Chapter

Methods for Prenatal Sex Determination and Their Importance in Understanding and Prevention of Gender-Related Birth Defects

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Abstract

Various hormones, chemicals, and teratogenic agents exhibit gender-related effects in utero as well as postnatally. Among such gender-specific teratogens are endocrine disruptors, especially phthalates that affect male gonads, diabetes-induced oxidative stress with more deleterious effects on male offspring, procarbazine-induced cleft palate affecting more male fetal rats compared to females, and VPA-induced autism-like behavior that affects differently males than females. Hence, there are many needs for the accurate determination of genetic gender. In newborn animals, the morphological methods that exist for sex determination (i.e., anogenital distance) are generally inaccurate. Hence, an accurate and simple method for the prenatal and early postnatal assessment of the genetic sex, prior to reliable evaluation from the external genitalia, is of utmost importance. Indeed, several methods have been developed for accurate assessment of genetic sex, which are discussed in this chapter. Findings from studies in our laboratory have shown that the method described by McFarlan et al. for the assessment of genetic sex in adult mice by PCR of Sly/Xlr genes can be reliably used for the genetic sex determination of any tissue, including embryos and fetuses, with an accuracy of about 100%.

Keywords: sex determination, sex differentiation, androgens, gender-related teratogenesis, methods for sex assessment

1. Introduction

The ability to accurately assess the genetic sex in tissues, embryos, fetuses, and newborns is crucial in animal models when gender has specific impacts on development and morbidity or whenever genetic and environmental effects are gender-related or gender-specific. For the human, gender assessment is crucial in all cases of ambiguous genitalia and intersex where the proper definition of the sex is of diagnostic and/or therapeutic importance.

Female and male embryos are morphologically and anatomically indistinguishable until the development of internal and external genitalia and secondary sex characteristics appear. In mice, for example, sexual differentiation starts around

prenatal day 11.5 when the male-determining gene Sry is expressed in the bipotential genital ridge and induces testes-specific gene expression. In the lack of Sry expression, female-determining gene expression is activated [1].

There are two basic phases of sexual development in mammals: sex determination at fertilization and sex differentiation that is associated with sex determination but may be influenced by a variety of internal factors (mainly hormones and their receptors) and external factors (hormones, endocrine disruptors, and a variety of environmental chemicals) [2]. We will therefore briefly describe in this chapter first the development of the sex organs and then in more details the teratogenic effects that are gender-specific and the different methods that are used for the discrimination between genders, assessing the genetic sex.

2. Development of the reproductive system

2.1 Development of internal genitalia

The reproductive system consists of the gonads, internal sex organs, and external genitalia [3]. In all mammals the initial stages of the development of reproductive organs are dimorphic (indifferent) since the precursor organs are similar in both genders [4]. During early development, both male and female primordial sex organs develop in every embryo, and with the advancement in development, depending on the genetic sex determined at fertilization and on endocrine function of the sex steroids, one of the two internal sex organs will regress and become nonfunctional. Hence, sex determination is genetically programmed during fertilization, but sex differentiation, the second phase of sexual development, is hormone-dependent [5]. SHH, FGF, and TGF signals are involved in the first phase, while androgen-dependent signaling and androgen receptors are mainly involved in the second phase [1–5].

In the human embryo, similarly to other mammals, there is initial development of an indifferent gonad, and both the Wolffian duct (mesonephric duct) and the Mullerian (paramesonephric) duct develop bilaterally in the primitive genital ridges. The presence of the Y chromosome (Sry) determines the persistence and further development of the Wolffian duct and derivatives, while its absence will cause regression (degeneration) of the Wolffian duct. The gonads will differentiate toward testes that will start secreting sex steroid hormones (androgens secreted by the interstitial [Leydig] cells of the testis), as well as the anti-Mullerian hormone secreted by the Sertoli cells that will induce regression of the Mullerian ducts [6, 7]. In the absence of the Sry, the Mullerian ducts will continue their differentiation to uterus and fallopian tubes, the gonad will be female, and the Wolffian duct will regress. In the human embryo, the gender-specific morphologic differentiation of the reproductive organs occurs during weeks 7–10 of gestation (5–8 postfertilization) with the establishment of endocrine function of the gonads [3]. The development of the external genitalia in the area of the urogenital sinus occurs slightly later.

2.2 Development of the gonads

The gonadal primordia appear in the human embryo around the fourth–fifth week postfertilization (weeks 6–7 of pregnancy), initially without the germ cells (gametes). The germ cells, apparently originating from the dorsal part of yolk sac epithelium that is later incorporated into the gut, migrate in the primitive hindgut into the dorsal mesentery alongside nerve fibers [3, 8] to the gonads. They start to invade the gonad during the fifth week postfertilization [9]. Migration of primordial germ cells may

continue up to postfertilization week 14. The molecular basis for the formation and migration of the germ cells is poorly understood [9]. The male gonad starts its morphologic differentiation before the female gonad, occurring during the end of week 6 postfertilization, at which time it also starts to secrete its hormones [3, 10].

2.3 Development of external genitalia

The first phase of the differentiation of external genitalia occurs during the fifth postfertilization week as an "indifferent stage" where the cloacal folds and genital tubercle, the area of future development of external genitalia, are similar in male and female embryos (ambisexual stage that extend to 9–10 weeks postfertilization). There are androgen-independent and androgen-dependent phases of development within the cloacal folds that unite and enlarge to form the genital tubercle which is located cranial to the urogenital opening (ostium) and composed of mesoderm of the urogenital sinus. The final development of the external genitalia is largely affected by environmental factors (i.e., endocrine disruptors) [11]. Sonic hedgehog (SHH) regulates the early development of the external genitalia. Under the influence of androgens (5-dihydrotestosterone), the genial fold will fuse to form the scrotum in the male. Androgen deficiency will induce the development of female external genitalia even in genetic males [10–12]. The inability to transform testosterone to 5-dihydrotestosterone, i.e., 5α-reductase deficiency and sometimes 17β-hydroxysteroid dehydrogenase deficiency, may lead in genetic males to the formation of female genitalia [11, 13].

3. The importance of sex identification in biology and in teratology

Teratogens might be gender-specific and might cause lethality or congenital malformations that are dependent on embryonic sex. Possible gender-specific effect of teratogens is not always established because in most studies embryonic and fetal genetic sex is not determined. The ability to determine fetal sex will allow a better understanding of the possible gender-related effects of teratogens and their mechanism of action.

It is important that sex identification techniques will be noninvasive and when needed will be performed even on highly degraded noninvasive samples such as feces and hair or different organs from which some tissue can be spared [14]. Nongenetic methods to determine fetal and neonatal sex were proven to be to a large extent inaccurate. Evaluation of anogenital distance difference is subjective, has an overlap zone, and is accurate only in about 50% of the cases [15]. Although Barr bodies were detected in the amnion and liver cells of rat embryos and fetuses during days 12.5–20.5, this cannot serve for accurate sex determination since they were detected in a relatively small proportion of subjects and in both sexes. They were detected in 20–50% in the amnion and 10–51% in the liver of females. Moreover, they were also detected in a very small proportion of males: 0–7% in the amnion and 0–8% in the liver [16]. Hence, genetic methods for the detection of gender-related genes and/or chromosomal studies are the most reliable methods.

4. Gender-related effects in biology and in teratology

4.1 Gender-related teratogenic effects

Gender-related biological effects have been shown at early stages of development. Schwartz et al. [17] examined the effect of the sex hormones—estradiol (E2)

and testosterone—on the modeling of cultured fetal mouse long bones separated according to their sex. They reported specific sex-dependent response of fetal mouse long bones to E2 and testosterone, bones from female fetuses responding to E2 and from male fetuses responding to testosterone. In a subsequent study, the authors described similar gender-specific effect of testosterone on growth plate chondrocytes in culture (see **Table 1**) [18].

