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Monozygotic twinning in infertility clinic patients

Olomouc, 2017 MUDr. Aleš Sobek

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DECLARATION

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TABLE OF CONTENTS

SOUHRN

U žen léčených pro neplodnost je 2-3 x vyšší výskyt monozygotních (MZ) dvojčat v porovnání s běžnou populací. Bylo navrženo několik možných hypotéz, ale žádná nedokázala tento jev přesvědčivě vysvětlit. Vliv dědičnosti nebyl dosud příliš studován, a pokud ano, byl považován za nedůležitý. Hlavním cílem práce bylo vyhodnotit souvislost mezi zvýšeným výskytem monozygotních dvojčat u pacientek léčených pro neplodnost a použitými technikami asistované reprodukce. Druhým cílem bylo zjistit, zda není výskyt monozygotních dvojčat u těchto pacientek ovlivněn dědičností. Dalším cílem bylo vyhodnotit optimální management u trojčetných těhotenství s monochoriální komponentou vzniklých po léčbě v rámci metod asistované reprodukce.

Prvním specifickým cílem současné studie bylo vyhodnotit riziko vzniku monozygotních dvojčat po in vitro fertilizaci (IVF) vzhledem k věku, ovariální funkci a použité technice asistované reprodukce u žen léčených pro neplodnost. Vyhodnotili jsme data od 1876 pacientek léčených v centru pro léčbu neplodnosti Fertimed v období 12 let. U každé pacientky jsme zaznamenali použitou mikromanipulační techniku, délku kultivace a typ kultivačního media. Skupinu žen jsme rozdělili na ženy s monozygotním dvojčetným těhotenstvím (skupina A), dizygotním dvojčetným těhotenstvím (skupina B), jednočetným těhotenstvím (skupina C) a potraty (skupina D). Výsledky ukázaly, že souvislost mezi použitou mikromanipulační technikou, délkou kultivace a typem kultivačního média nebyla v naší studii statisticky významná. Ženy s MZ dvojčaty měly oproti ženám v ostatních skupinách nižší věk a vykazovaly také nadprůměrné výsledky v hodnocení ovariální funkce.

Druhým specifickým cílem studie bylo vyhodnotit vliv dědičnosti. Pomocí dotazníků jsme oslovili pacientky s výskytem monozygotních těhotenství v rodinách a vyhodnotili možný vliv dědičnosti. Zjistili jsme, že ženy s monozygotními dvojčaty (skupina A) měly významně vyšší četnost MZ vícečetných těhotenství ve svých rodinách oproti ženám s dizygotními dvojčaty (skupina B) nebo jednočetnými graviditami (skupina C).

Třetím specifickým cílem bylo porovnat průběh těhotenství dvou trojčetných gravidit s monochoriální komponentou vzniklých po transferu dvou blastocyst v rámci léčby asistované reprodukce. U obou pacientek byla po předchozí konzultaci s pacientkami zvolena různá strategie managementu těhotenství. U první pacientky byla monozygotní komponenta ukončena fetocidou v 15. týdnu gravidity a pacientka porodila v termínu bez dalších komplikací. Druhá pacientka se rozhodla pro observaci těhotenství bez další intervence. Toto těhotenství bylo komplikováno rozvojem těžké mateřské morbidity a ukončeno císařským řezem ve 28. týdnu gravidity po úmrtí jednoho z plodů.

Hlavním závěrem práce je, že vyšší výskyt monozygotních těhotenství u pacientek léčených pro neplodnost může souviset s hereditárními vlivy a vliv technik asistované reprodukce není možná tolik významný. Dále bylo zjištěno, že počet monozygotních dvojčetných těhotenství byl vyšší u mladších žen, které vykazovaly parametry dobré ovariální funkce. Z práce vyplývá, že mladé ženy s výskytem monozygotních vícečetných těhotenství v rodinách by měly být tedy náležitě poučeny o zvýšeném riziku vícečetné gravidity a měl by jim být nabídnut transfer pouze jednoho embrya. Na základě výsledků další naší práce se dále domníváme, že u trojčat se smíšenou chorionicitou je vhodné zvážit redukci monochoriální komponenty a předejít tak riziku, které navíc představuje sdílená placenta.

SUMMARY

There is evidence for at least a two- to three-fold rise in the incidence of monozygotic twinning (MZT) after assisted reproductive techniques (ART) compared to spontaneous conception. Although the etiology underlying human MZ twinning in infertility patients remains a mystery, several mechanisms for the occurrence of MZ twinning have been proposed. A genetic contribution to MZT has been dismissed or considered to be unimportant, but families with MZT have been described. However, no clear connection between the incidence of MZT in infertility patients and genetics has been demonstrated. The main aim of this thesis was to investigate the association between ART and MZT with focus on genetics and to examine the management of high risk triple pregnancies with monozygotic pair produced after assisted reproduction techniques.

The first specific aim of the current study was to examine the probability of MZT in IVF (in vitro fertilization) patients relative to their age, ovarian function, and the ART techniques used in a population of women treated for infertility. We evaluated 1876 patients treated for infertility in our IVF center in a period of 12 years. We recorded the micromanipulation technique used, the length of embryo cultivation, and type of cultivation media. The results in a group of MZT pregnancies were compared with the results of women who delivered dizygotic twins, singleton pregnancies, or aborted. In contrary to other studies, we found that micromanipulation techniques, length of cultivation, and type of cultivation media did not influence the incidence of MZ twinning. The group of patients with MZT was characterised by lower age and excellent ovarian function.

Second specific aim was to assess the effect of inheritance. We organised a survey among IVF patients with multiple pregnancies in their families to evaluate the possible role of genetic factors. We recorded information up to the third generation. The incidence of MZT in families of group A (monozygotic twins) was significantly higher than groups B (dizygotic twins) and C (singleton pregnancies). We proposed that the

high incidence of MZT in infertility clinic patients is conditioned by hereditary factors with no significant influence of assisted reproduction techniques used.

Third specific aim was to compare pregnancy outcome and management options of monozygotic twins in triple pregnancies which resulted from IVF treatment. We reported on two triple pregnancies with mixed chorionicity with a monochorionic-diamniotic twin pair and a singleton following a transfer of two embryos after IVF treatment. In patient A the monochorionic component was ended by a selective fetocide which was performed in 15th week and remaining single pregnancy was delivered without complications at term. Patient B decided for an expectant management. The pregnancy was complicated by severe maternal morbidity and it was terminated in 28th week following the death of one fetus. We suggested, that fetal reduction in triplets should be offered as a management tool to improve perinatal survival. In triplets with mixed chorionicity the reduction of monochorionic twins is emphasized to prevent additional risk posed by shared placenta.

The overall conclusion of the thesis is that the high incidence of MZT in infertility clinic patients is conditioned by hereditary factors with no significant influence of assisted reproduction techniques used. Monozygotic twinning was more often in women with excellent ovarian function which led us to conclusion, that young women with an MZT history should undergo single embryo transfer because of the higher risk of MZT. We also suggest, that fetal reduction in triplets should be offered as a management tool to improve perinatal survival. In triplets with mixed chorionicity the reduction of monochorionic twins is emphasized to prevent additional risk posed by shared placenta.

ABBREVIATIONS

AH	Assisted hatching
ART	Assisted reproductive techniques
DCTA	Dichorionic triamniotic
E2	Estradiol
FR	Fetal reduction
GLMM	Generalised linear mixed models
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilization
MC	Monochorionic
MCA-PSV	Middle cerebral artery peak systolic velocity
MZ	Monozygotní
MZT	Monozygotic twinning
PCR	Polymerase chain reaction
PGD	Preimplantation genetic diagnosis
TTTS	Twin-to-twin transfusion syndrome
US	Ultrasound
ZP	Zona pellucida

1. BACKGROUND

1.1. Incidence of monozygotic twins

It is well known that multiple births occur more often after assisted reproductive technologies (ART) than after spontaneous conceptions. The higher incidence of dizygotic twins arising from the transfer of two embryos is an understandable consequence of ART but the mechanism of monozygotic twinning after ART is still unclear.

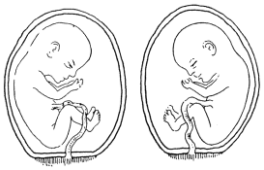
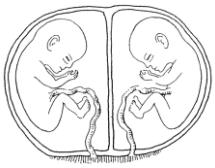
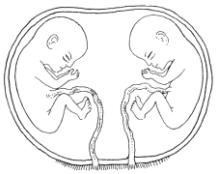
Dizygotic twins occur after the fertilization of two oocytes with two sperm. The twins differ both physically and genetically, and have separate amnion and chorion. We assume that 2/3 of all twins are dizygotic and 1/3 of twins are monozygotic.

Monozygotic twins occur when the fertilization of one oocyte by one sperm produces genetically identical twins. Monozygotic twinning (MZT) is a relatively rare phenomenon. The rate of monozygotic twinning after natural conception varies only slightly in the literature, from 0.4% [23] to 1.6% [64], most studies reporting the incidence of 0.4-0.5 %. In contrast, studies of assisted conceptions show wider variance, ranging from 0.72% [71] to 12.5%[80]. Generally, the incidence of monozygotic twinning after assisted reproductive techniques is about twice as high as after natural conception[86].

