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Evaluation the Effect of Female Age Factor on Human Oocyte Aneuploidy and Its Relation to P73 Gene Expression

Farhang Abed[1,2,3], Marefat Ghaffari Novin [*1,2], Mir Davod Omrani[*4], Mojgan Bandehpour [5,6], Mohsen Norozian[2], Ahmad Hosseini [5], Mohammad Hassan Heidari [5], Zahra Heidar[1,3]

[1]Infertility and Reproductive Health Research Center Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2]Department of Biology and Anatomical Sciences, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3]Saboohi Infertility Center, Mahdie Hospirtal, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[4]Department of Genetics, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[5]Cellular and Molecular Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[6]Department of Biotechnology, School of Advanced Technology in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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ABSTRACT

Objectives: TAP73 is a gene that encodes the cell cycle regulator protein. There is evidence that shows this gene has important role in infertility. In this study we evaluate relationship between gene expression of P73 and the emergence of aneuploidy.
Material and methods: 50 GV from people who were male factor and candidates of ICSI were divided into two groups, younger than 35 years (20 cases) and older than 35 (30 cases). The oocytes of both groups were cultivated in vitro for 24 hours. Polar body of matured oocyte was aspirated and evaluated for chromosome 21,18, 13 and X using of FISH technique. At the same time was evaluated the expression of P73 gene in their cytoplasm using the Real time PCR.
Results: The results showed that the 77% (15 of 20) of GV oocyte from under 35 years and 66% (20 of 30) of above 35 years were matured. Also aneuploidy was not seen in the polar body of the oocytes of the female under 35, but in oocytes of the female above the age of 35, there was chromosomal defect in 8 cases out of the total of 20. Also our results show that the rate of P73 gene expression in oocytes of female over 35 years in compare with the oocyte of female under 35 have significantly down regulated (P=0.003)
Conclusion: According to our knowledge, if we consider the prevalence of gene expression and the rate of aneuploidy simultaneously, this is for the first time that such case is reported. It can explain the incidence of aneuploidy among embryos that were created from elderly females' oocytes
KEYWORDS: P73, P53, Aneuploidy, Oocyte, Infertility, Cancer

INTRODUCTION

The diagnosis of aneuploidy dates back to 50 years ago (Jacobs 1959). Since then greater number of studies has been performed to identify its causes. Environmental factors, Medical treatments, Radiation, contraception and smoking have been concerned for its causes. The age is the only factor which is proved that can cause aneuploidy (Warbueton 2004). As a matter of fact the rate of mutation in females' germinal cells is less than males', Haldane suggested that there is a mechanism in female that it works as genome guard.

One of the human cancer syndromes is instability genome particularly chromosomal instability (CIN). CIN can cause numerical changes in a whole chromosome (aneuploidy) and/or structural changes such as translocation. Aneuploidy can be analyzed almost in all human cancer types (Merten 1994). Regarding this reason, about one century earlier, it was thought that aneuploidy leads to cancer (Boveri 2008).

In fact all human cancer cells are affected by aneuploidy (Mortens 1994). The evidences show that common causes of cancer and aneuploidy are the same and therefore the ways of approaching to them must be the same, too (Min 2010).

***Co-corresponding Authors:** Marefat Ghaffari Novin, Department of Biology and Anatomical Sciences, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, email: mghaffarin@yahoo.com
Mir Davod Omrani, Department of Genetics, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, email: davood_omrani@yahoo.co.uk

TAP53 family, which is also named tumor protein P53, is a gene that encodes the cell cycle regulator protein and therefore it works as a tumor suppressor. This protein has crucial impact in some cells' organisms and it prevents cancer in them. P53 is known as a genome guard and it prevents mutation (Strachan 1999).

Also there are evidences that show these protein family have an important role in fertility. It is suggested that these proteins can regulate female fertility in vertebrates such as human beings (Levine 2011). Recently the role of P53 in regulation of embryo's implantation by controlling in transcription level of gene expression *LIF* (Leukemia Inhibitory Factor) has been proved (Hu 2009).in addition it is clear that destruction of P53 family in female mouse can cause severe increase in its fertility (Levine 2011). Moreover, it is denoted that single nucleotide polymorphisms (SNPs), human genes P73 and P53 have an important role in female fertility difficulties (Feng 2011).