Exposures to substances, such as cigarettes, cocaine, and alcohol, have been implicated as causes of developmental problems, but only few studies have investigated the gender aspect of their teratogenicity.

Bahado-Singh et al. [19] reviewed data from the Center for Disease Control and Prevention, USA, for 2006, covering more than 2 million births from 19 reporting states. They found that first trimester cigarette smoking increased the risk of cleft lip and cleft palate only in males, OR 1.431 (95% CI 1.241, 1.651), while male gender also appeared to be an independent risk factor for some types of congenital anomalies [19]. A strong association between male gender and the presence of cleft lip and/or palate (OR = 3.51; 95% CI 2.83–4.37) was also found by Strange et al. [20].

Male gender as a gestational risk factor was also reported by Radin et al. [21] who investigated the effect of preconception intake of low-dose aspirin (LDA) on male live birth. They followed two groups of women with prior pregnancy loss: one group was treated with daily intake of LDA, and the second group was treated with placebo. They detected a low proportion of males at birth in the placebo group (44%) that may be related to a disordered inflammatory milieu that is harmful for

Substance	Teratogenic effect	References
Human studies		
Cigarette smoking	Increased risk for cleft lip and cleft palate in males	Bahado-Singh et al. [19], Strange et al. [20]
Cocaine	High risk for attention and inhibitory control problems, emotional modulation difficulties, health risk behaviors, and antisocial behavior in males	Bennett et al. [22, 23]
Alcohol	Fetal alcohol syndrome disorder is more prevalent in young boys	Thanh et al. [24]
	Childhood mental health problems are more prevalent in girls	Sayal et al. [25]
Endocrine disruptors	Exposure to endocrine disruptors, especially substances with estrogenic or antiandrogenic affects, such as 2-ethylhexyl phthalate and bisphenol A, might adversely affect embryonic sex organ development	Lambrot et al.
Rodent studies		
Alcohol	Impaired social recognition memory in a sexually dimorphic manner in prenatally exposed mice	Kelly et al. [26]
Procarbazine	Cleft palate and micrognathia were significantly more frequently in the male fetuses	Malek et al. [27]
VPA	Mice exposed to VPA during pregnancy demonstrated gender-dependent changes in social behavior, oxidative stress markers, and gene expression	Ornoy et al. [28, 30]
Endocrine disruptors	Exposure to endocrine disruptors, especially substances with estrogenic or antiandrogenic effects, such as 2-ethylhexyl phthalate and bisphenol A, might adversely affect embryonic sex organ development	Lambrot et al. [28], Rouiller-Fabre et al. [29]

Table 1.
Reported gender-related teratogenic effects in human and rodents.

male conception or survival. Preconception low-dose aspirin increased male live birth (first tertile: 48% male in LDA vs. 52% in placebo, intention-to-treat relative risk (ITT RR) ratio = 0.97, 95% CI: 0.70–1.35; second tertile: 57% male in LDA vs. 43% in placebo, ITT RR = 1.36, 95% CI: 0.98–1.90; third tertile: 53% male in LDA vs. 35% in placebo, ITT RR = 1.70, 95% CI: 1.13–2.57; P interaction = 0.03). Their results suggest that maternal inflammation may be hazardous to the conceptus or survival of male embryos.

Long-term gender differences between males and females exposed to illicit substances during pregnancy were also detected in neurodevelopmental studies. Bennett et al. [22] reported that males prenatally exposed to cocaine, especially if raised in high-risk environments, appeared to be at greater risk for attention and inhibitory control problems, emotional modulation difficulties, health risk behaviors, and antisocial behavior. Similarly, exposed males had mild cognitive deficit manifested by lower IQ scores and more difficulty with abstract/visual reasoning tasks than exposed females [23].

Thanh et al. [24] estimated the prevalence of fetal alcohol syndrome disorder among patients recorded at Alberta provincial health databases. They found that fetal alcohol spectrum disorder (FASD) was more prevalent in young boys than in young girls (on average 12.9 out of 1000 male births compared to 10.4 out of 1000 female births); however, there were no sex difference in the rate of FASD diagnosis when the children were diagnosed later in life.

In contrast, in a prospective, population-based study, Sayal et al. [25] investigated the relationship between maternal self-reports of the amount and frequency of alcohol use during the first trimester of pregnancy and the presence of clinically significant mental health (behavioral and emotional) problems at 4 and 6.5 years (parental report: n = 9086 and 8046, respectively) and at 7.7–9 years (teacher report: n = 5648). They reported an association between low levels of alcohol consumption in the first trimester (1 glass per week) and clinically significant childhood mental health problems, more prevalent in girls. This pattern was replicated with both parent and teacher data collected at two later time points, suggesting that the association persisted into middle childhood.

Sex-dependent neurodevelopmental effect of prenatal alcohol exposure was also described in rodent studies; Kelly et al. [26] exposed rats to ethanol during the prenatal and early postnatal periods. Ethanol exposure during development impaired social recognition memory in a sexually dimorphic manner; male rats showed a deficit in social recognition memory impaired in all variations of the test, while females had deficit only when the task was more challenging. They suggested that the deficit in ethanol-exposed females may be related to changes in oxytocin receptors in the amygdala.

Procarbazine is an alkylating antineoplastic substance used for the treatment of Hodgkin's lymphoma and brain cancers. Malek et al. [27] investigated the sex-related differences of procarbazine teratogenicity treatment in rats exposed to this substance during pregnancy. They reported that procarbazine induced clefts of the secondary palate in 90% of the fetuses. Gender-specific analysis of the results obtained from the fetuses of the procarbazine-exposed group showed that cleft palates were present in all males (100%) but only in 78.5% of female fetuses. Furthermore, micrognathia was observed significantly more frequently in the male fetuses. The authors suggested that these may be attributed to sex-related differences in the critical period for organogenesis.

Another example is the increased risk of oxidative stress-related congenital malformations in male infants of nondiabetic women compared to females [19].

Endocrine disruptors: It is well documented for years that prenatal exposure to endocrine disruptors, especially substances with estrogenic or antiandrogenic

affects, might adversely affect embryonic sex organ development [28, 29]. Indeed, there are many experimental animal studies showing the effects of these agents on the gonads and on the internal and external genitalia. Of special concern are the effects of substances with estrogenic effects on the development of the testes. For example, Lambrot et al. [28] studied the possible effects of phthalates, which are known to reduce testosterone secretion in the fetal rat, on first trimester human fetal testes in culture. They found that mono-2-ethylhexyl phthalate decreased the expression of the mRNA of anti-Mullerian hormone by the Sertoli cells and increased the apoptosis of the germ cells [28]. Later, the same group [29] reported that bisphenol A decreased the production of testosterone in the human fetal testis.

Valproic acid (VPA): Valproic acid is a highly teratogenic anticonvulsant that may also induce autistic-like behavior in human and in rodents. It is therefore used for the experimental induction of autistic-like behavior in mice and rats. Prenatal or early postnatal administration of valproic acid in mice or rats is known to induce neurobehavioral deficits. The affected animals (either offspring of the VPA-treated dams or the animals following early postnatal injection of VPA) will exhibit autistic-like behavioral changes and increased oxidative stress in their brains [30, 31]. We injected 4-day-old mice with 300 mg/kg of VPA and performed neurobehavioral studies during postnatal days 50–60. On day 60 we euthanized the animals and carried out biochemical and molecular studies on the prefrontal cortex. VPA induced changes in the redox potential and gene expression in relation to treatment and gender. VPA-induced oxidative stress was manifested by increased lipid peroxidation and activity of antioxidant enzymes and upregulation of antioxidant gene expression. There were significant differences between males and females, oxidative stress markers being more prominent in females. VPA also induced gender-dependent changes in the expression of many genes related to brain function. In addition there were behavioral changes typical of autistic-like behavior, but female mice were better than males in social behavior while they were poorer in learning [30, 32].