1.2. Types of monozygotic twin in relation to the time of division

The time at which the embryo divides is a critical factor in subsequent placental development, and the risk of complications is related to placental sharing. Division on days 1–4 (morula), before the chorion has differentiated, results in dichoriotic–di-amniotic twins (20%), which are indistinguishable from dizygotic twins resulting from two embryos. Division on 4–8 days (blastocyst) results in monochorionic–diamniotic twins (75%), and division on 1–2 weeks results in monochorionic–monoamniotic twins, which share one placenta and one amniotic sac. (Fig.1) Conjoined twins result from division after day 12.

Figure 1 - Types of monozygotic twins depend on the time of division

Days after fertilization	1–4	4–8	8–12
Twins produced	dichorionic–diamniotic 	monochorionic–diamniotic 	monochorionic–monoamniotic 

1.3. Monozygotic twinning in animal models

Monozygotic twinning has been induced experimentally in animal models. In 1921, Stockard[77]demonstrated an increased incidence of MZT in fish after either the available oxygen or the incubation temperature was reduced. Similarly, increased MZT has been identified in mice under in vitro conditions, although mice do not normally show MZ twinning in natural cycles[53]. MZT were also induced by delayed fertilization in rabbits[34]. Another study reported the occurrence of double ICMs at a rate of 0.6 and 3.0% respectively for in vivo and in vitro fertilized mouse embryos prior to hatching[42].

1.4. Etiology of monozygotic twinning in IVF

Twins usually result from one of the two scenarios. Dizygotic twins develop after the fertilization of two oocytes with two sperm. Monozygotic twins occur, when one embryo splits at some stage of its development to produce two identical embryos.

With ART treatment, the primary risk factor for a dizygotic multiple birth outcome is the transfer of more than one embryo. In the past, the replacement of two or more embryos was a common approach to increase the pregnancy rates after IVF. In an effort to reduce the incidence of multiple pregnancies, the ESHRE (European society of human reproduction) issued guidelines on the number of embryos to transfer. These recommendations have been periodically revised with a clear downward trend towards the transfer of only one embryo in patients with good prognosis. As a result of this, the incidence of multiples among ART pregnancies has declined by 10% to 15% over the past decade.[51]

In contrary, the etiology and risk factors for monozygotic twinning in IVF patients are less clear and the incidence of MZT in IVF treatment does not show a downward trend as in the case of dizygotic twins. The reason for this phenomenon has been discussed for almost three decades and several potential mechanisms have been proposed. Earlier studies examined the effect of ovulation induction and ovarian stimulation, changes in culture media and temperature changes, whereas later research concentrated on the effects of micromanipulation techniques, including mechanical manipulation of the zona pellucida and the use of intracytoplasmic sperm injection (ICSI). Current research concentrates on the length of culture and possible genetic etiology[76].

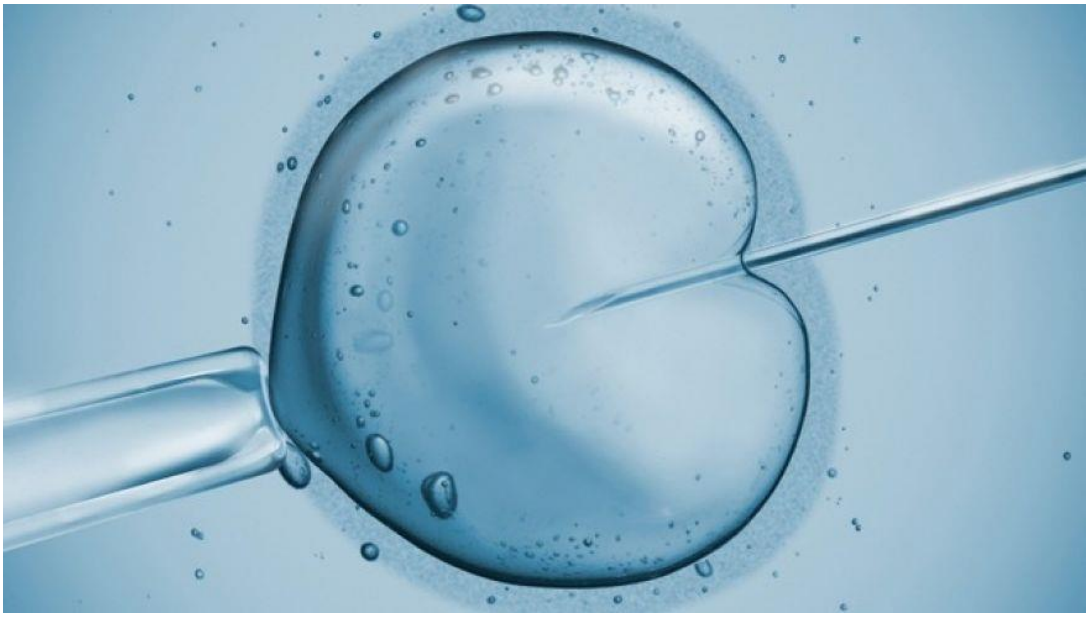
1.4.1. Micromanipulation techniques

The routine application of intracytoplasmic sperm injection (ICSI) and assisted hatching (AH) has prompted discussion of a possible connection between micromanipulation techniques and increased MZT. Once mature oocytes have been obtained from the woman, the ICSI procedure is performed under a microscope using multiple specialized micromanipulation devices that enable the embryologist to select and then pick up individual sperm with best morphology and motility. Once loaded in the needle, the sperm is injected into the cytoplasm of the oocyte. After the procedure, the injected oocyte is placed into cell culture inside an incubator and checked approximately 18 hours later for signs of fertilization.

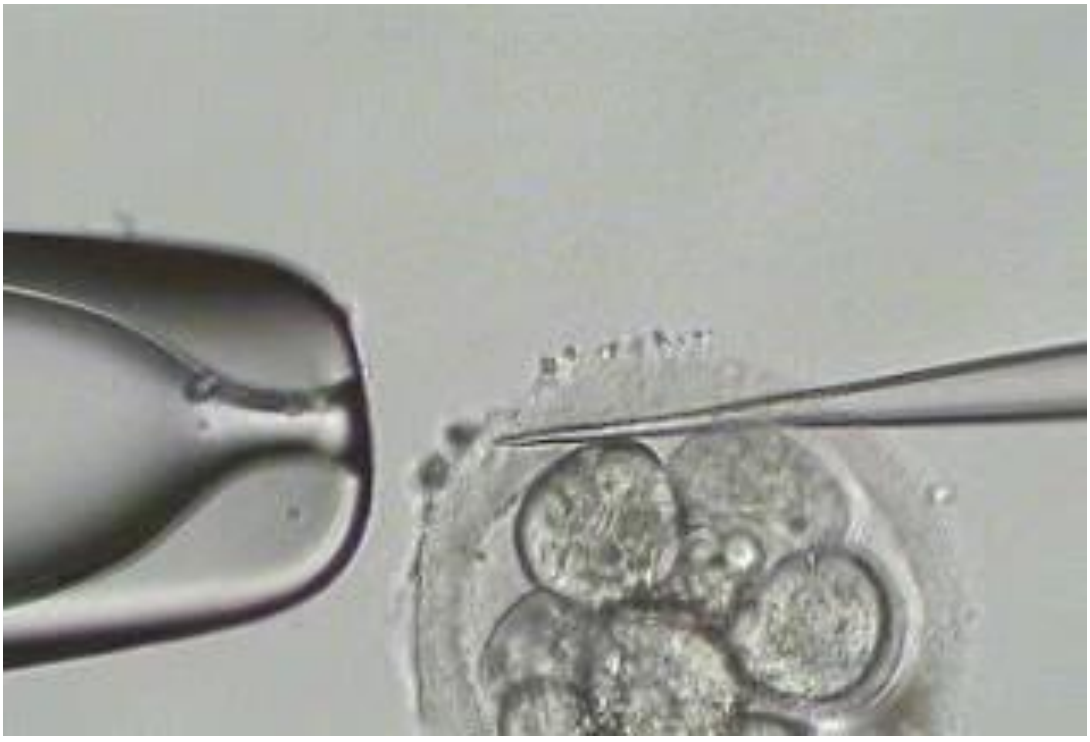
To successfully implant into the uterine wall, an embryo must hatch out of the zona pellucida (ZP), a protein layer covering the embryo in the initial stages of development, and attach to the inner lining of the uterus. Both techniques (ICSI and AH) manipulate the ZP in different ways. ICSI involves direct injection of a spermatozoon into the oocyte. The technique requires a very small number of sperm and allows the use of sperm with limited motility.

The other technique, AH, involves mechanical, chemical, or laser incision of the ZP of a fertilized embryo. AH has developed based on the observation that embryos with a thin ZP have a higher rate of implantation after in vitro fertilization (IVF). It is often performed in an effort to improve implantation rates among patients with a poor prognosis or on embryos noted to have a thick zona pellucida.[15] The American Society for Reproductive Medicine (ASRM) suggest that AH may be clinically useful among women who have failed at least two IVF cycles, are older than 38 years, or have poor-quality embryos[82]. A recent Cochrane review that included 31 randomized controlled trials found small statistical significance in the clinical pregnancy rate among women for whom assisted hatching was used compared with controls although a wide variation in the results among the trials was noted[15].

