicine (Group) Shanghai Chemical Reagent Corporation (Shanghai, China).

Study protocol

Ethics approval for this project was obtained from the ethics committee of Tongji Hospital. Enrolment occurred between March 2013 and October 2013. All patients who signed written informed consent were undergoing IVF treatment at the Reproductive Medicine Centre, Tongji Hospital.

This was a noninterventional study of patients undergoing routine practice. In order to exclude the influence of other infertility reasons on the antioxidant activity of FF, the reason for infertility of all women was tubal factor. And, all women from 22 to 39 years of age (mean, 30.1 ± 3.2) were non-smokers and had been unable to be pregnant naturally for at least one year. Patients underwent controlled ovarian stimulation COS with the use of a GnRH agonist long protocol [22]. The pituitary suppression was achieved by subcutaneous injection of triptorelin acetate (Decapeptyl, Ferring; Diphereline, Ipsen) daily starting in the midluteal phase of the preceding cycle [23]. When complete pituitary desensitization was confirmed by a low plasma E_2 level of ~ 30 pg/mL and an LH level of ~ 2 mIU/mL, ovarian stimulation was initiated by intramuscularly administering 150-300 IU/d recombinant FSH (Gonal-F, Serono; Puregon, MSD). The FSH dosage was adjusted according to the ovarian response, which was assessed by the transvaginal ultrasound and serum E_2 level. Recombinant hCG (250 mg; Ovidrel; Serono) was given to trigger ovulation when two leading

follicles reached a mean diameter of 18 mm. Oocytes were retrieved transvaginally 34-36 hours after hCG administration.

Collection and processing of FF

FF were collected during oocyte retrieval by follicle aspiration under ultrasonographic control. Care was taken to completely aspirate each follicle within one tube. Each follicle was aspirated separately. FF from follicles containing more than one oocyte or no eggs was excluded from the analysis. Specimens that were contaminated with blood were discarded. Samples were centrifuged at $1000 \times g$ for 10 min. The clear supernatant was divided into aliquots and frozen immediately at -80°C . A total of 407 FF samples of 52 infertile women were collected for further analyzed.

IVF procedure

The methods for sperm preparation, IVF and embryo culture have been described previously [24]. Semen was collected in sterile container by masturbation after 3-5 d of sexual abstinence and then kept for 30 min at 37°C . After liquefaction, samples were analyzed for sperm concentration, motility and morphology according to the World Health Organization criteria [25]. The oocytes were incubated in G-IVF medium (Vitrolife, Sweden). Normally fertilized oocytes were continuously cultured in G1 medium for two more days. Usually fewer than two best-quality embryos were transferred on day 2 or 3 after oocyte retrieval, and excess good-quality embryos were cryopreserved for subsequent frozen-embryo transfer (FET) cycles. Morphological evaluation of the embryos was performed on day 3 (72 h) based on number of blastomeres, rate of fragmentation, multinucleation of the blastomeres and early compaction [26]. Day 3 embryos were scored on a scale of I (high grade) to IV (low grade) [27, 28].

Measurement of DPPH radical scavenging capacity

The DPPH free-radical scavenging capacity of FF was tested according to the published method [29] with slight modifications. 0.4 mL FF sample was mixed with 3.6 mL 0.2 mM DPPH in methanol. The mixture was centrifuged at $1000 \times g$ for 10 min. The absorbance of clear

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Table 1. The relationship between antioxidant activities in follicular fluid of infertile women undergoing IVF and their embryo quality

Embryo quality	DPPH radical scavenging percentage (%)	Sig.	Reducing power	Sig.
Grades I and II embryos	35.8 ± 6.8	P < 0.05	0.428 ± 0.067	P < 0.05
Grades III and IV embryos	32.3 ± 8.3		0.396 ± 0.073	

Determination of reducing power

The reducing power of FF sample was determined according to the procedure described by published method [30]. FF sample was mixed with 2.5 mL potassiumferricyanide (1%), 2.5 mL trichloroacetic acid (10%) and 2.5 mL sodium phosphate buffer. Mixture was centrifuged at 1000×g for 10 min. 2.5 mL Distilled water and 0.5 mL ferric chloride (0.1%) were mixed with 2.5 mL upper layer, and absorbance was determined at 700 nm. Higher absorbance of the sample indicated stronger reducing power.