4.2 Sex-associated genetic disorders

Diseases associated with X chromosome, such as fragile X, Duchenne muscular dystrophy, and Rett syndrome, are more common in males than in females due to the X-linked inheritance pattern. Therefore in the last decades, the use of gender selection due to preimplantation genetic diagnosis has been significantly increased. These procedures test the polar bodies of eggs or cells from preimplantation embryos following IVF, to diagnose the sex of the embryo or the specific disorder in those affected and select for implantation those that are not, thus preventing the transmission of X-linked genetic disorders [33]. For example, in a case control study by Ye et al. [34], the authors described preimplantation gender selection of embryos of women whose first child was diagnosed with Duchenne muscular dystrophy. Sex-specific selection of female embryos after in vitro fertilization was developed for prevention of the disease in the patient's future children. However since about 10% of the women carriers for the Duchenne muscular dystrophy gene are symptomatic due to the pattern of X chromosome inactivation, a preimplantation gene analysis by PCR can nowadays allow the birth of normal offspring, both male and female. However, as this can only be done when the mutation is known, it is not feasible for some of the X-linked diseases, in which sexing is still important to prevent morbidity [35].

Nonmedical gender selection is merely performed to satisfy the parent's desire to breed a specific sex. In many countries gender selection of nonmedical purpose is prohibited for ethical reasons.

4.3 Discussion

Teratogens might be gender-specific and might cause lethality or congenital malformations that are dependent on embryonic sex. Possible gender-specific effect of teratogens is not always established because in most studies embryonic and fetal genetic sex is not determined. The paucity of data relating teratogenic effects to gender seems to result from the difficulties in the accurate anatomical assessment of sex in fetuses or newborns.

Indeed, most studies that investigated the teratogenic effects of drugs or teratogenic substances in pregnancy did not look for gender differences. Although gender is sometimes included as a covariate for the statistical analysis, generally, the biological differences between males and females are barely taken as a factor in such analyses. The ability to easily identify fetal sex will allow a better understanding of the possible gender-related effects of teratogens.

As stated above, there are many hereditary diseases that are gender-specific. The importance of sex identification in these cases was described above. These data emphasize that gender, being male or female, is an important factor that can influence both the vulnerability and the adaptive response of the fetus to prenatal teratogenic exposure or, in cases of sex-associated genetic disorders, to enable choosing the normal embryos.

We will therefore describe the existing methods of sex determination including those developed for clinical purposes and those mainly used for research purposes.

5. Methods for sex determination

5.1 Preconception sperm

Evaluation and controlling the sex of the embryo prior to conception by separation of the X and Y sperm may have an uttermost importance for prevention of X-linked diseases. Preselection of the desired sex sperm can reduce the number of animals used in research of diseases that are either gender associated or have different manifestations in each gender. Among humans, it allows the prevention of pregnancies with X-linked diseases. The different methods of sperm selection are based on the difference between the X and the Y chromosomes. The X chromosome is bigger and has increased DNA content than the Y chromosome. Additionally, the X chromosome has a negative charge, while the Y chromosome has a positive charge. The different sperms also have different antigens, and the Y chromosome swims in a straighter path. Methods for sperm separation should be safe and should not affect the chromatin integrity. The methods include flow cytometry, swim up, percoll and albumin gradient centrifugation, sephadex columns, and presence of H-Y antigen (see **Table 2**) [36].

At present, only flow cytometry was proven to effectively sort X and Y sperm. This method can use either fresh or frozen-thawed sperm. The greater amount of DNA in the X sperm allows sperm separation by this method [37]. The X chromosome has 2.8% more DNA than the Y chromosome. When a DNA-specific fluorochrome is used, the absorbed and then emitted light signal band of wavelengths varies according to the DNA content, so that the sperm can be sorted by flow cytometry instrument. Variations in the sperm head size, shape, and number of vacuoles may affect the sorting process. Only motile sperm can be used, and the multiple processing steps decrease the number of sperm available for assisted reproduction [38]. A risk of cytotoxicity by oxidative stress was shown in semen from horses [39]. When boars' semen was evaluated, the fluorescent dye (Hoechst)

	Method	Feasibility	Reference
Human studies			
Preconception sperm separation	Flow cytometry	Safe, about 85% accuracy, higher accuracy when sorting for X than from Y chromosome, can be used to prevent X-linked diseases	Bianco et al. [33], Hendriksen [35], Karabinus et al. [36]
	Sperm motility	Low accuracy, not in use for sex determination	Ericsson et al. [42]
	Gradient and centrifugation	Low accuracy, not in use for sex determination	Pearson et al. [43], Esmaeilpour et al. [44]
Barr bodies	Diagnosis of the inactivated X chromosome by light microscopy	Low accuracy, not in use for sex determination	Miller [46], Sharma et al. [47]
Chromosomal analysis	Karyotype	High accuracy, widely used in utero from amniotic fluid, chorionic villous sampling; postdelivery from blood for genetic evaluation	Gadd [48], Nadler et al. [49], Borrell et al. [50]
Ultrasonography	Ultrasonography	Safe, highly used, cost-effective and accurate	Grande et al. [51], Manzanares et al. [54]
Genetic methods	Sry and the Zfy genes	By FISH or PCR analysis	Cho [61]
	Cell-free DNA in maternal blood	Safe, can be done from 10 weeks gestation, diagnosis by PCR analysis, 98% accuracy	Koumbaris et al. [62], Ordonez et al. [63]
	Amelogenin gene	Based on deletion in intron 1 on chromosome X compared to the Y and analyzed by PCR, commercial kit, easy to use, low accuracy. Used in forensic medicine	Chowdhury et al. [68], von Wurmb-Schwark et al. [69]
	The TriXY method	Amplicons of known X and Y single nucleotide polymorphism (SNP), analyzed by PCR. Can be used in various tissues including hair shafts. High accuracy. Used in forensic medicine	Madel et al. [72]
	Y short tandem repeat (STR)	Y-STR can be used to determine sex by PCR and discriminate between paternal genealogical relationships. Can be used in various tissues, about 90% accuracy, used in forensic medicine	Pilli et al. [70], Delfin [71]
Animal studies			
Preconception sperm separation	Flow cytometry	Safe, a risk of oxidative stress was shown in horses' semen in vitro, about 90% accuracy, used in agriculture with normal offspring	Balao da Silva et al. [37], Spinaci [38] et al., Moore et al. [39], Gonzalez-Marin et al. [40], Tubman et al. [41]
	Immunological sperm sexing	Low accuracy, not in use for sex determination	Yadav et al. [34]
Barr bodies	Diagnosis of the inactivated X chromosome by light microscopy	Low accuracy, not in use for sex determination	Lyon et al. [45]

	Method	Feasibility	Reference
Physical examination	Anogenital distance	Inaccurate, cannot be used during pregnancy, valid in fetuses near term and offspring	Greenham [15], Griffith et al. [52]
	External genitalia score	High accuracy, cannot be used during pregnancy, valid in fetuses near term and offspring	Murdaugh [53]
Ultrasonography	Ultrasonography	Safe, no side effects, successfully used in large animals. Accuracy decreases with litter size	Aurich et al. [55], Quintela [56], Gil [57]
Genetic methods	Multiplex PCR	By two-step PCR analysis, the Sry gene sequence and internal control sequences, high accuracy	Yano [58], Prantner et al. [59], Kunieda et al. [60]
	Simplex PCR	Amplification of homologous genes on the X and Y chromosome that have an intron of different lengths, high accuracy	McFarlane et al. [1], Fontanesi et al. [64], Chuma et al. [65], Clapcote et al. [66], Tunster et al. [67]
	Cell-free DNA in maternal blood	Safe, PCR analysis among horses: 85% accuracy	Aurich et al. [55]

Table 2.
Studies assessing gender in human and animals.

decreased the rate of live spermatozoa; however, the sorting process did not affect the number of live spermatozoa or formed blastocysts [40].