Both of these techniques (ICSI and AH) leave small defects in the ZP, which may complicate the natural process of embryo hatching. The embryo may bypass its own mechanism of ZP lysis and herniate through these defects, thus resulting in MZT[86]. If so, the size of the defect will probably affect the rate of MZT. There is a distinct difference in the size of the artificial breach in the ZP when ICSI and AH are used. The zona opening formed by AH is 25–30 μm in diameter, whereas the puncture site following ICSI is much smaller (7–8 μm in diameter) [74]. In theory, a smaller hole would not allow the embryo to hatch appropriately and MZT would be more common in ICSI than AH patients. This finding is in agreement with the results of Vitthala[68], who collected data from 27 studies in a meta-analysis, and concluded that couples who underwent ICSI had a higher MZT rate than couples who underwent conventional IVF or AH. In contrast, Luke et al.[51] reported a greater effect after AH, although only in 2–3 day embryos (cleavage stage). The same observation was recently reported by Kanter et al.[47], who showed that early stage embryos may be more vulnerable to the effect of AH than blastocysts. Unlike the previous results, there are also reports, although involving a smaller number of patients, which show that micromanipulation techniques have no effect on MZT[26, 55]. It is apparent that larger studies with statistical power are needed to determine whether or not micromanipulation of the ZP increases the rate of monozygotic pregnancies in IVF patients.



Picture 1 - ICSI (intracytoplasmic sperm injection)



Picture 2 - Assisted hatching

1.4.2. Length of culture

It has previously been reported that culture to the blastocyst stage (day 5) in women with good-quality embryos may facilitate embryo selection, reduce aneuploidy embryos[4] and improve live birth rates[32]. Rijnders[66] and Peramo[63] were the first to report an association between the incidence of MZT and blastocyst transfer. They observed a significant difference between the rate of MZT after embryo transfer on day 3 (0.68%) and transfer on day 5 (2.7%). Da Costa[20] reported that 3.9% of pregnancies generated by blastocyst transfer were complicated by MZT gestation and Behr[9] detected an incidence of 5%. Wrigth et al.[89] examined the 1999–2000 data from the American Society of Assisted Reproductive Technologies (SART) for 39,198 pregnancies. The incidence of MZT pregnancies was four times higher after embryo transfer on day 5 than after embryo transfer on day 3. Similarly, a more recent and larger study that examined data from SART showed that MZT were more likely to develop from embryos transferred on day 5–6 than from cleavage embryos[51].

Picture 3- Blastocyst

Conversely, Franasiak *et al.*[29] reported recently on a cohort of 342 monozygotic pregnancies, that transfer in blastocyst stage was not associated with increased rates of monozygotic twins when controlling for embryo cohort quality. To explain the possible mechanism by which prolonged cultivation possibly affects the rates of MZT, most theories suspect the hardening of the zona pellucida [20]. Zona hardening may squeeze the inner cell mass (ICM) and induce embryo splitting during hatching[7]. Increased MZT might also correlate with the transfer of high-quality embryos, which are more often transferred after prolonged culture. These embryos are more sensitive to the effects of mechanical manipulation in the laboratory or to changes in temperature and pH during monitoring[51] which might result in the higher rates of MZT in after the blastocyst transfer.

1.4.3. Culture medium conditions

Studies of animals have shown that mice blastocysts duplicate their ICM more frequently in vitro than in vivo[40]. A similar phenomenon can be expected in humans. Steinman[78] speculated that the prolonged exposure of blastocysts to lower calcium levels in the culture medium could enhance ICM division because the intercellular bonds are destabilized. Others believe that changes in the culture medium, including an absence of growth factors and higher glucose content, could produce free radicals, which induce apoptosis, leading to the disruption of the ICM and presumably zygotic splitting[89]. Similarly, Behr[9] suggested that the current culture media cause perturbations of the cell-to-cell adhesions, facilitating the splitting of the ICM.

1.4.4. Genetics

Autosomal dominant inheritance with reduced penetrance was proposed as the possible etiology for familial MZ twinning. Inheritance was reported to occur through both the maternal and paternal lines[39]. Hamamy[35] reported high incidence of MZT (n=13) in an extended Jordanese family. Shapiro[70] investigated 10 families with multiple pairs of monozygotic twins born to parents who were also born as monozygotic

twins. In a recent study, Sobek *et al.*[76] documented, that 45% of the group of women who had monozygotic twins also had a family history of MZT. The results of those studies suggested that the incidence of MZT might be under the control of hereditary factors with genes transferred similarly by both the male and female parents.

1.4.5. Ovarian stimulation

The first study to report on increased MZ twinning following ovarian stimulation was initiated in 1964 in Belgium based on data collected from the East Flanders Prospective Twin Study (EFPTS) Evaluation of 2648 multiple births indicated a MZ twinning rate of 1.2% following ovarian stimulation[23]. In 2002, Kallen et al.[46] stated that women undergoing ovarian stimulation delivered MZ twins at nearly twice the rate of controls. More recently, Derom et al.[24] indicated that a higher proportion of twins conceived following ovarian stimulation with clomiphene citrate were MZ compared with iatrogenic cases where no clomiphene citrate was used. In agreement with this study Schachter et al.[71] found out that MZ frequency was more closely associated with controlled ovarian stimulation and ovulation induction by gonadotropin therapy than other ART procedures. The rate of MZT was 0.95% for all ART procedures and 1.5% following ovulation induction or ovarian stimulation alone. It has been suggested MZT twinning may be induced by hormonal stimulation causing hardening of the zona pellucida[23] or as a result of delayed implantation[78].

1.4.6. Age

Later maternal age is considered by many as the only factor that increases the frequency of monozygotic twinning in natural cycles[14]. A gradual reduction in the thickness of the ZP with increasing age in women has been reported Cohen[18]. A thinner ZP could be more vulnerable to inner cell protrusion at multiple sites during zona lysis, facilitating the division of the ICM. On the contrary, a recent study by Knopman et al.[49] found increased MZT rates in women < 35 years old. However, it must be noted that the rate of blastocyst transfer, which is believed to increase monozygotic twinning, is

higher in younger women with good-quality embryos than in women over 35 years old, which could confound these results.

1.4.7. Preimplantation genetic diagnosis (PGD)

Preimplantation genetic diagnosis (PGD) is a widely established reproductive alternative for couples with a high-risk of transmitting an inherited disorder. With respect to monogenic diseases, PGD can theoretically be applied for any genetic disease with a definitive molecular diagnosis and/or defined marker linkage within a family[25]. Currently all methods for PGD to are based on the polymerase chain reaction (PCR) Genetic analysis may be performed at various stages post fertilization, including the oocyte/zygote biopsied on the first day post-insemination (polar body analysis), on 1–2 blastomeres from cleavage-stage embryos biopsied on the third day post-insemination (blastomere biopsy) or on 5–10 trophectoderm cells biopsied from blastocysts on the fifth day post-insemination (blastocyst biopsy). Currently, most PGD cycles use blastomere biopsy[25]. Verpoest et al.[85] were the first to report the incidence of MZT after preimplantation genetic diagnosis (PGD). They assumed that the incidence of MZT was increased by breaks in the ZP associated with blastomere biopsy, similar to those formed by micromanipulation techniques. They found higher an incidence in the group treated with PGD than in the group treated without it, but the difference was not significant (2.1% vs 1.5%, respectively).

1.4.8. Temperature effect

It has been mentioned before that temperature fluctuations in developing fish embryos may result in increased MZT[77]. Similarly, it has been proposed that there may be a temperature effect in ART-associated MZ twinning. In 2005 Toledo et al.[81] proposed a possible association between temperature changes following the transfer of frozen-thawed embryos and MZ twinning. Conversely, other reports indicated no relationship between the transfer of frozen-thawed embryos and MZ twinning rate[5, 11]. If at all, embryo cryopreservation results in increased MZ twinning, the increase would be likely associated with zona hardening rather than a direct temperature effect[5].

1.5. Complications of monozygotic twins

Monozygotic twin pregnancies are high-risk pregnancies with increased perinatal morbidity and mortality. Monozygotic twins have an 80% chance of being monochorionic and have a 6-fold higher abortion rate before 24 weeks of gestation and a 3-fold greater risk of stillbirth and early neonatal death than dichorionic twins[31]. The perinatal complications include intrauterine growth restriction, prematurity, fetal demise, poly-and oligohydramnios and fetal anomalies. These problems predominantly arise because the placenta is not always shared equally between such twins and by the presence of arterio-venous shunts[31]. These are, in addition subject to unique complications such as Twin-twin transfusion syndrome (TTTS), Twin reversed arterial perfusion (TRAP), Twin anemia-polycythemia sequence (TAPS), or twin oligohydramnios-polyhydramnios sequence (TOPS).[48]

1.5.1. Twin-twin transfusion syndrome

Twin-twin transfusion syndrome (TTTS) is one of the most serious obstetric complications specific to MC twinning, occurring in about 10 to 20% of MC twin pregnancies[69]. It is now clear that TTTS is the result of a chronic imbalanced unidirectional blood flow from one twin to the other, through placental arterio-venous anastomoses between the two fetal circulations sharing the same placental mass. Such anastomoses are present to some degree in virtually all MC placentas. The donor twin becomes progressively hypovolemic, and develops anemia, growth restriction, oliguria and severe oligohydramnios. In TTTS, the recipient twin becomes hypervolemic and develops progressive cardiac failure and polyhydramnios. Further deterioration may result in in-utero death of both twins. In addition, polyhydramnios could be severe, leading to preterm rupture of membranes, preterm labor, and extreme preterm delivery. If left untreated, the perinatal mortality of affected pregnancies exceeds 80%. Close monitoring by antenatal ultrasound examinations enables early detection of TTTS in the mid-trimester. The earliest signs are discordance in liquor volume between the two gestational sacs, one with oligohydramnios and the other with polyhydramnios

(Quintero stage I). In stage II disease, the donor twin has severe oligohydramnios with non-visualisation of the fetal bladder. In more severe cases, there will be Doppler blood flow abnormalities (stage III), fetal hydrops (stage IV), and eventually fetal demise (stage V). If one of the fetuses dies in utero, there will be sudden exsanguinations from the remaining fetus to the dead fetus through the vascular communication, resulting in either death or cerebral damage to the remaining fetus.