Statistical analysis

All data analysis was performed using Statistical Package for Social Sciences (SPSS) version 13.0. The method validation data of all experiments was following: DPPH assay: mean, 35.10%; CV, 1.0%; 95% CI, 34.21-35.99. Reducing power assay: mean, 0.425; CV, 0.9%; 95% CI, 0.415-0.435. The control chart (3 sigma) was a time plot of sub-sample statistic with a central line, upper control limit (UCL) and lower control limit (LCL). Between-groups data was analyzed using the nonparametric Mann-Whitney U-test and χ^2 tests. Statistical significance was determined at P < 0.05.

Results

Data concerning two methods of antioxidant activity were presented in **Table 1**. The antioxidant activity of FF was statistically related to embryo quality in IVF. In this study, the embryos were graded according to the criteria established [26, 27]. Briefly, embryo grading I: Even,

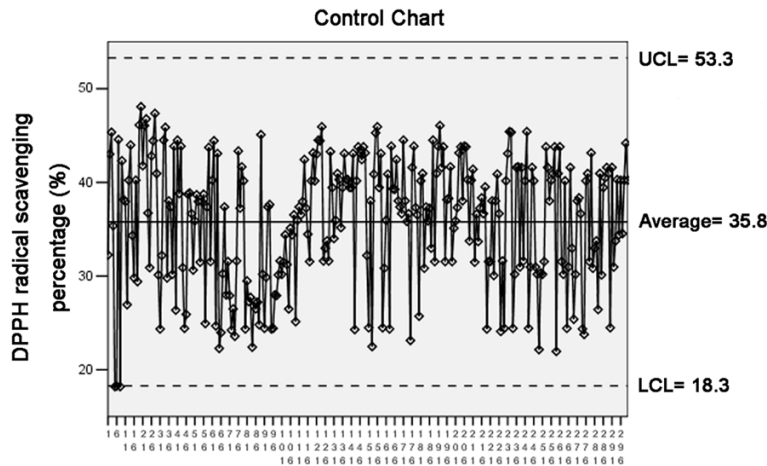


Figure 1. The control chart (3 sigma) of lower cut-off level of DPPH radical scavenging ability in follicular fluid from infertile women undergoing IVF. UCL, upper control limit; LCL, lower control limit.

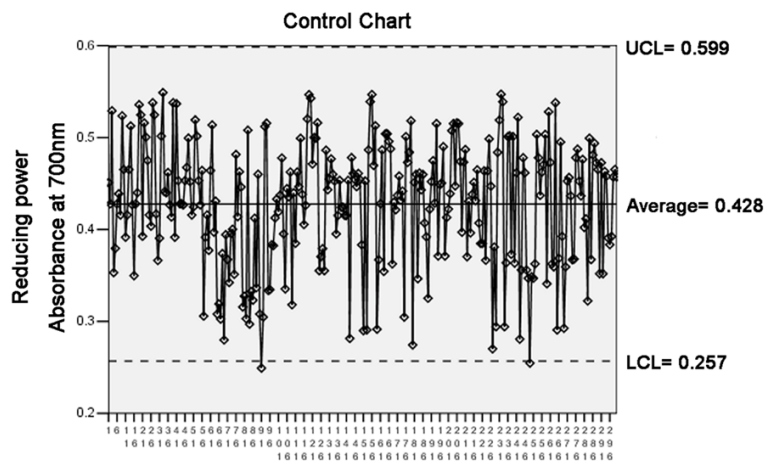


Figure 2. The control chart (3 sigma) of lower cut-off level of reducing power in follicular fluid from infertile women undergoing IVF. UCL, upper control limit; LCL, lower control limit.

supernatant was measured at 517 nm. DPPH radical scavenging activity was calculated as the following percentage: $[(R_{DPPH} - R_s)/R_{DPPH}] \times 100$ (R_{DPPH} = result of DPPH alone and R_s = result of DPPH in the presence of different FF sample).

