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The effect of cellular and physiological indicators on gender determination

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Abstract: Gender determination, in addition to having special value to parents, has particular importance in sex-linked diseases. This study aimed to investigate the cellular indicators (i.e. BMP-6 protein and PPARγ protein expression levels in granulosa cells) and the physiological indicators on gender determination. For this purpose, on 68 infertile patients referred to the clinic, ovarian stimulation was performed by different protocols and then ruptured by different HCG. Follow-up of patients was performed after they became pregnant after five months. U/S was done for knowing the gender of the baby then after labor rechecked another time. Also, granulosa-luteal cells (GLCs) were isolated from the follicular fluid of 68 women participating in the study. BMP-6 protein and PPARγ protein were measured using Western blotting. Results showed that the total number of delivered babies was 68, 41 males (60.3%) and 27 females (39.7%). About physiological indicators results, there was no significant association between the age of the mother and sex of the baby (*P*=0.934). No significant association was detected between the month during which the conception occurred and the sex of the baby (*P*=0.734). The same result was obtained for the follicle side (*P*=0.236), and follicle size (*P*=0.659), there was no significant association between the sex of the baby with the following factors: protocol of treatment (P=0.417), IVF after HCG (*P*=0.237), HCG type (*P*=0.572), parity (*P*=0.282), and type of infertility (*P*=0.376). The cellular indicators results showed that the BMP-6 protein level in granulosa cells of mothers with daughters was almost twice as high as mothers with sons (*P*=0.043). But there was no significant difference between mothers with daughters and mothers with sons in PPARγ protein level (*P*=0.12). It can be concluded that except for BMP-6 protein level, none of the cellular and physiological indicators affects gender determination. Therefore, this cell indicator can probably be evaluated as an effective indicator in determining gender.

Key words: Follicle size; Ovarian side; Age; Gender of baby; PPARγ; BMP-6.

Introduction

Most parents today, because they have fewer births, want to choose the sex of their children before pregnancy. In addition, today due to cultural, social, and economic problems, determining the sex of the child is one of the concerns of families and even governments. In addition, we significantly see that in some communities such as Germany (1), Sweden, Norway, and Finland (2), and Japan (3), reducing the sex ratio has become a problem. The mechanisms that cause this decrease in gender ratio are not well understood, but hypotheses have been proposed for it, including exposure to polluted and toxic environments such as exposure to tobacco smoke (4) or stress (5), what happened to the Kobe Earthquake in Japan (6).

Sex determination is a reproductive process that is performed before sex to select a gender (7). Currently, the sex selection is done for two medical and non-medical reasons that in medical reason preventing genetic disease to occur in a child and non-medical reason is done merely for the sake of parental interest and desire to have a son or daughter (8). Determining the sex of the fetus before fertilization is a topic that is very widespread today and many parents try to do it in various ways such as getting help from the diet, vaginal shower, and intimacy at the time of ovulation, their desired sex gets pregnant. Many mothers ask what the right size of follicle is for a boy. Or what should be the side and size of the follicle to have a boy? Or does having a daughter or son have anything to do with the size or number of follicles at the time of ovulation? Does the mother's age for pregnancy affect being a boy or a girl? Or what factors affect gender determination? Or does even the use of treatment protocols for fertility effects being a boy or a girl? And many more of these questions. People use fetal sex determination technology for a variety of reasons. We know that some diseases are inherited, and parents use this technology to prevent their babies from having X-linked diseases. Another reason is that families, although they do not differentiate between girls and boys, like to have children of both sexes. And finally, sometimes parents want to have a child of a certain gender. Sex determination before pregnancy is usually done by special nutrition for men and women and vaginal ultrasounds to determine the time of intercourse and the time of ovulation (7, 9). Experience and research show that receiving more calcium and magnesium ions increases the probability of giving birth to girls and receiving sodium and potassium ions increases the probability of giving birth to boys (10). Vaginal cleansing to acidify or alkalize it is another way to help determine pre-pregnancy sex (10, 11). Accordingly, in an alkaline environment, the probability of Y-containing sperm surviving and procreation increases, and in an acidic environment, the probability of X-containing sperm survi-

ving and procreation increases(10, 11). According to Schettles method, if intercourse takes place during ovulation, the probability of having a son increases, and 2 to 3 days after ovulation, the probability of giving birth to a girl increase (11). LH test or ultrasound can be used to determine the time of ovulation. Today, one of the in vivo methods used to determine the sex of the baby is Preimplantation Genetic Diagnosis (12). The use of PGD leads to the formation of embryos of the desired sex and normal growth, that have no abnormal chromosomes, and looks good under a microscope. In this procedure, after ovarian stimulation, multiple oocytes are removed from the mother (12). The eggs are fertilized in the laboratory using the father's sperm in a technique called in vitro fertilization (IVF). As the embryos develop through cleavage, a blastomere is removed from each embryo and then assessed for the presence of Y and X chromosome and separated by sexual chromosome. Embryos of the desired gender are transferred back in the mother's uterus (13).