In the past, the only treatment option of polyhydramnios, the most prominent feature of TTTS, were the series of amnioreductions. Survival rate significantly improved after the introduction of laser coagulation, making it the accepted treatment of choice for TTTS. However, results are still not very satisfactory, with high mortality rates and significant complications, including iatrogenic preterm premature rupture of membranes resulting in preterm delivery before 32 weeks gestation.

TTTS syndrome

- 10-20% MC twin pregnancies,
- Chronic imbalanced unidirectional blood flow,
- Arterio-venous anastomoses,
- Hypoperfusion of the donor, hyperperfusion of the recipient twin,
- Donor: hypovolemic, anemic, develops growth restriction, oliguria and severe oligohydramnion,
- Recipient: hypervolemic, develops progressive cardiac failure and polyhydramnion.

1.5.2. Twin reversed arterial perfusion

Twin reversed arterial perfusion (TRAP) sequence is a rare complication of monozygotic multiple pregnancy. In this disorder, there is a normally formed donor and a recipient (the acardiac twin) who lacks a heart structure. The pump or donor twin may develop cardiac failure because of the high-output to the recipient twin. Overall, the

perinatal mortality rate for the pump twin is 35-55% as a result of heart failure[27]. Treatment options include fetoscopic ligation of the umbilical cord, bipolar forceps, embolization of the acardiac twin's umbilical artery with absolute alcohol, platinum coils, or thrombogenic coils of umbilical cord. Neodymium/yttrium-aluminum-garnet (Nd:YAG) laser or monopolar thermocoagulation of the cord are also being used[27].

TRAP

- 1% of monochorionic twins,
- Arterio-arterial anastomosis,
- Acardiac twin receives blood supply via “pump” twin,
- 55% mortality in pump twin as a result of heart failure.

1.5.3. Twin anemia-polycythemia sequence

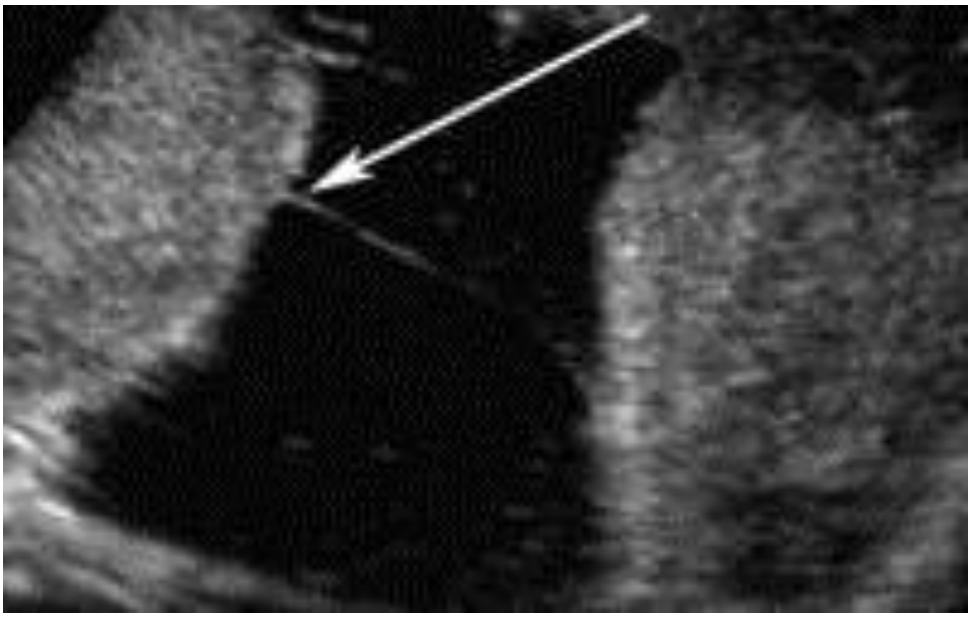
Twin anemia-polycythemia sequence (TAPS) is a complication of monochorionic placentation characterized by discordance in hemoglobin levels in the absence of amniotic fluid abnormality characteristic of classical twin-twin transfusion syndrome (TTTS). TAPS may occur spontaneously or following laser treatment for twin–twin transfusion syndrome (TTTS), defined as post laser TAPS. The diagnosis is based on measurement of the middle cerebral artery peak systolic velocity (MCA-PSV). In mild form of the disease, the anemic donor twin has increased MCA-PSV and the recipient has decreased MCA-PSV. In severe disease, critical Doppler findings, including hydrops or single twin demise are present. Treatment options include fetoscopic laser, fetal blood transfusion, conservative management, and often preterm delivery.

TAPS

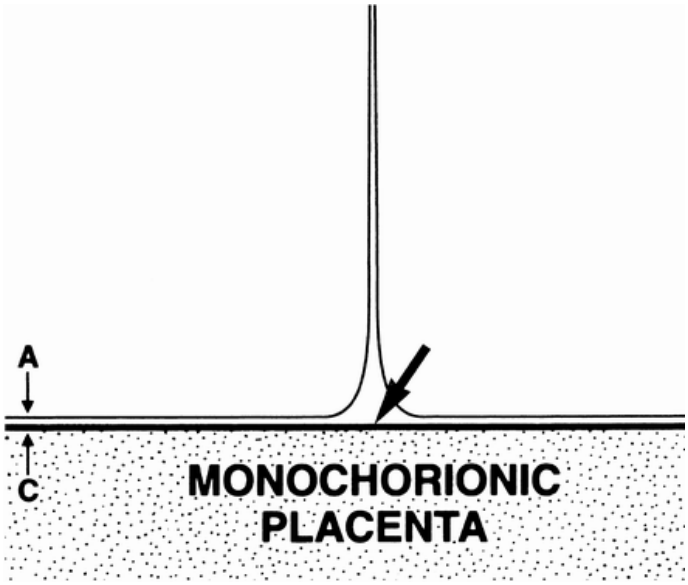
- Discordance in hemoglobin levels,
- Donor develops anemia, recipient develops polycythemia,
- Measurement of MCA-PSV.

1.6. Management of monozygotic twins

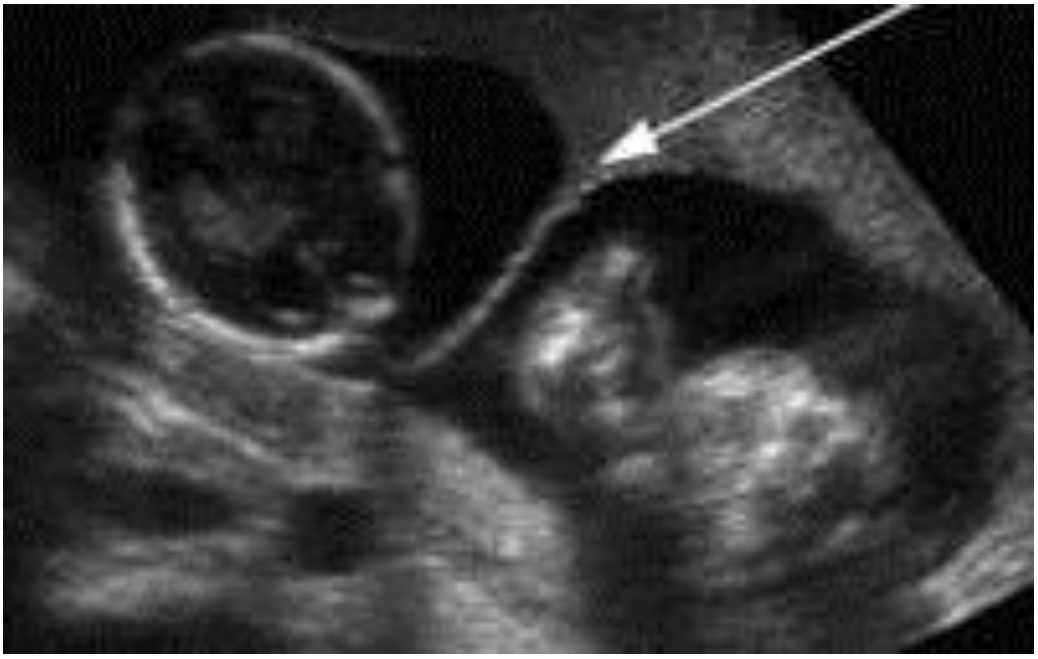
The crucial step in the management of twins is the detection of chorionicity. Placental type can be determined as early as in 6 th week of pregnancy. The appearance of so called T-sign or „empty lambda“ leads us to the diagnosis of monochorionic twins. The lambda sign confirms dichorionic pregnancy.