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Table 2. Identification of the relationship between lower cut-off level in antioxidant activity of follicular fluid from women undergoing IVF and embryo quality

	Cut-off level	Grades I and II embryo formation (%)	Sig.
DPPH radical scavenging ability	> 18.3% (382)	77.7% (297/382)	P < 0.001
	< 18.3% (25)	12.0% (3/25)	
Reducing Power	> 0.257 (396)	75.0% (297/396)	P < 0.001
	< 0.257 (11)	27.3% (3/11)	

regular, spherical and equal size blastomeres, no fragmentation or < 5% fragments. Embryo grading II: Uneven or irregular, unequal size blastomeres, < 10% fragments. Embryo grades III: Defects of cytoplasm, < 50% fragments. Embryo grades IV: > 50% fragmentation with gross. As shown in **Table 1**, the DPPH radical scavenging ability of FF from embryos grading I and II were significant higher than that of embryos grading III and IV ($P < 0.05$). Similar results were obtained in reducing power assay. The reducing power of FF from embryos grading I and II were significant stronger than embryos grading III and IV ($P < 0.05$).

Then, the lower limit of antioxidant activity in FF was investigated by using control chart. As shown in **Figures 1** and **2**, all the FF samples showed different DPPH radical scavenging capability and reducing power (mean, $35.8 \pm 6.8\%$ and 0.428 ± 0.067 , respectively). The LCL of DPPH radical scavenging percentage and reducing power were 18.3% and 0.257, respectively. It means FF may adversely affect the embryo development when the DPPH radical scavenging ability or reducing power below the LCL. This estimated LCL was further validated in FF samples of embryo grading I-IV.

As shown in **Table 2**, in DPPH assays, percentage of grades I and II embryo formation were detected to be significant higher (77.7%) in patients with DPPH radical scavenging activity > 18.3% compared with those of < 18.3% ($P < 0.001$). Similar results were obtained in reducing power assay. In the patients with reducing power > 0.257, the percentage of grades I and II embryo formation were 75.0%, which significant higher than that of < 0.257 ($P < 0.001$).

Discussion

In this work we investigated, for the first time, the lower limit of antioxidant activity of FF from

patients undergoing an IVF. Various researches showed that antioxidant activity of FF was positively correlated with embryo quality. Paszkowski and Clarke [31] and Yang et al. [32] found that decreased antioxidant activity was associated with impaired embryo development. Hosseini et al. [33] shown

that for vitrified embryos, the best effect was found when antioxidant was added from day 1 of in vitro culture in continuation with post-warming culture period. Day 1-8 supplementation significantly increased the rates of cleavage, day 7 and day 8 blastocyst production. Jana et al. [9] investigated the antioxidant capacity of 803 FF samples from 128 infertile women. They found that significantly decreased total antioxidant capacity correlate with poor oocyte and embryo quality. However, conflicts with the results of a study by Fujimoto et al. [12] in which no significant correlations between antioxidant activities of FF from 39 women undergoing IVF and embryo quality. In our study, we demonstrated a positive correlation between antioxidant activities of FF and embryo quality (**Table 1**). Our results also conflict with the findings of Attaran et al. [21], who obtained a positive correlation between FF reactive oxygen species (ROS) levels and embryo quality. The reasons for this discrepancy were also not clear. However, we do deem that a particular threshold of antioxidant activity in FF may be required for conception in IVF, just like the opinion of Wiener-Megnazi et al. [18]. Therefore, the goal of the current study was to describe the lower limit below which embryo development is not favorable.

In this work we determined the antioxidant activity of FF from patients undergoing an IVF by DPPH radical scavenging assay and reducing power assay. To calculate antioxidant efficiency in vitro, several methods were used to measure a radical scavenging activity of sample against free radicals: superoxide anion radical (O_2^-), hydroxyl radical (OH) or peroxy radical (ROO) [34, 35]. Among the several methods, a rapid, simple and inexpensive method based on a free DPPH radical scavenging effect was often used to quantify antioxidants in complex