This method is used in order to affect fetal sex among humans [38]. In a large cohort study of 4993 couples, it reached about 87% accuracy when sorting for X sperm and 74% when sorting for Y sperm. Sperm was used in various assisted reproduction methods including intrauterine insemination (IUI), in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), and frozen embryo transfer. Following sperm sorting, the pregnancy rate for female sex was 93.5%, while for male sex the pregnancy rate was 85.3%.

Sperm sorting by flow cytometry technology is commercially used in animals. It was first used in rabbits and now mostly in bovines. Commercially bovine semen is available, and in 2017 this technique was used in 15 countries [41]. The analysis of sorted bovine sperm by the flow cytometry method (SexedULTRA™) by evaluating motility, DNA fragmentation rate, and plasma membrane and acrosome (a body containing enzymes on the sperm head derived from the Golgi apparatus) integrity showed that the semen quality was not affected by the sorting process [42]. The calves produced following flow cytometry selection did not vary from controls in prenatal and postnatal death rate and in anthropometric parameters. None of the offsprings had gross anatomical abnormalities [43]. When used for assisted reproduction in humans, the rate of major congenital anomalies was not statistically indistinguishable from the general population [38].

The progressive sperm motility of the Y sperm was used by Ericsson et al. [44] to divide human sperm according to sex. This method selected a population of spermatozoa that were mainly (85%) but not totally Y sperm. Since the slower fraction contained, beside X sperm, also non-motile and abnormal sperm, it could not be used for X sperm selection.

Immunological sperm sexing method was offered as one of the choices to separate X- and Y-chromosome-bearing sperm. It is based on the development of antibodies to antigens and proteins that are differently expressed between genders. The anti-H-Y antibody was not suitable since it did not preferentially adhere to the

Y sperm. Other proteins differentially expressed between sexes were also found inappropriate due to the low levels of membrane proteins of the sperm [36].

The X and Y sperm can also be separated by the use of albumin gradient [45], the PureSperm centrifugation method, and a combination of both [46]. However none of the methods is valid due to their inaccuracy.

5.2 The Barr body

Based on the Lyon hypothesis, all but one X chromosome is randomly inactivated early in embryogenesis, after implantation. The end result is that, when evaluated, female cells have one Barr body, while the male cell has none. In 1948 Barr et al. proved, first in cats and then in humans, that female and not male cells consist of deeply stained body in the nucleus. Since the correct number of human chromosomes was only found in 1956, this method allowed indirect evidence on the human sex, especially in complicated cases like neonates with ambiguous genitalia. The origin of the Barr body was established by Lyon [47], who claimed that the Barr body is a heteropyknotic material (pyknosis-irreversible condensation of chromatin in the nucleus) originating from the X chromosome that is randomly inactivated and can be from either maternal or paternal origin in the same species.

This method was used to differentiate between female and male embryos and fetuses at the stage when anatomical discrimination is not feasible. Although it also allowed the use of discarded tissues which is especially important in embryos who have limited amount of tissue material, it cannot be trusted. Barr bodies were detected in the amnion and liver cells of rat embryos and fetuses during days 12.5–20.5, in a relatively small proportion of subjects and in both sexes. They were detected in 20–50% in the amnion and 10–51% in the liver of females. Moreover, they were also detected in a very small proportion of males: 0–7% in the amnion and 0–8% in the liver [16].

The Barr body had a tremendous importance when it was discovered, when there were no other ways to assess the sex of animals and humans. This test may be misleading in cases of abnormalities of sex chromosomes like in XO women (Turner) or XXY males (Klinefelter) [48]. This method is no more feasible in clinical research due to its limitations and the discovery of much more accurate methods like chromosomal analysis which is generally one of the simplest and very accurate ways for gender detection.

Lately Barr body became a marker in some malignancies since it was found that Barr body disappearance happens in some malignant cells [49]. The X chromosome has tumor suppressor genes, and the disappearance of the Barr body results in misregulation of the centromere-associated heterochromatin and epigenetic instability.

5.3 Chromosomal analysis

It was developed only in 1956 and became of clinical use slightly later. Chromosomal analysis by light microscopy is feasible during the metaphase of cell division. This discovery allowed the diagnosis of the origin of many of the known syndromes, and when intrauterine chromosomal analysis of fetal origin cells by amniocentesis was established in 1965, prenatal genetic evaluation of the developing fetus was allowed [50, 51]. The understanding of the mammalian genome and the development of more accurate, easily used, and cheap methods for genetic evaluation improved the understanding of diseases. Microarray genetic methods are now commonly used for prenatal evaluation of fetuses [52, 53].

5.4 Physical (anatomical) examination

As described earlier, until the appearance of sexual characteristics, male and female embryos are morphologically indistinguishable [1]. Different methods have been developed, especially in rodents, for accurate sex determination after the appearance of gender-related sexual characteristics.

Farris et al. showed in 1942 that in the newborn rat, sex can be distinguished based on the larger genital papilla of the male and its longer distance from the anus. The female rat did not have nipples up to postnatal days (PND) 8–15. The average anogenital distance (AGD) at PND 1 was 2.8 mm in the male and 1.2 mm in the female [54]. This method, which was found feasible in pups, was also feasible right after delivery. Greenham et al. [15] evaluated the method in albino mice pups on the first 3 days of life. Sex was verified at 3 weeks by visual examination. They found 14.5% sexing error in the females and 1.8% sexing errors in the males. When pups with non-determined AGD (1.6–2.1 mm) were excluded, the mistake rate dropped to 2.1% in females and 6.3% in males. They concluded that accurate sex discrimination cannot be reached at this age group by this method.

Lately Murdaugh et al. [55] offered a method of prenatal sex discrimination in mouse fetuses on 16.5–18 gestational day (GD) based on morphological features of the external genitalia. They based their method on the development of three areas from caudal to rostral: the scrotal/labial swelling, the preputial swelling, and the distal glans. By evaluating the urethral plate which is located between the glans and the base of the tubercle, they found two major criteria: the urethral seam in males or meatus in females (which will later be the vaginal opening) and the shape of the meatus. They found, following verification by PCR of Sry from the tail tissue, that sexing was successful when experienced raters evaluated fixed and unfixed fetuses and also from photographs. The seam vs. meatus at GD 17 was the most accurate method with 96% accuracy. Evaluation of the prepuce attachment to the genital folds (92.5%) and the shape of the area of the ventral midline where the prepuce swellings meet (62%) increased the accuracy to 99.5%. Raters with no experience performed best when evaluating the genital shape (93% accuracy). Full evaluation increased their accuracy rate to 95%. However, morphological sex discrimination does not give 100% accuracy. Although this method can be used while performing an experiment, it is not practical for tissues that were kept for further investigations.