The recent results show that the expression of TAP53 would be decreased in oocytes of old female (Guglielmino 2011). And is suggested that there may be a relationship between down regulation of gene expression of P73 protein and emergence of aneuploidy (Guglielmino 2011).

In this study we tried to find out the down regulation of this gene expression, that has a relation with aging, whether or not is a predisposition factor for aneuploidy?"

MATERIALS AND METHODS

This study has been approved in the research ethic committee of Shahid Beheshti University of Medical Sciences and the permission has been implemented. This research is done in infertility clinic of Mahdieh hospital; the GV oocytes that were used for this research came from people who were male factor and candidates of ICSI, before harvesting their oocytes and having GV oocyte, the candidate's asked to sign the consent.

The long protocol was implemented for the all patients who participated in this study. They took oral contraceptive, daily in previous cycle and from 19 to 21 days of cycle they administrated buserline (Superfact; Hochest, Germany) 50 IU subcutaneous daily. From the third day, they administrated HMG (Meryonal; Serono, Italy) 150-300 IU daily for 6-7 days. When their major follicle became 15 millimeter, they administrated 10000 IU HCG IM and then after 36 hours follicular aspiration was done.

The cumulus and corona cells were removed using enzymatic digestion after a variable timing followed by oocyte retrieval then incubated in Quinn's Advantage Medium w/HEPES(Sage, USA) till evaluating of oocytes. 50 GV oocyte were divided into two groups, younger than 35 years (20 cases) and older than 35(30 cases). The oocytes of both groups were cultivated in Quinn's Advantage Cleavage Media at 37°C, 5% CO2 in air for 24 hours. After they got mature and we saw the first polar body with inverted microscope along with the help of mechanical way, their polar body was aspirated (for more details look at in Gianaroli 2000) the aspirated polar body was fixed on a slide using Carnoy's fixative (Methanol: Glacial Acetic Acid (3:1)) and the aneuploidy of chromosome 21,18, 13 and X with the help of FISH method were checked and evaluated. Then the oocyte was washed in cooled media then it was fixed in a lasing buffer micro tube when it was free of albumin. The RNA extraction and its change to cDNA were done based on the protocol of Agilent company (Agilent, USA). The Real time PCR experiments were done with using of Rotter-gene 6000(Corbett) set. The reaction results were analyzed by REST9 program. In this research GAPDH gene is used as a normalizer.

Data that included the rates of maturation, rate of aneuploidy of oocytes were analyzed with SPSS version 18 software.

RESULTS

As it is seen in the table 1, from the 20 GV oocytes that were under the age of 35 group, 15 oocytes (75 %) reached to metaphase ll. However, the 30 GV oocytes above the age of 35, 20 oocytes (66%) reached to metaphase ll. The difference between them was significant.

Table1.Is shown the rate of mature oocytes in IVM cases.

	Under the age of 35	Above the age of 35
No. of GV oocyte	20	30
No. of matured oocyte	15	20
Maturation rate	75%	66%

FISH experiments

The results of FISH technique showed that chromosomal defect and aneuploidy was not seen in the polar body of the oocytes of female(s) under 35,whereas in oocytes of female above the age of 35, (in 8 cases from total 20

cases there were chromosomal defect such as trisomy 21. (N=4)(fig.1), trisomy 18 (N=2) and monosomy 18, monosomy 13 , XO one of each ones (N=1).That difference was significant.

The table 2. of the chromosomal situation of females under the age of 35 years

Chromosome	13	18	21	X
Total cases	15	15	15	15
Normal	15	15	15	15
Abnormal	0	0	0	0

The table 3. of the chromosomal situation of females above the age of 35 years

Chromosome	13	18	21	X
Total cases	20	20	20	20
Normal	19	18	16	19
Abnormal	1	2	4	1