biological systems in recent years [36]. DPPH was deep violet in color and characterized by an absorption band in ethanol solution at about 517 nm. When a solution of DPPH was mixed with antioxidants, there was a bleaching of purple-colored in solution. The decreasing absorbance levels indicated increasing antioxidant activity of FF. The reducing power assay was another important determination of the antioxidant activities of samples. The color change was investigated by monitoring the increase in absorbance at 700 nm in the presence of antioxidants [37]. Increase in absorbance indicates an increase in the reducing power of the FF samples. In our study, the FF showed a wide range in activity of scavenging DPPH radical (13.54%-48.07%) and reducing power (0.235-0.548). Our results also showed that stronger antioxidant capacity in FF indicated better embryos grade, which was similar with previous reports [9, 31-33]. This phenomenon also occurred in animal embryos. There were some reports revealed antioxidant may promote the in vitro development of embryos from cows, pigs and other livestock [38-40]. In addition, FF had its highly complex and integrated antioxidant systems. First of all, FF had evolved a variety of interrelated enzymatic antioxidant mechanisms which enable them to cope with oxidative environments [13]. Besides enzymatic systems, the thiol tripeptide glutathione (GSH) was considered to be pivotal in protecting cells from ROS-induced oxidative damage [15]. FF was also protected from oxidative damage by various non-enzymatic dietary antioxidants, including vitamins and polyphenols [6, 41].

Conclusion

In this study, our results showed the lower limit of antioxidant activity of FF below which embryo development is not favorable (**Figures 1 and 2**). These results were in good agreement with published reports which obtained a direct relationship between decreased antioxidant activity and poor embryo quality. This cut off value of antioxidant activity of FF is expected to assist embryologists and clinicians in predicting development of embryo from infertile patients undergoing IVF. Furthermore, the antioxidant activity of FF needs to be better evaluated in our future studies on larger series. And it was also needed to better understand the mechanism of antioxidant activity in FF responsible for their effects on the embryo quality.

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Disclosure of conflict of interest

None.

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References

- [1] de Mouzon J, Goossens V, Bhattacharya S, Castilla JA, Ferraretti AP, Korsak V, Kupka M, Nygren KG and Nyboe Andersen A; European IVF-monitoring (EIM) Consortium, for the European Society of Human Reproduction and Embryology (ESHRE). Assisted reproductive technology in Europe, 2006: results generated from European registers by ESHRE. *Hum Reprod* 2010; 25: 1851-1862.
- [2] Zheng Y, Dong X, Wang R, Yang W, Zhang H, Zhu G and Ai J. The criteria for optimal down-regulation with gonadotropin-releasing hormone-agonist: a retrospective cohort study. *Gynecol Endocrinol* 2015; 31: 959-965.
- [3] Gerris J, De Neubourg D, Mangelschots K, Van Royen E, Van de Meerssche M and Valkenburg M. Prevention of twin pregnancy after in-vitro fertilization or intracytoplasmic sperm injection based on strict embryo criteria: a prospective randomized clinical trial. *Hum Reprod* 1999; 14: 2581-2587.
- [4] Zheng Y, Dong X, Huang B, Zhang H and Ai J. The artificial cycle method improves the pregnancy outcome in frozen-thawed embryo transfer: a retrospective cohort study. *Gynecol Endocrinol* 2015; 31: 70-74.
- [5] Ajduk A and Zernicka-Goetz M. Quality control of embryo development. *Mol Aspects Med* 2013; 34: 903-918.
- [6] Palhares Junior MB, Romão GS, Da Broi MG, Martins WdP and Jordão Junior AA. Serum and follicular oxidative stress biomarkers levels