Ultrasonography: Prediction of the fetal sex by ultrasonography is based on the assessment of the external genitalia. Among humans it is safe and cost-effective and was shown lately to be accurate even in the first trimester [56]. It is successfully used in animals including horses [57], cows [58], and other large animals. In multiple pregnancies the method is less accurate. Gil et al. found in canine pregnancy that the accuracy was 100% when there were up to two fetuses but decreased with the litter size [59]. This method is not in use in small animals where there are several fetuses in each litter.

5.5 Genetic methods

Genetic sex determination methods are not related to subjective physical examination, are accurate, require small samples, and do not necessitate the evaluation of a specific tissue, and any organ can be used. Their applicability depends on the specific methods. Successful assays are simple, need small amount of tissue, and are accurate during the entire pregnancy. Measuring the activity of X chromosome-linked enzymes [60] or RNA-based PCRs is complicated by the presence of some gene products only at certain developmental stages [61]. However, this problem is not present when the test is based on DNA (see **Table 2**).

5.6 The Sry and the Zfy genes

The Sry and the Zfy genes are located on the mammalian Y chromosome and were detected by simple PCR analysis in mammalian tissues including preimplantation embryos [62]. Their FISH analysis in gonadal tissue of male and female hermaphrodite patients was in agreement with chromosomal analysis [63]. Since it is characteristic to male gender, it can be used for sex determination.

Lately, molecular analysis of free fetal DNA extracted from maternal plasma became a safe noninvasive approach to fetal sex determination. Fetal cell-free DNA can be found in maternal blood at about 10 week's gestation [64]. The Sry sequence can be detected in the maternal plasma by real-time PCR [65].

However, determining the Y chromosome genes Sry and Zfy may be misleading since female genetic sex is concluded based on the absence of a PCR amplicon. To overcome this, some studies evaluated both genes and/or evaluated autosomal genes as internal control.

Multiplex PCR: Multiplex PCR simultaneously amplifies a Y chromosome gene (e.g., Sry) in combination with an endogenous control gene to confirm that the inability to amplify the Y chromosome gene is a true negative for that gene.

Simplex PCR: Simplex PCR assays for the determination of the genetic sex in mice amplify homologous genes on the X and Y chromosome that have an intron of different lengths. Determination of two primers is complicated; simple PCR using orthologous genes on sex chromosomes which requires only one set of primer is therefore preferential.

To determine the sex of European rabbits and hares, Fontanesi et al. [66] used the simplex PCR. By co-amplification of the orthologous sexual chromosome genes zinc finger protein (ZFX) and Zfy genes, they used the same pair of PCR primers. The method was based on the analysis of a point mutation that differentiates the size of the ZFX and the Zfy genes. They used the hair, muscle, and ear tissue [66].

Chuma and Nakatsuji [67] used the Uba1 and Ube1y1 genes on the X and Y chromosomes, respectively. Primers were designed to cover deleted regions within the Ube1y1 gene, resulting in two amplification products in males, a small and large amplicon, but only the larger product in females [67].

Clapcote and Roder [68] used as an alternative method a single set of primers to amplify the X chromosomal gene Kdm5c (synonyms: Jarid1d, Smcy) and the Y chromosomal gene Kdm5d (synonyms: Jarid1d, Smcy), resulting in two amplicons in the male and one in the female [68] . However, in both cases, the size differences of the amplicons were relatively small, 19 bp for Uba1/Ube1y1 and 29 bp for Kdm5c/Kdm5d resulting in difficulties in accurately determining the sex while assessing the results by gel electrophoresis.

Tunster [69] offered to amplify the two-copy Y-linked Rbm31y and the single-copy X-linked Rbm31x. Their sequence alignment identified a high degree of sequence homology and revealed an 84 bp deletion in Rbm31x compared with Rbm31y. The analysis revealed a 269 and 353 bp products in male samples and only the 269 bp product in female samples [69]. However, since the accurate analysis depends on the difference between one and two products and in the common product there is no size difference, it may sometimes be hard to interpret.

To overcome the small size difference, qPCR with melting point analysis was used by Prantner et al. [61] to determine the sex of mice blastocysts demonstrating that this method, although technically complicated, is accurate in all the stages of the pregnancy since DNA, which does not change during the pregnancy, was evaluated. They used the primer pair previously used by Chuma and Nakatsuji [67] that amplified the portion of the X chromosome gene Kdm5c (synonyms: Jarid1c, Smcx)

and the corresponding Y chromosome gene Kdm5d (synonyms: Jarid1d, Smcy). The different sizes of the fragments (X 331 bp, Y 302 bp) resulted in distinguishable melting curves of the qPCR product. Following temperature increment of the PCR product, the dsDNA was denaturized, resulting in two melting points in the male and one in the female [61].

A single PCR probe of the pseudoautosomal genes Xlr and Sly was offered by McFarlane et al. [1] for sex determination with the advantage of a larger difference of 405 bases between genders. This method uses lysate, does not need DNA purification [1], and can be carried out in any laboratory that is equipped for basic molecular studies. The accuracy of the method was proven in tail tissue among different adult mouse strains.

By using the method published by McFarlane et al., we evaluated liver tissue that was collected from newborns of the outbred ICR CD1 mice. The method was verified by evaluating liver tissue from 60-day-old male and female mice with known sex. To further verify the offspring male genetic sex, we also determined the Zfy gene by PCR analysis.

The genetic sex was accurately determined in all the adult mice by Sly/Xlr genes and in 91% by Zfy gene (**Figure 1**). In the genomic DNA samples from the newborn mice, the sex was identified easily by both Sly/Xlr and Zfy in most samples. However, Sly/Xlr (97.5%) appeared slightly superior to the Zfy (94.9%) gene. In about 7% of the samples, we could not assess the sex from Sly/Xlr or Zfy after the first run and had to repeat the analysis. This allowed accurate determination of the genetic sex from both genes in all samples, except one where the DNA was inappropriate for study. Hence, the method described by McFarlane et al. [1] for sexing mice by PCR using a single primer pair for both sexes (Sly/Xlr) seems simple and accurate, as the differentiation between genders is determined by a size difference between the amplicons. If the data is not clear in the first PCR, then a second PCR will enable accuracy of almost 100% of the cases. Hence, there seems to be no need to carry out concomitant studies on the Zfy gene (see **Figures 1** and **2**). It should be noted that other investigators have also shown that this method can also be applied to younger fetuses and embryos and to any tissue.

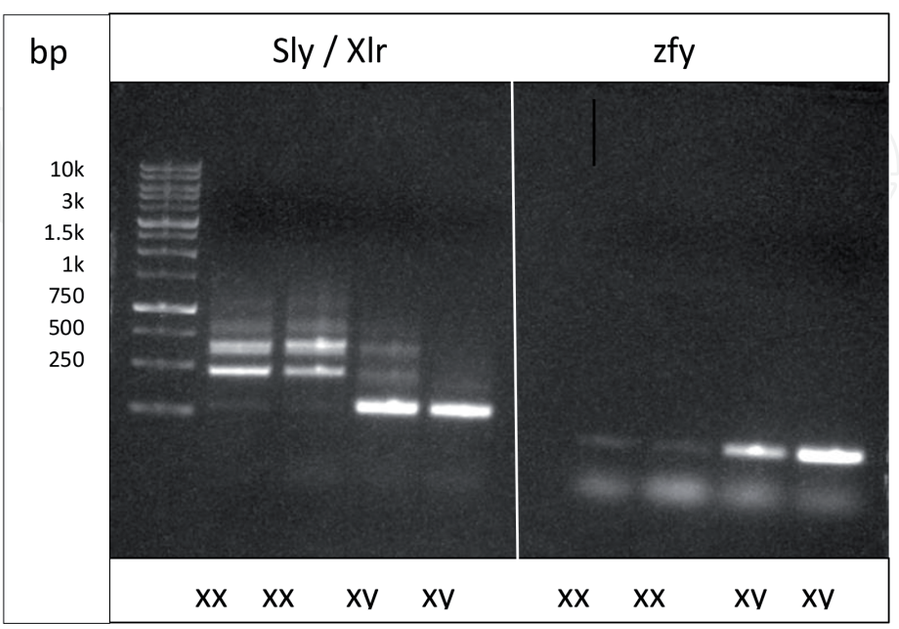

Figure 1.
Mice sex determination using Sly/Xlr and Zfy genes. PCR sex determination results. Sly/Xlr: 280 bp product in males, 685 bp and approximately 480 and 660 bp products in females. Zfy: clear product in males, almost no visible DNA product in females.