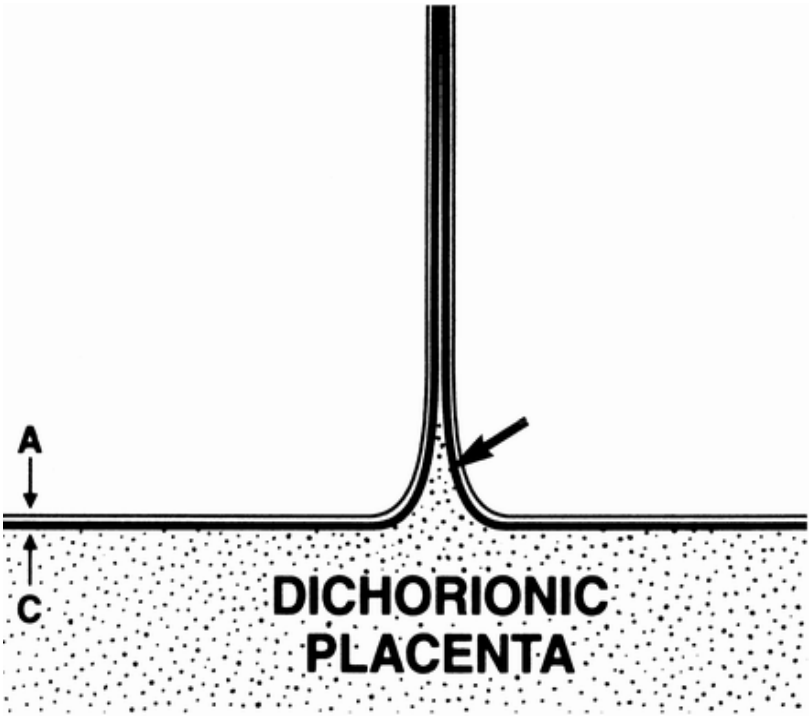
Picture 4 - T-sign - monochorionic pregnancy



Picture 5- T-sign scheme



Picture 6- Lambda sign - dichorionic pregnancy



Picture 7- Lambda sign – scheme

The management of dichorionic diamniotic twins which arise from one zygote is similar to dizygotic twins. Outcomes and management of monochorionic diamniotic (MCDA) and monochorionic monoamniotic twin pregnancies, which are less common, have been the subject of much research and speculation over the past decades. Monochorionic twin pregnancies are affected by a unique set of complications, and therefore have a higher burden of associated morbidity and mortality for both mother and child. Series of ultrasound scans is recommended from 16 th gestational week. We should record growth of the fetuses, position if the placenta, cervix length and bladder filling. Special attention has to be payed to the difference in the amount of amniotic fluid, growth discordance and to abnormal Doppler parameters. Management options include observation (serial cardiotocography, ultrasonography and echocardiography), selective surgical (laser coagulation) and nonsurgical interventions (indomethacin, tocolysis) or elective termination of the pregnancy.

2. OBJECTIVE OF THE THESIS

The main aim of the thesis was to examine the causes of higher incidence of MZT in patients treated for infertility.

Specific Aims:

1. To examine patients treated for infertility relative to their age, ovarian function, and the ART techniques used in relation to the incidence of monozygotic pregnancies.

2. To investigate the genetic background of monozygotic twinning in a population of women treated for infertility.

3. To compare pregnancy outcome and management options of monozygotic twins in triple pregnancies which resulted from IVF treatment.

3. SET OF PATIENTS

3.1. Specific aim 1 and 2

The study group included 1876 patients treated for infertility in IVF center Fertimed, Olomouc, Czech Republic between 2000 and 2012. All patients came from the same region and were treated by three clinicians following the same clinical and therapeutic guidelines. The diagnosis of MZT was made by ultrasound (US), or following the information about the delivery of more babies than the number of embryos transferred. We divided patients into four groups according to the pregnancy outcome, as follows:

(A) monozygotic twins (MZT);

(B) dizygotic twins (DZT);

(C) singleton pregnancies;

(D) abortions.

In group A, we were able to contact 20 patients (87%). All women from this group were personally interviewed. The patients from groups B and C were administered a genetic questionnaire. We received 65% of the questionnaires from group B and 55% from group C. Patients from group D were not contacted from ethical reasons.

3.2. Specific aim 3

In 2016, two patients with dichorionic triamniotic (DCTA) pregnancies were referred to The Fetal Medicine Center at Palacky University during the 12 th week of gestation following IVF treatment. In both cases, embryo-transfer (ET) was carried out at the blastocyst stage and two embryos were transferred to each patient. For both patients, the embryonic disk split following ET, resulting in triamnial triplets with mixed chorionicity.

Patient A (0 Para) was 30 years of age,

Patient B (I Para) was 32 years of age.

4. METHODS

4.1. Specific aim 1

We recorded the patient age and implantation rate and the following five variables characterising ovarian function:
basal FSH,
estradiol (E2) level on the day of human chorionic gonadotropin administration,
number of oocytes,
total consumption of gonadotropins,
consumption of gonadotropins needed for recovery of one oocyte.

The serum FSH concentrations were measured using a commercially available immunoradiometric assay (IRMA) kit (Immunotech, Praque, Czech Republic). The FSH assay was standardized against the WHO second international standard 94/632 reference material. The sensitivity of the FSH assay was 0.2 IU/l and the intra- and inter-assay CVs were 2.6% and 6.3%, respectively. Estradiol concentrations were measured using an immunoassay (Advia Centaur; Bayer, Leverkusen, Denmark). The sensitivity of the E2 assay was 37 pmol/l and the intra- and inter-assay CVs were 9.7% and 10.2%, respectively. Furthermore, we recorded the following variables characterizing IVF: the micromanipulation technique used; length of embryo cultivation; and type of cultivation media. The parameters of ovarian function, age, implantation rate and characteristics of IVF (ICSI, assisted hatching, and cultivation media) were compared in the four groups of patients.

4.2. Specific aim 2

A genetic survey on the occurrence of multiple pregnancies in the close family was done for groups A, B, and C by personal questionnaire. We recorded information up to the third generation (brothers and sisters, parents, grandparents, grandparents' sisters and brothers and their children, and all nephews and granddaughters) The questionnaire

was sent to all of the patients. In the event that the answers were not clear, the patient was contacted personally. Group D was not interviewed for ethical reasons.

4.3. Specific aim 3

Both women were examined and carefully informed about the risk and prognosis of triple pregnancies with a monochorionic component. In both cases, we offered fetal reduction.

Patient A opted for selective fetal reduction of the monochorionic twins. The procedure was, however, delayed due to the presence of a sub-placental hematoma which led to the procedure being postponed until the 15th week of gestation. The procedure was subsequently carried out without complication via the intra-cardiac injection of 1 ml of 7.5% potassium chloride (KCl) using a transabdominal needle guided by ultrasound. Afterwards, serial ultrasound scans were used to screen the patient for potential aberrations in fetal growth and disturbances in the volume of amniotic fluid.

Patient B, however, opted to continue the pregnancy without intervention. Ultrasound diagnostic screening was performed on a weekly basis. We paid particular attention to a range of Doppler parameters, including the pulsatility index and peak systolic velocity of the middle cerebral artery, the pulsatility index of the umbilical artery and ductus venosus, the cerebroplacental ratio and amniotic fluid disturbances. In addition, we analysed the biometry of all fetuses on a bi-weekly basis.

5. STATISTICAL ANALYSIS

5.1. Statistical analysis for specific aim 1 and 2

For statistical analysis of the data, we only used the first records from patients who were pregnant more than one time (n = 1876). Out of such patients, 1715 couples (91%) had complete records for all parameters tested and were used in the statistical analysis of the data. We characterized the groups based on the mean and standard error (SE) values for all relevant predictors tested within the framework of generalised linear mixed models (GLMM). We assumed a normal error distribution and used the year of data collection as a random effect on both the intercept and slope. Where appropriate (E2 level and consumption of FSH/oocyte), variables were log-transformed prior to the analysis to stabilize variance. The number of oocytes retrieved and the number of embryos transferred were assumed to follow a Poisson distribution. Procedure glimmix of SAS was used to fit GLMM. Because we approximated the marginal log likelihood with an adaptive Gauss-Hermite quadrature, we selected the best model according to the lowest AICc (Burnham and Andersson, 2002). The difference in AICc (ΔAICc) between the best model and the second best model > 2 was considered as evidence for the effect. Tukey–Kramer adjustments were applied for multiple comparisons between groups. A genetic survey on multiple pregnancies in close families (up to the third generation) was conducted for groups A, B and C. Group D was not interviewed for ethical reasons. The differences in percentages of MZT among groups A, B and C were compared by a generalized linear model (GLM) for binary responses (presence/absence) and ΔAICc > 2 was used to identify the best model.

6. RESULTS

6.1. Specific aim 1

Of 1876 couples, we used data from 1715 couples (91%) for statistical evaluation in which all evaluated parameters were properly recorded and available. Among the 1715 couples investigated, we identified 23 couples in the MZT group (A). Sixteen MZT pregnancies (69.5 %) arose from the division of one of the transferred embryos. In 6 patients (26.1%), both of the transferred embryos divided into MZT embryos and resulted in 4 embryos. In 1 patient (4.3%), 1 transferred embryo divided into 3 monozygotic embryos. Three pregnancies (13%) ended during weeks 24–26 of pregnancy following embryo reduction. In group B there were 423 patients (24.66%), in group C there were 880 patients (51.31 %), and in group D there were 389 patients (22.68%).

With the exception of basal FSH, all parameters of ovarian function (E2 level, number of oocytes, total consumption of gonadotropins, and consumption of gonadotropins needed for recovery of 1 oocyte) and the implantation rate were better in group A (Table I); however, statistical support was obtained only for the E2 level (ΔAICc = 102.49; significant multiple comparisons: AB, AC, AD, BC, BD, and CD). The amount of FSH needed to recover 1 oocyte (ΔAICc = 3.94; significant multiple comparisons: AC) and the implantation rate were significantly different (ΔAICc = 76.45; significant multiple comparisons: AB, AC, and AD). The age of patients differed among the four groups (ΔAICc = 18.51; significant multiple pair comparisons existed for pairs: AD, BC, BD, and CD).