Ovarian follicle growth, granulosa cell differentiation, oocyte maturation, and ovulation are the most critical factors affecting fertility (14). Granulosa cells are also involved in oocyte growth and maturation through endocrine and signaling activities. The abnormal development of granulosa cells and their inefficiency are among the causes of infertility (15). Many factors have essential roles on granulosa cells. One of these factors is Bone Morphogenetic Protein-6 (BMP-6), which is produced by ovarian follicles. This protein, in addition to its effect on the regulation of bone development, has an important role in the process of ovarian follicle development (16). The other factor is Peroxisome Proliferative-Activated Receptor-gamma (PPARγ) has been known as an important regulatory factor in fertility (17). Therefore, evaluating these factors is very important in female fertility (18).

Now, according to these explanations, is it assumed that changing a part of the treatment protocol such as pregnancy time following ovarian stimulation and program of ovarian stimulation or other variables such as age, ovarian position, and follicle size will change the sex of the fetus? Therefore, this study aimed to determine whether the patient's age, follicle size, ovarian side, gestational age after ovarian stimulation, and ovarian stimulation program could determine the sex of the baby. The role of BMP-6 and PPARγ in granulosa cells was also evaluated in determining the gender of the child.

Materials and Methods

Physiological parameters

This study was done during 2017-2020 in the private clinic in Erbil city. 68 females in different age groups and in different days were included in the study seeking for pregnancy with different types of female infertility (primary and 2ndary), 2nd day of the cycle came for basal U/S then different protocol of ovarian soft stimulation done by (letrizole or letrizole + Gonal F or clomiphene, or clomiphene+ Gonal F or Gonal F, then follow up of follicles done by vaginal U/S the ovarian side and follicular size documented on their files when follicles reached the size of 18 mm and above HCG injection given to them for follicle rupturing then couples asked to do intercourse after injection after then asked about how many hours intercourse done between them, after 20 days B HCG done for them to ensure their pregnancy, follow up of the pregnancy done by U/S after 4th month of gestation for knowing the gender of the baby, then rechecking of the gender of the baby done after birth.

BMP-6 protein and PPARγ protein expression levels in granulosa cells

Granulosa-luteal cells (GLCs) were isolated from the follicular fluid of 68 women participating in the study. BMP-6 protein and PPARγ protein were measured using Western blotting. GLCs were Lysate with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholic acid,1% Nonidet P-40, and complete protease inhibitor mixture tablets (Roche Applied Science, Penzberg, Germany). From each sample, 10 ng of protein were separated by 10% SDS-PAGE and electro-transferred to a PVDF membrane (EMD Millipore, USA). Membranes were blocked with 5% milk for 2h at 25℃ and incubated 12h at 4℃ using the following primary antibodies: Rabbit anti-rat BMP-6 (1:1,000; cat No. ab155963; Abcam, UK), and Anti-PPARγ (Antibody (B-5): sc-271392, Santa Cruz Biotechnology, USA). The BMP-6 protein level and PPARγ protein level were normalized based on the level of β-actin protein. The primary antibody for β-actin was mouse anti-rat β-actin (1:1,000; cat no.sc-130656; Santa Cruz Biotechnology, USA). Membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000, cat no.611-1302; Rockland) for 2h at 25℃ and then evaluated with Enhanced Chemi-Luminescence, and Fusion Fx (Vilber Lourmat, France).

Statistical Analysis

Data were analyzed by t-test, Fisher's exact test, and Chi-square test at the significant level of $P<0.05$ and the data were presented as mean ± standard deviation.

Results

The total number of delivered babies was 68, 41 males (60.3%) and 27 females (39.7%). Table 1 shows no significant association between the age of the mother and sex of the baby (p = 0.934). No significant association was detected between the month during which the conception occurred and the sex of the baby (p = 0.734). The same can be applied for follicle side (p = 0.236), and follicle size (p = 0.659) as presented in Table 1.

It is evident in Table 2 that there was no significant association between the sex of the baby with the following factors: protocol of treatment (p = 0.417), IC after HCG (p = 0.237), HCG type (p = 0.572), parity (p = 0.282), and type of infertility (p = 0.376).