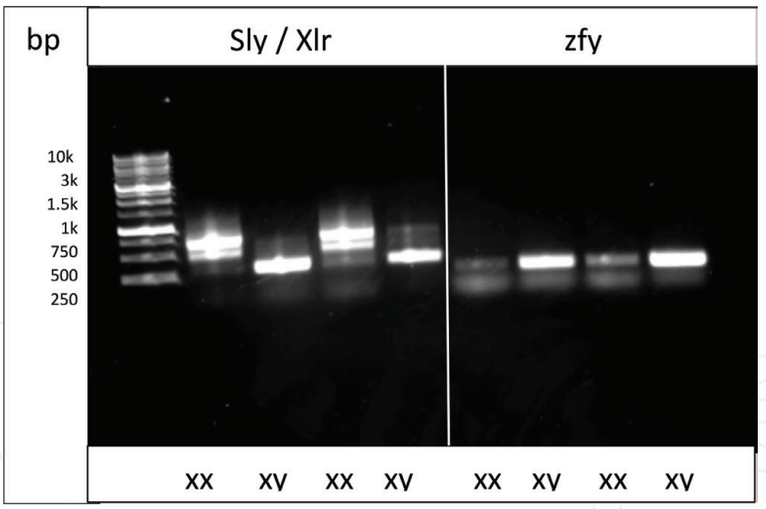

Figure 2.
Determination of the sex of newborn mice using Sly/Xlr and Zfy genes PCR sex determination results. Sly/Xlr: 280 bp product in males, 685 bp and approximately 480 and 660 bp products in females. Zfy: clear product in males, almost no visible DNA product in females.

5.7 Sex analysis in forensic medicine

The amelogenin gene which is found on both X and Y chromosomes is in common use for sex discrimination in forensic medicine. A 6 bp deletion in intron 1 on chromosome X compared to the Y chromosome can be detected by using a pair of PCR primers. It can be used in various tissues including long-lasting remnant tissues like dental pulp [70]. However, mutations and deletions in the amelogenin Y were reported to result in amplification failure. Additionally low DNA quality and quantity necessitated alternative molecular genetic assays [71].

DNA can be recovered from highly degraded tissues as happens in archeology or forensic medicine. The petrous bone was found suitable for short tandem repeat (STR) typing via electrophoresis. This method compares DNA loci from multiple samples. The probes that attach to special areas on the DNA measure the number of repeats of a special unit whose length can be detected by PCR analysis. The difference in autosomal repeated units can be used to discriminate between related and unrelated people, while Y-STR can be used to determine sex, and discriminate between paternal genealogical relationships [72]. Y-STR was used to differentiate between the assailant and victim in males and for proving male sexual harassment in females. Even small samples of vaginal and rectal swabs up to 72 hours post the insult were suitable for evaluation. This method can also prove multiple assailants and be used for matching with a reference sample [73].

The TriXY-Homogeneous genetic sexing [74] is another method that can use ancient DNA specimens from archeological excavations and hair shafts. This method uses three amplicons of known X and Y single nucleotide polymorphism (SNP) markers: one on the X chromosome and two on the Y chromosome detected by PCR. The different melting temperatures of the PCR products were used to discriminate between sexes.

5.8 Discussion

An ideal method for sex identification would be accurate, simple, and cheap, enabling its use in most laboratories. In addition, it should also be able as much as possible to be used for all animals as well as tissues and/or cells. We have described all available methods currently used for the identification of sex. It seems that the

most reliable and accurate methods are the determination of chromosomes and molecular determination of genes related to the sex chromosomes and/or gender.

For chromosomal analysis, we need viable cells that are able to divide, and if this is not possible, these methods cannot be used. On the other hand, genetic methods are reliable and do not need living cells, and it is easy to obtain DNA for these studies even in very ancient and nonviable tissues. These methods are therefore the most accepted ones.

As stated above, there are many methods for the genetic sex determination of tissues, generally using genes that are on the X or Y chromosome. Each of these methods has its advantages and disadvantages. Of all methods, the method described by McFarlane et al. [1] using the Sly/Xlr genes seems to be the simplest and most accurate one. As reported above, we used this method for the successful identification of sex in newborn mice and found it superior to the method using the detection of the male gene Zfy. Hence, this method can be used on embryonic and fetal tissues as well as isolated DNA obtained from any tissue.

6. Conclusions

Reliable and easy to perform sex determination methods are important for many medical and biological reasons, especially in situations where the physical examination is unable to be accurate. Hence, many methods have been developed to serve the purpose of accurate sex determination. In this chapter we described the main needs for the accurate sex determination and the methods that can be used. We should note that today there are many biological processes that are gender-dependent, but many of these gender-specific processes are still unknown, especially in teratology. A better understanding of these gender-related effects will enable us to find more appropriate ways for treatment and even for prevention.

Acknowledgements

This review was funded by the Irving Harris Foundation, Chicago, USA.

Conflict of interest

No potential conflict of interest is reported by the authors.

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References

[1] McFarlane L, Truong V, Palmer JS, Wilhelm D. Novel PCR assay for determining the genetic sex of mice. Sexual Development. 2013;**7**(4):207-211

[2] Biason-Lauber A. Human sex development: From basic science to clinical practice and Back. Pediatric Endocrinology Reviews. 2017;**15**(1):8-20

[3] Gondos B. Development of the reproductive organs. Annals of Clinical and Laboratory Science. 1985;**15**(5):363-373

[4] Murashima A, Kishigami S, Thomson A, Yamada G. Androgens and mammalian male reproductive tract development. Biochimica et Biophysica Acta. 2015;**1849**(2):163-170

[5] Eid W, Biason-Lauber A. Why boys will be boys and girls will be girls: Human sex development and its defects. Birth Defects Research Part C, Embryo Today. 2016;**108**(4):365-379

[6] Rey RA, Grinspon RP. Normal male sexual differentiation and aetiology of disorders of sex development. Best Practice & Research Clinical Endocrinology & Metabolism. 2011;**25**(2):221-238

[7] Josso N, Rey R, Picard JY. Testicular anti-Mullerian hormone: Clinical applications in DSD. Seminars in Reproductive Medicine. 2012;**30**(5):364-373

[8] Mollgard K, Jespersen A, Lutterodt MC, Yding Andersen C, Hoyer PE, Byskov AG. Human primordial germ cells migrate along nerve fibers and Schwann cells from the dorsal hind gut mesentery to the gonadal ridge. Molecular Human Reproduction. 2010;**16**(9):621-631