When analyzing differences between response groups in terms of the micromanipulation technique used (ICSI: A, 100%; B, 98%; C, 97.8 %; and D, 96%), we could not decide which of the two models compared (models with and without effect) was better (ΔAICc = 0.28). The same was true with the percentage of MZT in cycles with (n = 1754) or without (n = 122) assisted hatching (1.20% vs. 1.64%; ΔAICc = 1.84). No differences were found owing to the length of embryo cultivation (1.34%

after 48 h of cultivation, n = 283 IVF cycles; 0.96% after 72 h, n = 840 IVF cycles; 1.40% after 96 h, n = 753 IVF cycles; and 1.81% after 120 h, n = 110 IVF cycles; ΔAICc = 3.66). When assessing the effect of the type of cultivation medium, no difference was observed among the 4 groups (ΔAICc = 3.39). The incidence of MZT in the 3 media was as follows: Sage, 1.55% MZT (n = 513); Medicult, 1.08% MZT (n = 185); and Scandinavian Science, 1.10% MZT (n = 1178). The number of transferred embryos did not differ among the four groups (A:2.28; B:2.72; C: 2.58; D:2.70), nor did the FSH consumption (A:1987; B:2237; C:2257; D:2263)

6.2. Specific aim 2

We interviewed all women in group A, but were able to contact only 20 (87%) regarding the incidence of MZT in their families. In 7 families, no twins were identified. In 9 families, monozygotic twins were previously born without question; 2 families reported twins without an understanding of monozygosity, and in the other 2 families, the occurrence of dizygotic twins was reported. One man had monozygotic twins twice in his life. The first time, he and his former wife had monozygotic twins after IVF with ICSI in our center, and subsequently, he had MZT spontaneously with his then wife. In 9 group-A couples with positive MZT anamnesis, we reported 51 families with a total of 19 (37%) monozygotic twins. Using a generalized linear model for binary responses, we revealed among-group differences in the percentages of MZT in patient families (DAICc ¼ 21.04). The group-A patient families had 45% proven cases of MZT up to the third generation. We received 65% of the genetic questionnaires for group B, and 55% for group C. In groups B and C, the occurrences of MZT in families were 13.4% and 9.5%, respectively. Genes for monozygosity were more often transferred through women (8) than through men (4).

6.3. Specific aim 3

Fetal reduction (FR) was performed upon Patient A in the 15th week of gestation without complications. Subsequently, serial ultrasound scans detected no pathology and the remaining fetal material was absorbed. The remaining pregnancy was terminated at

40+1 weeks of gestation via the induction of labor owing to a suspicion of placental insufficiency, as indicated by abnormal Doppler parameters. A healthy child, weighing 3220 g, with an APGAR score of 10-10-10, was born without complication and postnatal adaptation was excellent.

The pregnancy of patient B was complicated by hypertension from the 16th week of gestation and by the development of gestational diabetes mellitus from the 24th week. Growth restriction stage I and anhydramnion were detected in the fetus with its own placenta and the patient was hospitalized in the 25th week due to signs indicative of preeclampsia. We administered two intra-muscular doses of Betamethasone (14 mg) to help the fetal lungs mature in accordance with the standard protocol. Following extensive counselling, Patient B opted for the expectant management of her pregnancy. During the 28th week, we confirmed the intrauterine death of the fetus with its own placenta, and diagnosed growth restriction stage I of the remaining monochorionic fetuses. Ophthalmological examination of Patient B revealed hypertonic retinopathy grade II. After consultation with a gynaecologist, neonatologist and psychologist, Patient B decided to terminate her pregnancy and Caesarean section was performed during the 28th week. Monochorionic twins were delivered, with birth weights of 940g and 780g, both with an APGAR score of 8-8-10. Afterwards, Patient B was monitored for preeclampsia and diabetes; medication was adjusted repeatedly before the patient was discharged from hospital care.

7. DISCUSSION

7.1. Specific aim 1 and 2

Although the increased incidence of MZT after assisted reproduction is widely recognised, general mechanisms underlying this phenomenon are poorly understood[7]. Using an extensive data set involving the performance of IVF patients, we focused on the effects of ovarian function, micromanipulation, and genetic factors. We found that the incidence of MZT is associated with elevated levels of E2 and low FSH consumption per oocyte. In contrast, micromanipulation techniques or the type of cultivation media did not have an effect on the frequency of MZT. Most importantly, a detailed inquiry into the hereditary components in families of patients revealed evidence for a genetic background underlying MZT.

A questionnaire given to all patients is unlikely to be absolutely correct, but the failures are expected to be equally common in all interviewed groups. It is important to keep in mind that patients at 30–35 years of age were born at a time when US was not available to all pregnant women, and even now US detection of MZT and DZT may be in error. A deceased grandmother who was born as a twin and survived could not be confirmed. We believe that biases of this kind were equal in all groups.

We are also aware of the fact that twinning resulting from a single embryo transfer may not necessarily be monozygotic. The reason for this could be a spontaneous conception superimposed upon the pregnancy resulting from IVF, as has been previously described by Mains[46]; the risk of this phenomenon is probably low.

The genetic background of MZT has been described in the general population[39,50] but the genetic background of MZT has rarely been discussed in the context of ART[68]. MZT division of embryos follows an autosomal dominant pattern of inheritance with low expression and transfer by males or females.

In agreement with the higher incidence of gonosomal mosaicism after ICSI, which is not related to ART procedures but correlates with genetic information in the oocyte[44,73,12,59,17] we found that the incidence of MZT is not increased by ART techniques. Instead, the main cause of this phenomenon is, as we believe, a characteristic of the patient. Autosomal dominant genetic information "to produce MZT" is enhanced in ART by stimulation, a higher number of oocytes with good quality, a higher implantation rate, and a low abortion rate.

A close link between genes for embryo division, zona pellucida architecture, and abnormal mucopolysacharide synthesis in the zona could account for female-only participation in MZT[6]; however, the genes for MZT are also transferred through males and we have to seek an explanation for this phenomenon in the embryo. The embryo can divide into two and sometimes three parts[83]. This phenomenon has been observed in animals[11]. The genes for MZT were expressed in approximately 37% of all families of our group of 20 MZT pregnancies in which a response on the survey was obtained. The same expression was reported by Harvey[39] in one Indian family. The low penetrance of this attribute can be explained by the Menezo theory[54] of linear polarization of embryos by apoptosis[61]. Apoptosis can be regulated by genes[38]. We hypothesise that embryos which divide 50:50 undergo MZT. In embryos dividing unequally (30:70), only the larger embryo survives. The smaller embryo grows a limited time and eventually vanishes before clinical detection. Such unequal division and separation of blastoceles was first described by Malter and Cohen[58], and documented by Aston[7]. Further biological MZT reduction can be expected during implantation[11]. A higher incidence of MZT in the group of women with high fecundity was likely caused by the high implantation rate of embryos of high quality[11].

The autosomal dominant transfer of genes for MZT can be documented by the fact that 22% of all couples had >1 embryo divide. The same experience has been described by Abusheika[2] . In one case, one embryo divided into three fetuses. Salat-Baroux[68] reported three MZ embryos in one gestational sac.

The incidence of monozygotic twins in women treated for infertility was three-fold higher than women who conceived spontaneously. This finding can be explained by a higher number of transferred embryos, as previously described by Sills[74] [3.6 embryos/ET], (Da Costa[20]) [2.5–3.9/ET], (Salat[68]) [4/ET], (Abusheika[2]) [2–3/ET], (Milki[55]) [2.8/ET], (Alikani[5]) [3.2/ET], (Wright[89]) [1–6/ET], and/or by a higher quality of embryos generated by stimulation[23].

The group of patients with MZT was characterised by lower age and excellent ovarian function (high E2 level, high number of oocytes recovered, low consumption of FSH needed for stimulation of one oocyte, and high implantation rate). The E2 level was significantly higher in the MZT group, which is an expression of good ovarian function and, as we believe, not the trigger for embryo division. Indeed, embryo division is also possible in natural cycles with average E2 levels.

High-quality oocytes produced in the MZT group resulted in premium quality embryos with a high implantation rate (in 7 couples, >1 embryo divided and in 1 patient 1 embryo split into 3 fetuses). We assume that such embryos have a better chance to survive MZT reduction than non-selected embryos from a natural cycle. If those embryos are "genetically" prone to produce MZT and if we implant more of such embryos, there may be more MZTs born after IVF. In the last 2 years we most often performed single embryo transfers in our center and we did not detect an MZT during this period, which strengthens this idea. The high implantation rate of embryos in highly fertile women in our group of MZT patients was in agreement with Blickstein[11] and Bortolus[13].

The results suggest that the incidence of MZT is under the control of hereditary factors and is enhanced in ART by excellent ovarian function. To reduce the number of MZT, it is advisable to collect information on the family history of MZT before the actual stimulation process and perform elective single embryo transfer in women with excellent ovarian function and a positive family history of MZT. It would be appropriate to consider individually whether or not to recruit such women as oocyte donors and proper counselling is advisable.

7.2. Specific aim 3

The prevalence of monochorionic twins in triplet pregnancies following spontaneous conception is approximately 40%[1] and 10% after assisted conception[41]. Dichorionic triplets have a perinatal mortality rate which is eight times higher than trichorionic pregnancies[8] and thus represent a specific subgroup for triplets which is associated with the highest complication rate.