Western blotting results showed that the BMP-6 protein level in granulosa cells of mothers with daughters was almost twice as high as mothers with sons (P=0.043) (Table 3). But there was no significant difference between mothers with daughters and mothers with sons in PPARγ protein level (P=0.12).

Table1. Baby sex by age of mother, the month of the year, and follicle size.

	Male baby		Female baby		Total		
	No.	**(%)**	**No.**	**(%)**	**No.**	**(%)**	***P*-value**
Age (years)							
< 20	6	(66.7)	3	(33.3)	9	(100.0)	
20-24	13	(61.9)	8	(38.1)	21	(100.0)	
25-29	11	(55.0)	9	(45.0)	20	(100.0)	
30-34	6	(54.5)	5	(45.5)	11	(100.0)	
≥ 35	5	(71.4)	2	(28.6)	7	(100.0)	0.934*
Month							
Winter	13	(65.0)	7	(35.0)	20	(100.0)	
Spring	8	(57.1)	6	(42.9)	14	(100.0)	
Summer	10	(66.7)	5	(33.3)	15	(100.0)	
Autumn	9	(50.0)	9	(50.0)	18	(100.0)	0.734
Follicle side							
Right	23	(54.8)	19	(45.2)	42	(100.0)	
Left	18	(69.2)	8	(30.8)	26	(100.0)	0.236
Follicle size Mean±SD	20.22	(±2.41)	20.50)	(±2.69)			0.659†

*By Fisher's exact test. † By t-test for two independent samples.

Table2. Baby gender by the treatment plan, parity, and type of infertility.

	Male baby		Female baby		Total		***P*-value**
	No.	**%**	**No.**	**%**	**No.**	**%**	
Protocol							
Femara	2	(40.0)	3	(60.0)	5	(100.0)	
Femara + GF	27	(62.8)	16	(37.2)	43	(100.0)	
Natural	0	(0.0)	2	(100.0)	2	(100.0)	
Clomid	2	(66.7)	1	(33.3)	3	(100.0)	
Clomid + GF	2	(100.0)	0	(0.0)	2	(100.0)	
GF	3	(75.0)	1	(25.0)	4	(100.0)	0.417*
IC after HCG							
< 36	26	(65.0)	14	(35.0)	40	(100.0)	
> 36	12	(50.0)	12	(50.0)	24	(100.0)	0.237**
HCG type							
Pregnyl	11	(73.3)	4	(26.7)	15	(100.0)	
Choriomon	17	(63.0)	10	(37.0)	27	(100.0)	
Ovitrelle	10	(55.6)	8	(44.4)	18	(100.0)	0.572**
Parity							
Nullipara	24	(68.6)	11	(31.4)	35	(100.0)	
1-3	12	(48.0)	13	(52.0)	25	(100.0)	
4-5	4	(57.1)	3	(42.9)	7	(100.0)	0.282*
Type of infertility							
Primary	17	(70.8)	7	(29.2)	24	(100.0)	
Secondary	19	(59.4)	13	(40.6)	32	(100.0)	0.376**

*By Fisher's exact test. **By Chi-square test.

Table 3. Comparison of BMP-6 protein and PPARγ protein expression in Granulosa cells between the Mothers with female baby Mothers with a male baby.

	Mothers with a female baby	Mothers with a male baby	***P*-value**
BMP-6 protein	0.95±0.31*	0.41±0.22	0.043
PPARγ protein	0.53±0.09	0.61±0.13	0.12