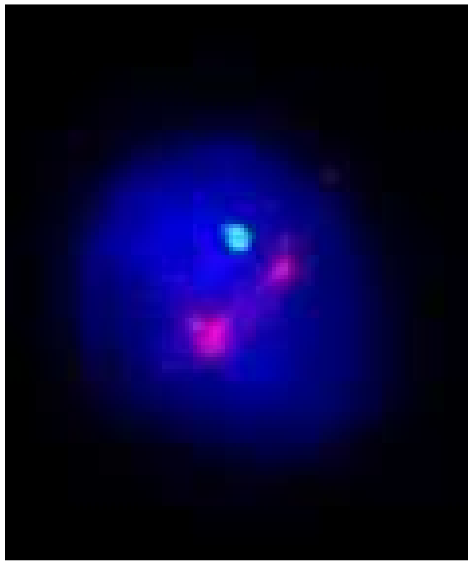
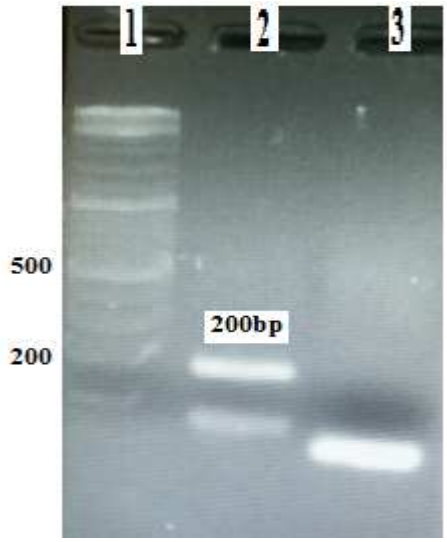


Fig.(1) In this picture that came from polar body, a signal (Green) is seen for Chromosome 21 and two signals (red) for Chromosome 13.

Real time experiments

To approve the accuracy of well-made cDNA, about 5 micro liter of lysate production was used as template in normal PCR reaction. In this reaction, the PCR reaction was done with the help of GAPDH primers and the results were observed with 3% agarose gel (Fig.2).

Fig.(2) cDNA PCR product on 3% gel agarose



In this study the rate of P73 gene expression was evaluated and examined by Real Time PCR experiments with Rotor gene 6000 set. The reaction results were analyzed by using $\Delta\Delta Ct$Formula and REST9 program.The used primers in Real Time PCR reactions were shown in table (1).

Table 4. The used primers in Real Time PCR reactions

Gene Name	Primer Sequence	Gene Name	Primer Sequence
P73	R:CTTCGTTGAAGTCCCTCC F:TACTCCCCGCTCTTGAAG	GAPDH	R:TGAGCCATAGAGTTTCCCCTTC F:AAGGTGAAGGTCGGAGTCAAC

The results have shown that the rate of P73 gene expression in oocyte of female over35 years to compare with the oocyte of female under 35 has significantly down regulated (P=0.003) fig.(2).

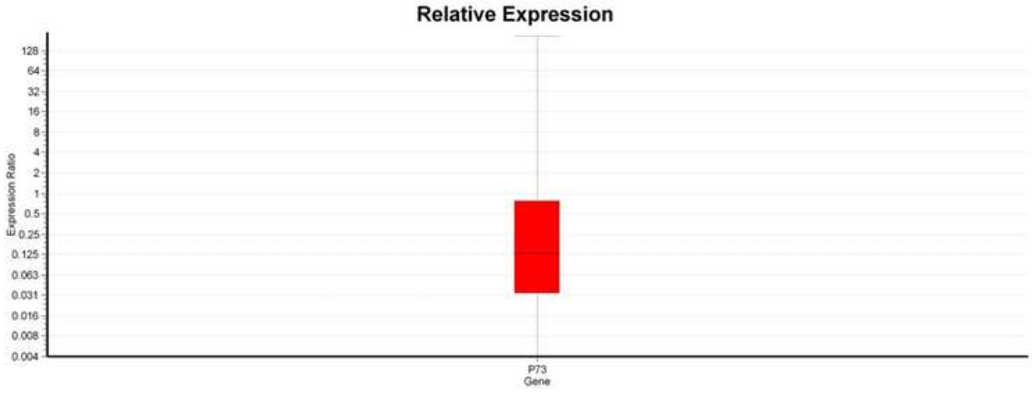


Fig.(3) The proportion of P73 gene expression in oocyte of female(s) above the age of 35 in compare tothe oocyte of female(s) under 35

DISCUSSION

In this study, the rate of aneuploidy of all chromosomes in the group with the age above 35 were significantly higher. Our finding in this study confirms the previous studies (Verlinsky 1999; Kahraman 2000 ; Pehlivan 2003) which addressed the rate of aneuploidy of oocytes would increase along with ageing. Current study, the rate of aneuploidy of chromosome No. 21is clearly and significantly more than other aneuploidies.