This case report focuses upon the clinical management and outcome of two patient cases, each with triple pregnancies with a monochorionic-diamniotic twin pair and a singleton following the transfer of two embryos during IVF treatment. When couples are faced with the dilemma of dichorionic triamniotic (DCTA) pregnancy, the first option is to adopt a conservative approach. Attempting to continue a pregnancy with all fetuses is associated with a high risk of perinatal morbidity and mortality caused mainly due to premature delivery[1]. Abel et al.[1] evaluated data from 47 triplet pregnancies with monochorionic twin pairs and found that the risk of premature delivery <30 weeks was significantly higher in non-reduced pregnancies compared to pregnancies which had been reduced (25% versus 0%) and in deliveries <34 weeks (88% versus 3%). A high proportion of triplets (28 %), managed in an expectant manner, were also shown to be complicated by twin to twin transfusion syndrome. In contrast, FR has been associated with a rate of miscarriage approximately two times higher than that seen in pregnancies which are managed expectantly[57]. A slightly higher early miscarriage rate was found in cases involving selective fetal reduction from 3 to 1 than in cases involving 3 to 2 reductions (22% versus 17%)[1]. There are two predominant mechanisms underlying miscarriage following FR. The first of these relates to procedure-related trauma or infection, in which miscarriage would be expected within 2 weeks of FR. The second relates to the consequence of the mother resorbing dead feto-placental tissue, which is likely to result in miscarriage several weeks, or even months, after FR[41].

A variety of techniques have been proposed for the reduction of multifetal pregnancies, including the transvaginal[33] or transabdominal[10] administration of KCl or NaCl directly into the fetal heart[90], the aspiration of embryos during the early weeks of

gestation[43] or umbilical cord coagulation[33]. Pregnancy loss from such procedures is known to vary from 5% to 30%[33]. In dichorionic triplets the most common approach is the reduction of a monochorionic pair to prevent the negative impact of a shared placenta. Reduction of the singleton, and maintenance of the monochorionic pair, is a less common option, as this procedure is associated with the higher rate of complications. Furthermore, Rong et al.[67] investigated 35 pregnancies and showed that monochorionic twins were associated with a higher rate of late miscarriage than retained singletons (18.5% versus 0 %); retained twins also suffered from a premature birth rate (11%) and lower birth weight in comparison to a retained singleton. retained singleton.

The first visit was planned in the 12 th week to meet criteria for the first trimester screening. After the results were obtained, we planned the strategy for ongoing pregnancy. The policy in our own department is to offer fetal reduction of a monochorionic pair to patients with DCTA. In each case, however, the final decision rests with the patient. In cases of trichorionic triplets, we offer fetal reduction for twin pregnancies, which is in line with the recommendations of Wimalasundera and Van de Mheen[87,84] who indicated that the reduction of triplets to twins resulted in a significant reduction of risk associated with preterm delivery and intrauterine fetal death.

8. CONCLUSION

Micromanipulation techniques, length of cultivation, and type of cultivation media did not influence the incidence of MZ twinning. We believe that the incidence of MZT is under control of hereditary factors and it is enhanced in ART by excellent ovarian function. MZT gene is inherited autosomally dominantly and the gene is transferred from both parents. It is important to collect information on the family history of MZT before the actual stimulation and make this a part of decision making process on the stimulation policy. In order to reduce the incidence of MZT, it is advisable to perform elective single embryo transfer in women with excellent ovarian function and positive family history of MZT. It would be appropriate to consider individually whether or not to recruit such women as oocyte donors and proper counselling is advisable.

We also suggest that fetal reduction should be offered to patients with triplets as a clinical management tool to improve perinatal survival. In triplets with mixed chorionicity, which are associated with the highest rate of complications, we emphasize the reduction of monochorionic twins to prevent the additional risks posed by the shared placenta. While previous studies have reported higher miscarriage rates for pregnancies undergoing multifetal reduction compared to an expectant management plan, it is important that these concerns are balanced against the lower risk of prematurity and fetal morbidity.

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10. PUBLICATIONS

10.1. Publications in relation to the study

10.1.1. Publications with impact factor as first author

Sobek A Jr, Zbořilová B, Procházka M, Šilhánová E, Koutná O, Klásková E, Tkadlec E, Sobek A. High incidence of monozygotic twinning after assisted reproduction is related to genetic information, but not to assisted reproduction technology itself. Fert Ster 2015;3:756-60. **IF 4.426**

Ales Sobek Jr., Martin Prochazka, Eva Klaskova, Marek Lubusky, Radovan Pilka. High incidence of monozygotic twinning in infertility treatment. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2016 11;160,3:358-362. **IF 0.924**

10.1.2. Publication in a reviewed Journal without impact factor as first author

Sobek A Jr., Procházka M., Kláskova E., Zbořilová B., Ľubuský M., Sobek A. Triple pregnancy with mixed chorionicity following in vitro fertilization (IVF): is fetal reduction neccesary ? Ceska Gyn, 2017; ahead of print

10.2. Other publications

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10.2.1. Publications in Journals with impact factor as first author

Sobek A Jr, Hammadeh M, Vodicka J, Sobek A. Ultrasonographically guided transvaginal hydrolaparoscopy. Acta Obstetricia et Gynecologica Scandinavica 2008;87(10):1077-80. **IF 2.191**

A. Sobek Jr, E. Tkadlec, B. Hladíková, and A. Sobek Is there a declining trend in ovarian function among infertility clinic patients? Hum Rep 2010;25,1:127–132. **IF 4.621**

10.2.2. Publications in reviewed Journals without impact factor as first author

Sobek A Jr., Vodicka J., Sobek A. Transvaginal hydrolaparoscopy and Ultrasonographically guided transvaginal hydrolaparoscopy-two outpatient methods of pelvis examination Česká gynekol 2007;1,72:11-5.

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10.2.3. Publications with impact factor as a co-author

Klásková E., Tüdös Z., Sobek A., Dostál J., Procházka M., Zbořilová B., **Sobek A. Jr.,** Dostálová Z., Zapletalová J., Adamová K., Lattová V. : Low-level 45,X/46,XX mosaicism is not associated with congenital heart disease and thoracic aorta dilatation: prospective magnetic resonance imaging and ultrasound study, Ultrasound Obstet Gynecol 2015; 45:722-727. **IF 4.254**

10.2.4. Publications in a reviewed Journal without impact factor as a co-author

Klásková E., Zapletalová J., Sobek A., Horák D., Wiedermann J., **Sobek A, Jr**. Postižení kardiovaskulárního systému u žen s Turnerovým syndromem, kardiovaskulární rizika spojená s těhotenstvím, Praktická gynekologie 2010;1801-8750.

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11. LECTURES

2016 Celostátní konference ultrazvukové diangostiky Brno. Ultrazvukově asistovaná transvaginální hydrolaparoskopie.

2016 Celostátní konference asistované reprodukce Brno. Ultrazvukově asistovaná transvaginální hydrolaparoskopie.

2012 Celostátní konference laparoskopické operativy Poděbrady. UTHL- nové instrumentarium.

2010 ISUOG World congress, Praha 2010. Ultrazvukově kontrolovaný vstup do dutiny břišní.

2006 FIGO World congress of Gynecology and Obstetrics, Kuala Lumpur, Malaysia. Decrease of ovarian function- new threat to human population.

2006 VIII congress of the ICGI & ambulatory gynecology, Praha. Operative treatment of infertile patients

2006 Annual National conference ČGPS ČLS JEP a SSG ČR, Brno. Ultrasonographically guided transvaginal hydrolaparoscopy- the learning curve.

2005 1st Beijing International conference on Obstetrics and gynecology, Peking, China
Ultrasonographically guided transvaginal hydrolaparoscopy (UTHL)-two options of pouch of Douglas puncture.

2005 3rd International egg donation conference, London. Treatment of infertile patients in Czech Republic

12. APPENDICES

12.1. Appendix 1

Mean (SE) values for several variables of ovarian function estimated for four response groups of patients by fitting the generalised linear mixed Model. Only the E2 and implantation rate affected the means among patients with monozygotic twins (MZT), dizygotic twins (DZT), singleton pregnancy and abortions.