Discussion

The results of this study showed that the size and side of the follicle, the age of the mother, the month of the year, the treatment program, the parity, and the type of infertility had no significant effect on the sex of the baby. In fact, what determines the size of the follicle are several life-related processes, each of which can undergo hormonal, nutritional and pharmacological changes (19, 20). During the embryonic period, primordial germ cells (PGCs) migrate from the primary stem to the developing gonads, where they proliferate and later form follicles along with cells lining the ovarian tissue (21). The follicle is an immature egg that is enclosed and nourished by several ovarian epithelial cells. Upon reaching puberty and almost sometime after the menstrual cycle is normalized, the hypothalamus produces and releases the gonadotropin-releasing hormone, which, when it reaches the anterior pituitary gland, produces FSH and LH (22). Initially, after menstrual bleeding stops, FSH affects a set of 15 to 20 follicles and causes them to grow. Of these 15 to 20 assemblages, only one is fully developed and becomes the dominant follicle. This follicle goes through most of the developmental stages, preantral and antral, and reaches its maximum size about 24 hours before ovulation(19). Ultrasound will be detectable and measurable around the time of ovulation. It may be interesting to know that the size of the follicle has nothing to do with your baby becoming a girl or a boy(23)! To clarify this issue, we need to explain to you about determining the sex of the fetus during fertilization: In the middle of their cycle, females produce one or more oocytes in the middle of their cycle, which are only X chromosomally (24). In contrast, males have two types of sperm that contain the X and Y sex chromosomes. In this way, if an egg containing the X sex chromosome is fertilized with a sperm containing the sex Y chromosome, the egg cell becomes male, and if the X egg is fertilized with an X-containing sperm, the egg cell becomes female. The sperm containing the Y chromosome is much smaller than sperm containing the X chromosome (24). Unlike sperm containing the X chromosome, which can survive in the vaginal environment for up to 4-5 days, sperm containing the Y chromosome survive in the vaginal environment for a maximum of 16-18 hours (25, 26). On the other hand, when an egg is released, it has 12 to 24 hours to be fertilized by sperm and then destroyed. Second, sperm containing the Y chromosome survive better in an alkaline environment and move faster than sperm containing the X chromosome (25, 26). Based on these characteristics, it is possible to intervene in determining the sex of the fetus (27). Sperm containing the Y chromosome survive better in an alkaline environment. In addition, the environment of the uterus at the time of ovulation, day 14 in a regular monthly cycle that is 28 days, has an alkaline pH, and if fertilization takes place on this day, the fetus will most likely be born a boy (25). And other times, because the pH of the cervix is low and acidic, the sperm containing the X chromosome is more viable and the fetus is more likely to become a girl. But if intercourse occurs 1 to 2 days after or before ovulation, the chances of becoming a boy are greatly reduced and the chances of a girl are high (25). So we can easily say that all eggs have the same X chromosome. Although at the time of ovulation, the dominant follicles in different cycles may be different sizes and on different sides of the other ovary, they all contain the same X chromosome. So, when you ask what the right follicle size is for a boy's pregnancy or what size it should be for a boy to become a boy, in a word, the size of the follicle or the location of the ovary has nothing to do with sex determination.

In this study, our results also showed that maternal age and month of the year did not have a significant effect on infant sex. Although most studies agree that maternal age does not affect a child's gender, it is important to note a few points. Fukuda *et al*., (2011) reported that the sex ratio of offspring is associated with the mothers' age at menarche (28). Misao Fukuda and colleagues at the MDKI Institute of Health in Hugo, Japan, have found a link between the sex of the fetus and the age at which the mother first menstruates. If the mother's first menstrual period is 10 years old, she has a 46% chance of having a son, if she is 12 years old Be 50%, and if 14 years old Is 53%. According to Fukuda, previous studies have shown that the level of the female hormone estradiol is higher in women who experience menstruation for the first time before the age of 12. (28). This may cause the male sperm in the body of these women to disappear spontaneously. According to Valerie Grant of the University of Auckland in New Zealand, the results of this study are plausible because we know those male fetuses are more vulnerable to hormonal imbalances than female fetuses.

Rueness *et al*., (2012) studied whether the sex ratio differs by maternal age in all pregnancies, and also assessed the sex ratio in pregnancies complicated by pre-eclampsia, fetal death, preterm delivery, or small for gestational age (SGA) offspring. They used data from all births in Norway from 1967 through 2006, to estimate sex ratios (the number of males per 100 female offspring) according to maternal age. The analyses were done among all pregnancies and within subgroups of complicated pregnancies. In addition, the odds ratio (OR) of having a male offspring by maternal age in all pregnancies with adjustment for pre-eclampsia, fetal death, preterm delivery, or SGA offspring were estimated. They showed that there was no relationship between maternal age with the human sex ratio. However in pregnancies with pre-eclampsia, the proportion of males decreased with increasing maternal age. In multivariable analyses including all pregnancies, with adjustment for complications, there was still no association of maternal age with offspring sex. However, in pregnancies with SGA offspring, the adjusted OR of delivering a boy at term was lower than expected (OR 0.87, 95% confidence interval 0.85-0.89). The lower proportion of male births at high maternal age in pregnancies with preeclampsia and pregnancies with live-born SGA offspring born at term supports the hypothesis that male fetuses are more vulnerable to maternal stress than female fetuses (29). In fact, at this age, one should pay attention to the level of estrogen hormones, especially estradiol (E2) and progesterone, and their changes in order to modulate the physiological stresses (30) and the appropriate time for Human Chorionic Gonadotropin (hCG) administration (31).