[9] Mamsen LS, Brochner CB, Byskov AG, Mollgard K. The migration and loss of human primordial germ stem cells from the hind gut epithelium towards the gonadal ridge. The International Journal of Developmental Biology. 2012;**56**(10-12):771-778

[10] Biason-Lauber A. Control of sex development. Best Practice & Research Clinical Endocrinology & Metabolism. 2010;**24**(2):163-186

[11] Blaschko SD, Cunha GR, Baskin LS. Molecular mechanisms of external genitalia development. Differentiation. 2012;**84**(3):261-268

[12] Yamada G, Suzuki K, Haraguchi R, Miyagawa S, Satoh Y, Kamimura M, et al. Molecular genetic cascades for external genitalia formation: An emerging organogenesis program. Developmental Dynamics. 2006;**235**(7):1738-1752

[13] Wilson JD, Rivarola MA, Mendonca BB, Warne GL, Josso N, Drop SL, et al. Advice on the management of ambiguous genitalia to a young endocrinologist from experienced clinicians. Seminars in Reproductive Medicine. 2012;**30**(5):339-350

[14] Hrovatin K, Kunej T. Genetic sex determination assays in 53 mammalian species: Literature analysis and guidelines for reporting standardization. Ecology and Evolution. 2018;**8**(2):1009-1018

[15] Greenham LW, Greenham V. Sexing mouse pups. Laboratory Animals. 1977;**11**(3):181-184

[16] Kajii T, Farias E, Gardner LI. Sex chromatin in embryonic rat liver. Experimental Cell Research. 1968;**50**(2):435-440

[17] Schwartz Z, Soskolne WA, Neubauer T, Goldstein M, Adi S, Ornoy A. Direct and sex-specific

enhancement of bone formation and calcification by sex steroids in fetal mice long bone in vitro (biochemical and morphometric study). Endocrinology. 1991;**129**(3):1167-1174

[18] Schwartz Z, Nasatzky E, Ornoy A, Brooks BP, Soskolne WA, Boyan BD. Gender-specific, maturation-dependent effects of testosterone on chondrocytes in culture. Endocrinology. 1994;**134**(4):1640-1647

[19] Bahado-Singh RO, Schenone M, Cordoba M, Shieh WS, Maulik D, Kruger M, et al. Male gender significantly increases risk of oxidative stress related congenital anomalies in the non-diabetic population. The Journal of Maternal-Fetal & Neonatal Medicine. 2011;**24**(5):687-691

[20] Martelli DR, Coletta RD, Oliveira EA, Swerts MS, Rodrigues LA, Oliveira MC, et al. Association between maternal smoking, gender, and cleft lip and palate. Brazilian Journal of Otorhinolaryngology. 2015;**81**(5):514-519

[21] Radin RG, Mumford SL, Silver RM, Lesher LL, Galai N, Faraggi D, et al. Sex ratio following preconception low-dose aspirin in women with prior pregnancy loss. The Journal of Clinical Investigation. 2015;**125**(9):3619-3626

[22] Bennett DS, Bendersky M, Lewis M. Children's intellectual and emotional-behavioral adjustment at 4 years as a function of cocaine exposure, maternal characteristics, and environmental risk. Developmental Psychology. 2002;**38**(5):648-658

[23] Bennett DS, Bendersky M, Lewis M. Children's cognitive ability from 4 to 9 years old as a function of prenatal cocaine exposure, environmental risk, and maternal verbal intelligence. Developmental Psychology. 2008;**44**(4):919-928

[24] Thanh NX, Jonsson E, Salmon A, Sebastianski M. Incidence and prevalence of fetal alcohol spectrum disorder by sex and age group in Alberta, Canada. Journal of Population Therapeutics and Clinical Pharmacology. 2014;**21**(3):e395-e404

[25] Sayal K, Heron J, Golding J, Emond A. Prenatal alcohol exposure and gender differences in childhood mental health problems: A longitudinal population-based study. Pediatrics. 2007;**119**(2):e426-e434

[26] Kelly SJ, Leggett DC, Cronise K. Sexually dimorphic effects of alcohol exposure during development on the processing of social cues. Alcohol and Alcoholism. 2009;**44**(6):555-560

[27] Malek FA, Moritz KU, Fanghanel J, Bienengraber V. Sex-related differences in procarbazine-induced cleft palate and microgenia and the anti-teratogenic effect of prenatal folic acid supplementation in rats. Annals of Anatomy. 2003;**185**(5):465-470

[28] Lambrot R, Muczynski V, Lecureuil C, Angenard G, Coffigny H, Pairault C, et al. Phthalates impair germ cell development in the human fetal testis in vitro without change in testosterone production. Environmental Health Perspectives. 2009;**117**(1):32-37

[29] Rouiller-Fabre V, Habert R, Livera G. Effects of endocrine disruptors on the human fetal testis. Annales d'endocrinologie. 2014;**75**(2):54-57

[30] Ornoy A, Weinstein-Fudim L, Tfilin M, Ergaz Z, Yanai J, Szyf M, et al. S-Adenosyl methionine prevents ASD like behaviors triggered by early postnatal valproic acid exposure in very young mice. Neurotoxicology and Teratology. 2019;**71**:64-74

[31] Rodier PM, Ingram JL, Tisdale B, Nelson S, Romano J. Embryological origin for autism: Developmental

anomalies of the cranial nerve motor nuclei. The Journal of Comparative Neurology. 1996;**370**(2):247-261

[32] Ornoy A, Weistein-Fudim L, Ergaz Z, Yanai J, Turgeman G. Gender differences in the expression of nervous system genes in a mouse model of valproic acid-induced autism-like behavior is corrected by S-adenosyl methionine. Birth Defects Research. 2018;**11**:791

[33] Ravitsky V, Nguyen MT, Birko S, Kleiderman E, Laberge AM, Knoppers BM. Pre-implantation genetic diagnosis: The road forward in Canada. Journal of Obstetrics and Gynaecology Canada. 2019;**41**(1):68-71

[34] Ye Y, Yu P, Yong J, Zhang T, Wei X, Qi M, et al. Preimplantational genetic diagnosis and mutation detection in a family with duplication mutation of DMD gene. Gynecologic and Obstetric Investigation. 2014;**78**(4):272-278

[35] Bianco B, Christofolini DM, Conceicao GS, Barbosa CP. Preimplantation genetic diagnosis associated to Duchenne muscular dystrophy. Einstein. 2017;**15**(4):489-491

[36] Yadav SK, Gangwar DK, Singh J, Tikadar CK, Khanna VV, Saini S, et al. An immunological approach of sperm sexing and different methods for identification of X- and Y-chromosome bearing sperm. Veterinary World. 2017;**10**(5):498-504

[37] Hendriksen PJ. Do X and Y spermatozoa differ in proteins? Theriogenology. 1999;**52**(8):1295-1307

[38] Karabinus DS, Marazzo DP, Stern HJ, Potter DA, Opanga CI, Cole ML, et al. The effectiveness of flow cytometric sorting of human sperm (MicroSort(R)) for influencing a child's sex. Reproductive Biology and Endocrinology. 2014;**12**:106

[39] Balao da Silva CM, Ortega-Ferrusola C, Morrell JM, Rodriguez Martinez H, Pena FJ. Flow cytometric chromosomal sex sorting of stallion spermatozoa induces oxidative stress on mitochondria and genomic DNA. Reproduction in Domestic Animals. 2016;**51**(1):18-25

[40] Spinaci M, De Ambrogi M, Volpe S, Penazzi P, Galeati G, Seren E. Assessment of some morphofunctional characteristics of flow-cytometrically sorted and stained boar spermatozoa. Veterinary Research Communications. 2005;**29**(Suppl 2): 207-209