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- and response to controlled ovarian hyperstimulation (COH) in IVF cycles. *Fertil Steril* 2012; 98: S241.
- [7] Singh R and Sinclair KD. Metabolomics: approaches to assessing oocyte and embryo quality. *Theriogenology* 2007; 68 Suppl 1: S56-62.
- [8] Combelles CM, Gupta S and Agarwal A. Could oxidative stress influence the in-vitro maturation of oocytes? *Reprod Biomed Online* 2009; 18: 864-880.
- [9] Jana SK, K NB, Chattopadhyay R, Chakravarty B and Chaudhury K. Upper control limit of reactive oxygen species in follicular fluid beyond which viable embryo formation is not favorable. *Reprod Toxicol* 2010; 29: 447-451.
- [10] Singh AK, Chattopadhyay R, Chakravarty B and Chaudhury K. Markers of oxidative stress in follicular fluid of women with endometriosis and tubal infertility undergoing IVF. *Reprod Toxicol* 2013; 42: 116-124.
- [11] de los Santos MJ, Gámiz P, Albert C, Galán A, Vilorio T, Pérez S, Romero JL and Remohí J. Reduced oxygen tension improves embryo quality but not clinical pregnancy rates: a randomized clinical study into ovum donation cycles. *Fertil Steril* 2013; 100: 402-407.
- [12] Fujimoto VY, Bloom MS, Huddleston HG, Shelley WB, Ocque AJ and Browne RW. Correlations of follicular fluid oxidative stress biomarkers and enzyme activities with embryo morphology parameters during in vitro fertilization. *Fertil Steril* 2011; 96: 1357-1361.
- [13] Carbone MC, Tatone C, Delle Monache S, Marci R, Caserta D, Colonna R and Amicarelli F. Antioxidant enzymatic defences in human follicular fluid: characterization and age-dependent changes. *Mol Hum Reprod* 2003; 9: 639-643.
- [14] Knapen MF, Zusterzeel PL, Peters WH and Steegers EA. Glutathione and glutathione-related enzymes in reproduction. A review. *Eur J Obstet Gynecol Reprod Biol* 1999; 82: 171-184.
- [15] Lambrinoudaki IV, Augoulea A, Christodoulakos GE, Economou EV, Kaparos G, Kontoravdis A, Papadias C and Creatsas G. Measurable serum markers of oxidative stress response in women with endometriosis. *Fertil Steril* 2009; 91: 46-50.
- [16] Ozkaya MO and Naziroglu M. Multivitamin and mineral supplementation modulates oxidative stress and antioxidant vitamin levels in serum and follicular fluid of women undergoing in vitro fertilization. *Fertil Steril* 2010; 94: 2465-2466.
- [17] Petean CC, Ferriani RA, dos Reis RM, de Moura MD, Jordao AA Jr and Navarro PA. Lipid peroxidation and vitamin E in serum and follicular fluid of infertile women with peritoneal endometriosis submitted to controlled ovarian hyperstimulation: a pilot study. *Fertil Steril* 2008; 90: 2080-2085.
- [18] Wiener-Megnazi Z, Vardi L, Lissak A, Shnizer S, Reznick AZ, Ishai D, Lahav-Baratz S, Shiloh H, Koifman M and Dirnfeld M. Oxidative stress indices in follicular fluid as measured by the thermochemiluminescence assay correlate with outcome parameters in in vitro fertilization. *Fertil Steril* 2004; 82 Suppl 3: 1171-1176.
- [19] Velthut A, Zilmer M, Zilmer K, Kaart T, Karro H and Salumets A. Elevated blood plasma antioxidant status is favourable for achieving IVF/ICSI pregnancy. *Reprod Biomed Online* 2013; 26: 345-352.
- [20] Das S, Chattopadhyay R, Ghosh S, Goswami SK, Chakravarty BN and Chaudhury K. Reactive oxygen species level in follicular fluid-embryo quality marker in IVF? *Hum Reprod* 2006; 21: 2403-2407.
- [21] Attaran M, Pasqualotto E, Falcone T, Goldberg JM, Miller KF, Agarwal A and Sharma RK. The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. *Int J Fertil Womens Med* 2000; 45: 314-320.
- [22] Huang B, Hu D, Qian K, Ai JH, Li YF, Jin L, Zhu GJ and Zhang HW. Is frozen embryo transfer cycle associated with a significantly lower incidence of ectopic pregnancy? An analysis of more than 30,000 cycles. *Fertil Steril* 2014; 102: 1345-9.
- [23] Huang B, Qian K, Li Z, Yue J, Yang W, Zhu G and Zhang H. Neonatal outcomes after early rescue intracytoplasmic sperm injection: an analysis of a 5-year period. *Fertil Steril* 2015; 103: 1432-1437, e1.
- [24] Huang B, Li Z, Zhu L, Hu D, Liu Q, Zhu G and Zhang H. Progesterone elevation on the day of HCG administration may affect rescue ICSI. *Reprod Biomed Online* 2014; 29: 88-93.
- [25] World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th edition. Cambridge, UK: Cambridge University Press; 2010.
- [26] Papanikolaou EG, D'haeseleer E, Verheyen G, Van de Velde H, Camus M, Van Steirteghem A, Devroey P and Tounaye H. Live birth rate is significantly higher after blastocyst transfer than after cleavage-stage embryo transfer when at least four embryos are available on day 3 of embryo culture. A randomized prospective study. *Hum Reprod* 2005; 20: 3198-3203.
- [27] Veeck LL. An atlas of human gametes and conceptuses: an illustrated reference for assisted reproductive technology. New York: Parthenon Publishing; 1999.