Our results also showed that the treatment program, the parity, and the type of infertility had no significant effect on the sex of the baby. There are some separation methods for selecting sperm sex to fertilize female gametes, such as micro-sorting and the Erickson method (32-34). In Ericsson, as sperm passes through an albumin gradient, the differences in mass between the X and Y chromosomes cause the females dragged down by the weight of the extra "leg" of the X sex chromosome (35). The method has a 70-72% success rate for boys and a 69-75% success rate for girls (35). The third method is the determination of sperm sex chromosomes in sperm sorting by flow cytometry. Sperm sorting is an advanced technique that sorts sperm "in vitro" by flow cytometry(36). This shines a laser at the sperm to distinguish X and Y chromosomes and can automatically separate the sperm into different samples. The technology is already in commercial use for animal farming (36). It claims a 90% success rate but is still considered experimental by the FDA (37). The treatment program to stimulate the ovaries as a next step in the in vitro fertilization or IVF process is taking daily injections of hormones. These hormones are regularly produced in your body; however, the injections you will be taking contain a higher dose than what would naturally occur. This allows multiple eggs to mature in the ovary, which increases the chance of pregnancy. Fertility drugs commonly given in this program include leuprolide, FSH, human menopausal gonadotropins (hMG), hCG, antagon, progesterone in oil, and estradiol tablets. Sometimes, we precede the stimulation cycle with birth control pills, depending on your IVF cycle protocol. Your physician will determine which protocol is best for you and detailed information will be reviewed with you. During your IVF cycle, it is tracked the development of eggs inside ovarian follicles by tracking your blood hormone levels and performing ultrasounds. Typically, a mother will need to be seen approximately three to four times during this stimulation to track progress. A cycle may be canceled if the ovaries are not responding optimally. The rate of cycle cancellation is approximately 5 to 10 percent and is age-dependent with older patients being canceled more often. Another possible complication during the stimulation process is called ovarian hyperstimulation. Ovarian hyperstimulation is a condition that develops when the ovaries become very enlarged and tender due to the stimulation medications used. Ovarian hyperstimulation can lead to the development of pelvic pain and the accumulation of pelvic fluid, which sometimes requires bed rest or hospitalization. To minimize this complication, which now occurs in less than 5% of cycles, efforts are made to select appropriate stimulation protocols and appropriate drug doses. It is an ovarian stimulation program that is used before other contraceptive methods to induce ovulation and to increase the number of eggs released. This can increase the chances of pregnancy and not affect the sex of the baby. In general, as mentioned earlier, the determination of fetal sex by in vitro and in vivo methods is mainly dependent on increasing the availability and viability of Y chromosome sperm. Here are some important points that are important to consider when determining a particular gender. The diet should be observed by the woman and the type of nutrition of the man has no effect on determining the sex, but it's done by the couple makes it easy to implement (38, 39). Diet should be started from two months before pregnancy (at least 4 to 6 weeks before pregnancy) and at this time, arbitrary use of drugs that upset the ionic balance of the uterus for the desired sex should be avoided (38-40). The factor of sperm count in each intercourse is important in determining the sex, so that the more sperm, the more likely it is to become a boy, and vice versa. In order for the fetus to become male, intercourse should not take place until ovulation (day 14 from the start of menstruation in 28-day cycles), which is the right time in terms of pH for having a boy(41, 42). In addition, the role of stress, living environment and lifestyle in determining the sex of the fetus cannot be ignored (25, 29). The inadequacy of each of these can ruin your efforts to have the baby you want. In addition, by considering the various factors affecting the choice of infant sex and the role of psychosocial factors, the couple's sexual knowledge can play an important role in achieving the desired sex and improving the quality of sexual relations (43).

Also, the western blotting results showed that the BMP-6 protein level in granulosa cells of mothers with daughters was almost twice as high as mothers with sons (P=0.043). But there was no significant difference between mothers with daughters and mothers with sons in PPARγ protein level (P=0.12). These two proteins are the most important indicators for determining fertility (17, 44, 45). Therefore, in the study, their effect on determining the gender of the child was evaluated. As mentioned, BMP-6 protein appears to be influential in gender determination. It seems that increasing the rate of BMP-6 protein increases the probability of becoming a girl. BMPs are a group of growth factors also known as cytokines and metabolites (46). The discovery of these proteins was due to their ability to induce bone and cartilage formation. BMPs are now thought of as a group of axial morphogenic messengers that harmonize tissue architecture throughout the body (47-50).

It can be concluded that the size of the follicle and the side of the ovary and the ovarian stimulation program do not affect the sex of the baby. Also, our data showed that the patient's age, the time close to pregnancy for ovarian stimulation, and PPARγ protein levels in granulosa cells did not affect the sex of the infant. BMP-6 protein expression in granulosa cells showed that levels of this protein were almost twice as high in mothers with daughters as in mothers with sons. Therefore, this cell indicator can probably be evaluated as an effective indicator in determining gender.

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