[41] Moore SG, Hasler JF. A 100-year review: Reproductive technologies in dairy science. Journal of Dairy Science. 2017;**100**(12):10314-10331

[42] Gonzalez-Marin C, Gongora CE, Gilligan TB, Evans KM, Moreno JF, Vishwanath R. In vitro sperm quality and DNA integrity of SexedULTRA™ sex-sorted sperm compared to non-sorted bovine sperm. Theriogenology. 2018;**114**:40-45

[43] Tubman LM, Brink Z, Suh TK, Seidel GE Jr. Characteristics of calves produced with sperm sexed by flow cytometry/cell sorting. Journal of Animal Science. 2004;**82**(4):1029-1036

[44] Ericsson RJ, Langevin CN, Nishino M. Isolation of fractions rich in human Y sperm. Nature. 1973;**246**(5433):421-424

[45] Pearson PL, Bobrow M, Vosa CG. Technique for identifying Y chromosomes in human interphase nuclei. Nature. 1970;**226**(5240):78-80

[46] Esmaeilpour T, Elyasi L, Bahmanpour S, Ghannadi A, Monabbati A, Dehghani F, et al. Effect of combined density gradient centrifugation on X- and Y- sperm separation and chromatin integrity. Iranian Journal of Reproductive Medicine. 2012;**10**(5):435-440

[47] Lyon MF. Gene action in the X-chromosome of the mouse (*Mus musculus* L.). Nature. 1961;**190**:372-373

[48] Miller FA. Your true and proper gender: The Barr body as a good enough science of sex. Studies in History and Philosophy of Biological and Biomedical Sciences. 2006;**37**(3):459-483

[49] Sharma D, Koshy G, Gupta S, Sharma B, Grover S. Deciphering the role of the Barr body in malignancy: An insight into head and neck cancer. Sultan Qaboos University Medical Journal. 2017;**17**(4):e389-ee97

[50] Gadd RL. The volume of the liquor amnii. Proceedings of the Royal Society of Medicine. 1966;**59**(11 Part 1):1131-1133

[51] Nadler HL, Gerbie AB. Role of amniocentesis in the intrauterine detection of genetic disorders. The New England Journal of Medicine. 1970;**282**(11):596-599

[52] Borrell A, Grande M, Pauta M, Rodriguez-Revenga L, Figueras F. Chromosomal microarray analysis in fetuses with growth restriction and normal karyotype: A systematic review and meta-analysis. Fetal Diagnosis and Therapy. 2018;**44**(1):1-9

[53] Grande M, Jansen FA, Blumenfeld YJ, Fisher A, Odibo AO, Haak MC, et al. Genomic microarray in fetuses with increased nuchal translucency and normal karyotype: A systematic review and meta-analysis. Ultrasound in Obstetrics & Gynecology. 2015;**46**(6):650-658

[54] Griffith JQ, Farris EJ. The Rat in Laboratory Investigation. Philadelphia: JB Lippincott Company; 1942

[55] Murdaugh LB, Mendoza-Romero HN, Fish EW, Parnell SE. A novel method for determining sex in late term gestational mice based on the external genitalia. PLoS One. 2018;**13**(4):e0194767

[56] Manzanares S, Benitez A, Naveiro-Fuentes M, Lopez-Criado MS, Sanchez-Gila M. Accuracy of fetal sex determination on ultrasound examination in the first trimester of pregnancy. Journal of Clinical Ultrasound. 2016;**44**(5):272-277

[57] Aurich C, Schneider J. Sex determination in horses-current status and future perspectives. Animal Reproduction Science. 2014;**146**(1-2):34-41

[58] Quintela LA, Becerra JJ, Perez-Marin CC, Barrio M, Cainzos J, Prieto A, et al. Fetal gender determination by first-trimester ultrasound in dairy cows under routine herd management in Northwest Spain. Animal Reproduction Science. 2011;**125**(1-4):13-19

[59] Gil EM, Garcia DA, Giannico AT, Froes TR. Use of B-mode ultrasonography for fetal sex determination in dogs. Theriogenology. 2015;**84**(6):875-879

[60] Yano T. Sexing of in vitro-fertilized preimplantation mouse embryos by the PCR method. The Japanese Journal of Human Genetics. 1993;**38**(3):277-288

[61] Prantner AM, Ord T, Medvedev S, Gerton GL. High-throughput sexing of mouse blastocysts by real-time PCR using dissociation curves. Molecular Reproduction and Development. 2016;**83**(1):6-7

[62] Kunieda T, Xian M, Kobayashi E, Imamichi T, Moriwaki K, Toyoda Y. Sexing of mouse preimplantation embryos by detection of Y chromosome-specific sequences using polymerase chain reaction. Biology of Reproduction. 1992;**46**(4):692-697

[63] Cho NH, Han SW, Ha MJ. Sry gene detection in gonads of intersex patients

using FISH. Journal of Korean Medical Science. 1997;**12**(3):204-209

[64] Koumbaris G, Kypri E, Tsangaras K, Achilleos A, Mina P, Neofytou M, et al. Cell-free DNA analysis of targeted genomic regions in maternal plasma for non-invasive prenatal testing of trisomy 21, trisomy 18, trisomy 13, and fetal sex. Clinical Chemistry. 2016;**62**(6):848-855

[65] Ordonez E, Rueda L, Canadas MP, Fuster C, Cirigliano V. Evaluation of sample stability and automated DNA extraction for fetal sex determination using cell-free fetal DNA in maternal plasma. BioMed Research International. 2013;**2013**:195363

[66] Fontanesi L, Tazzoli M, Pecchioli E, Hauffe HC, Robinson TJ, Russo V. Sexing European rabbits (*Oryctolagus cuniculus*), European brown hares (*Lepus europaeus*) and mountain hares (*Lepus timidus*) with ZFX and ZFY loci. Molecular Ecology Resources. 2008;**8**(6):1294-1296

[67] Chuma S, Nakatsuji N. Autonomous transition into meiosis of mouse fetal germ cells in vitro and its inhibition by gp130-mediated signaling. Developmental Biology. 2001;**229**(2):468-479

[68] Clapcote SJ, Roder JC. Simplex PCR assay for sex determination in mice. BioTechniques. 2005;**33**(5). 702, 704, 706 p

[69] Tunster SJ. Genetic sex determination of mice by simplex PCR. Biology of Sex Differences. 2017;**8**(1):31

[70] Chowdhury RM, Singhvi A, Bagul N, Bhatia S, Singh G, Goswami S. Sex determination by amplification of amelogenin gene from dental pulp tissue by polymerase chain reaction. Indian Journal of Dental Research. 2018;**29**(4):470-476

[71] von Wurmb-Schwark N, Bosinski H, Ritz-Timme S. What do the X and Y chromosomes tell us about sex and gender in forensic case analysis? Journal of Forensic and Legal Medicine. 2007;**14**(1):27-30

[72] Pilli E, Vai S, Caruso MG, D'Errico G, Berti A, Caramelli D. Neither femur nor tooth: Petrous bone for identifying archaeological bone samples via forensic approach. Forensic Science International. 2018;**283**:144-149

[73] Delfin FC, Madrid BJ, Tan MP, De Ungria MC. Y-STR analysis for detection and objective confirmation of child sexual abuse. International Journal of Legal Medicine. 2005;**119**(3):158-163

[74] Madel MB, Niederstatter H, Parson W. TriXY-homogeneous genetic sexing of highly degraded forensic samples including hair shafts. Forensic Science International Genetics. 2016;**25**:166-174