About the P73 gene, this study shows that this gene expression in oocytes of females above the age of 35 years is significantly less than females' younger than 35. Although this subject was presented in Guglielmino et al study (Guglielmino 2011, Levine 2011), according to our knowledge, if we consider the prevalence of gene expression the rate of aneuploidy simultaneously, this is for the first time that such case is reported. Our study can explain the incidence of aneuploidy among embryos that were created from elderly females' oocytes (Kajii 2008; Takeuchi 2008).

Nowadays, it is proved that there is a direct relation between aneuploidy and aging. In 20 decades of living the incidence is 2%-3% and this incidence will be increased in 40 decades to 35% (Hassold 2001).

Also it has been proved that aging has a negative effect on female fertility, as so this has an important impact on quality and quantity of oocytes. In this situation ovarian reserve throughout ageing has tendency to decrease until they reach their menopause when the amount of oocytes get zero. In elderly female the numbers of oocytes and fertility will decrease and the abortion will increase, too (Broekmans 2007). Also in ART for elderly females, the numbers of oocytes that are taken are a few and their embryos are not cable of to implantation easily (Jones 2008).

The chromosome segregation errors depend on the age would occur during the meiosis. In elderly female, not only the prevalence of trisomy 21, but also the other types of autosomal aneuploidies will increase that can cause an increase in abortion by them. It is indicated that 93% of trisomy 18, 95% trisomy 21 and 100% trisomy 16, the ageing is the only risk factor (Otter.2010; Hassold 1996) .A study concluded that fertilization failure in oocytes has a relation with aneuploidy (Pellestor 2003).

The PGS techniques are used because of the high prevalence of aneuploidy in ART particularly in females above the age of 35 (Verlinsky 1999; Kahraman 2000; Pehlivan 2003). By these techniques were identified that 70% of embryos of female above the age of 35 suffer from aneuploidy (Voullaire 2000; Wells 2000).

As for the P53 family such as P63 and P73 that have an important role in control of cell cycle, Apoptosis , genomic stability like ploidy , their difficulties must be consider as one of the etiologic factor of such disorders (Collavin 2010, Levine 2011).

It is shown that TAp 73 involved with SAC protein and it controls its activities. In addition, the down regulation of p73 gene is a long up regulation of spinal assembly complex (SAC) gene in lung cancers (Min 2010). The results shown that TAP73 is as a regulator of SAC reply and destroying of TAp73 can cause some problems in stopping of cell mitosis (Tomasini 2009). In addition, when the mouse cells of Trp73-/- become genome instability, aneuploidy will increase on them. And may be this situation causes spontaneous cancers on them (Tomasini 2008). In fact, in mice of $TAp73^{-/-}$damage to the structure of spindle apparatus during the mitosis and meiosis then finally will causes tumor, and also it causes some difficulties in oocytes that even the oocytes fertilize the embryos will not stay alive (Tomasini 2008).

The studies that were done in Sweden about the health of children born with IVF are represented that the prevalence of cancer among these children particularly those that had elderly mothers is more than normal children. Probably this risk factor is not related to IVF but it is related to other predisposition factors (Kallen 2010). This risk factor may be for the reason of decrease in P73 gene expression of their mothers that are transmitted to their children, of course this must be proved and clear in details through more studies in future.

In today world, it seems that the some of diseases and disorders that related to ageing are concerned to P53 protein family, so that it is shown in hair loss and white hair difficulties (Botchkarev 2001), retina and eye disorders (Vuong 2012), arthro disease (Lee 2012), memory disorders (Flores 2011) and Alzheimer (Lanni 2012) all concerned to this protein family.

Recently, Nomikos and his co-workers, in a study on mouse, injected the PLC that its existence for fertilization was proved and identified that its nonexistence can cause the inactivity of oocyte and fertilization failure, they injected it with sperm to oocyte and then the injected sperm was activated and caused fertilization and normal cell divisions (Nomikos 2012). May be in future, we can inject the P73 protein to oocyte so that it would compensate the lack of fertilization and increase the efficiency of meiosis is check point. Probably with this work the damaged oocytes can go to Apoptosis phase and although the numbers of oocytes in ART will be a few, we will have just healthy oocytes that their capacity of fertility, cell division and implantation will be increased instead. Professor Evers previous Chairman of European Society of Human Reproduction and Embryology (ESHRE) in a conference in Tehran said that when we can say," we have improved on ART ,that we can take an oocyte from our patient and then we can have an embryo of that oocyte and we can give a healthy baby to his parents."

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