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- [28] Huang B, Ren X, Wu L, Zhu L, Xu B, Li Y, Ai J and Jin L. Elevated progesterone levels on the day of oocyte maturation may affect top quality embryo IVF cycles. *PLoS One* 2016; 11: e0145895.
- [29] Huang B, Ban X, He J, Tong J, Tian J and Wang Y. Comparative analysis of essential oil components and antioxidant activity of extracts of *Nelumbo nucifera* from various areas of China. *J Agric Food Chem* 2010; 58: 441-448.
- [30] Huang B, Li Z, Ai J, Zhu L, Li Y, Jin L and Zhang H. Antioxidant capacity of follicular fluid from patients undergoing in vitro fertilization. *Int J Clin Exp Pathol* 2014; 7: 2273-2282.
- [31] Paszkowski T and Clarke RN. Antioxidative capacity of preimplantation embryo culture medium declines following the incubation of poor quality embryos. *Hum Reprod* 1996; 11: 2493-2495.
- [32] Yang HW, Hwang KJ, Kwon HC, Kim HS, Choi KW and Oh KS. Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. *Hum Reprod* 1998; 13: 998-1002.
- [33] Hosseini SM, Forouzanfar M, Hajian M, Asgari V, Abedi P, Hosseini L, Ostadhosseini S, Moulavi F, Safahani Langroodi M, Sadeghi H, Bahramian H, Eghbalsaied S and Nasr-Esfahani MH. Antioxidant supplementation of culture medium during embryo development and/or after vitrification-warming; which is the most important? *J Assist Reprod Genet* 2009; 26: 355-364.
- [34] Niki E. Role of vitamin E as a lipid-soluble peroxyl radical scavenger: in vitro and in vivo evidence. *Free Radic Biol Med* 2014; 66: 3-12.
- [35] Fujita M, Tsuruta R, Kasaoka S, Fujimoto K, Tanaka R, Oda Y, Nanba M, Igarashi M, Yuasa M, Yoshikawa T and Maekawa T. In vivo real-time measurement of superoxide anion radical with a novel electrochemical sensor. *Free Radic Biol Med* 2009; 47: 1039-1048.
- [36] Laguerre M, Hugouvieux V, Cavusoglu N, Aubert F, Lafuma A, Fulcrand H and Poncet-Legrand C. Probing the micellar solubilisation and inter-micellar exchange of polyphenols using the DPPH center dot free radical. *Food Chem* 2014; 149: 114-120.
- [37] Kim H, Yang H and Kim J. Standardization of the reducing power of zero-valent iron using iodine. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 2014; 49: 514-523.
- [38] Fortier ME, Audet I, Giguere A, Laforest JP, Bilodeau JF, Quesnel H and Matte JJ. Effect of dietary organic and inorganic selenium on antioxidant status, embryo development, and reproductive performance in hyperovulatory first-parity gilts. *J Anim Sci* 2012; 90: 231-240.
- [39] Tarin J, Ten J, Vendrell FJ, de Oliveira MN and Cano A. Effects of maternal ageing and dietary antioxidant supplementation on ovulation, fertilisation and embryo development in vitro in the mouse. *Reprod Nutr Dev* 1998; 38: 499-508.
- [40] Lee KS, Kim EY, Jeon K, Cho SG, Han YJ, Yang BC, Lee SS, Ko MS, Riu KJ, Lee HT and Park SP. 3,4-Dihydroxyflavone acts as an antioxidant and antiapoptotic agent to support bovine embryo development in vitro. *J Reprod Dev* 2011; 57: 127-134.
- [41] Scalbert A, Johnson IT and Saltmarsh M. Polyphenols: antioxidants and beyond. *Am J Clin Nutr* 2005; 81: 215S-217S.