Variable	MZT (A)	DZT (B)	Singleton (C)	Abortions (D)
No of patients	23 (1.34%)	423 (24.66)	880 (51.31)	389 (22.68)
Age (years)	29.3 (0.9) NS	29.6 (0.3)	30.2 (0.3)	31.1 (0.3)
Basal FSH (IU/l)	6.97 (0.50) NS	6.56 (0.14)	6.79 (0.11)	6.86 (0.15)
E2 (pmol/l)	4192 (123) S*	1168 (38)	1520 (49)	1308 (42)
Oocytes retrieved	14.4 (1.0) NS	12.8 (0.5)	12.3 (0.4)	12.4 (0.5)
Total FSH consumption (IU)	1987 (154) NS	2237 (43)	2257 (34)	2263 (45)
FSH consumption/oocyte (IU)[a]	180.69 (5) NS	223.39 (2)	258.54 (2)	269.63 (2.5)
Implantation rate (%)	126 (0.05) S **	84 (0.03)	46 (0.02)	49 (0.03)
No of embryos transferred	2.28 (0.32) NS	2.72 (0.11)	2.58 (0.09)	2.70 (0.11)
Positive MZT family history (%)	45	13.4	9.5	NA

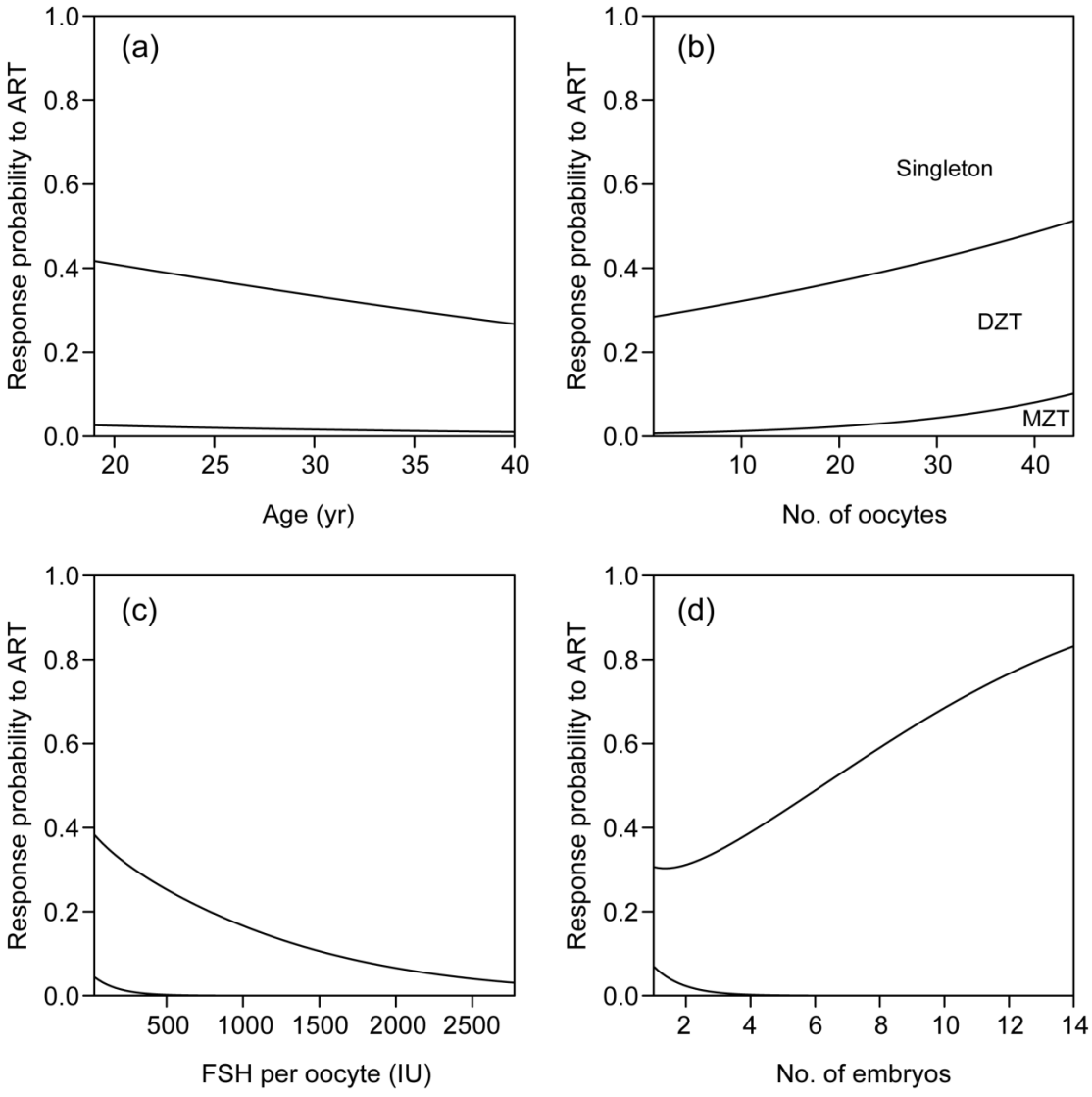
a The variable was logtransformed prior to parameter estimation.

* Multiple comparison significant for B,C,D

** Multiple comparison significant for A,B,C

12.2. Appendix 2

The cummulative probability plot showing the effects of age (a), number of oocytes retrieved (b), consumption of gonadotrophins per oocyte (c) and number of embryos transferred (d) on response probabilities to ART as predicted by single-effect generalised multinomial linear mixed models. The curves partition the total probability space into three areas indicating the probabilities of MZT (the lower part), DZT (the middle part) and singleton (the upper part).



12.3. Appendix 3

ORIGINAL ARTICLES: GENETICS

High incidence of monozygotic twinning after assisted reproduction is related to genetic information, but not to assisted reproduction technology itself

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Objective: To study the incidence of monozygotic twinning (MZT) in patients using in vitro fertilization, relative to their age, genetic background, ovarian function, and assisted reproductive techniques used.
Design: Analysis of a collected database.
Setting: Infertility treatment center.
Patient(s): A total of 1,876 patients receiving infertility treatment between 2000 and 2012. Pregnancies with monozygotic twins (A: 23) were compared with deliveries of dizygotic twins (B: 423), singleton pregnancies (C: 880), and aborted pregnancies (D: 389).
Intervention(s): None.
Main Outcome Measure(s): A genetic survey on multiple pregnancies in the extended family. Measures were micromanipulation technique, the length of embryo cultivation, type of cultivation media, basal follicle-stimulating hormone level, estradiol level on the day of human chorionic gonadotropin administration, number of oocytes, total consumption of gonadotropins, and consumption of gonadotropins needed for recovery of 1 oocyte.
Result(s): No differences were found between the incidence of MZT in cycles that did vs. did not use micromanipulation techniques. In addition, the length of embryo cultivation or type of cultivation media used did not affect the results. Estradiol levels and implantation rates were significantly higher in group A. The incidence of MZT in families in group A was significantly higher than that in groups B and C.
Conclusion(s): We propose that the high incidence of MZT in infertility-clinic patients is conditioned by hereditary factors, and good ovarian function only facilitates the expression. The resulting recommendation is that young women with a positive family history and good ovarian function undergo elective single-embryo transfer, and proper counseling is advisable. (Fertil Steril® 2015;103:756–60. ©2015 by American Society for Reproductive Medicine.)
Key Words: Monozygotic twins, incidence, genetics, assisted reproduction techniques, infertility

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Evidence shows at least a two- to threefold rise in the incidence of monozygotic twinning (MZT) after assisted reproductive techniques (ART), compared with spontaneous conception (1–5). Depending on the time of cell division, we can diagnose bichorionic–biamniotic, monochorionic–biamniotic, or monochorionic–monoamniotic twins (1, 6).

Very little is known about the mechanisms involved in the increased incidence of MZT in the group of patients treated with ART. Several possible causes of this phenomenon have been discussed, including ovulation induction (2), micromanipulation techniques (3, 7–9), length of cultivation (5, 10–12), age of the patient (6, 13, 14), and glucose (15, 16) or glutathione concentration in cultivation media; however, no general agreement on the cause has been reached to date (4, 17).

Monozygotic twinning carries a higher risk of placental perfusion disorders (18), umbilical cord accidents (19), and developmental anomalies (20, 21). Mortality rates are always higher with monozygotic twins than with singleton births (22), posing an urgent need to identify the underlying factors behind this phenomenon. The possibility of a genetic contribution to MZT has been dismissed (1) or considered to be unimportant (23), but families with MZT have been described (24–26). However, no clear connection between the incidence of MZT in infertility patients and genetics has been demonstrated. The aim of the current study was to examine the probability of MZT in patients utilizing in vitro fertilization (IVF) and/or intracytoplasmic sperm injection (ICSI), relative to their age, ovarian function, and the ART used. We simultaneously organized a survey among IVF patients with multiple pregnancies in their families, to evaluate the possible role of genetic factors.

MATERIALS AND METHODS

The study group included 1,876 patients treated for infertility in our IVF center between 2000 and 2012. All patients came from the same region and were treated by 3 clinicians, who followed the same clinical and therapeutic guidelines. The diagnosis of MZT was made with the use of ultrasound, or on the basis of delivery of more babies than embryos transferred. We divided patients into 4 groups, according to pregnancy outcome: group A, monozygotic twins; group B, dizygotic twins; group C, singleton pregnancy; and group D, abortion.

We recorded patient age and implantation rate and the following 5 variables characterizing ovarian function: basal follicle-stimulating hormone (FSH), estradiol (E_2) level on the day of human chorionic gonadotropin administration, number of oocytes, total consumption of gonadotropins, and consumption of gonadotropins needed for recovery of 1 oocyte. The serum FSH concentrations were measured using a commercially available immunoradiometric assay immunoradiometric assay kit (Immunotech). The FSH assay was standardized against the World Health Organization second international standard 94/632 reference material (27). The sensitivity of the FSH assay was 0.2 IU/l, and the intra- and inter-assay coefficients of variability were 2.6% and 6.3%, respectively. Estradiol concentrations were measured using an immunoassay (ADVIA Centaur, Bayer). The sensitivity of the E_2 assay was 37 pmol/l, and the intra- and inter-assay coefficients of variability were 9.7% and 10.2%, respectively. In addition, we recorded the following variables characterizing IVF: the micromanipulation technique used; length of embryo cultivation; and type of cultivation media.

The parameters of ovarian function, age, implantation rate, and characteristics of IVF (ICSI, assisted hatching, and cultivation media) were compared in the 4 groups of patients. A genetic survey was given on the occurrence of multiple pregnancies in the extended family, as was done for groups A, B, and C with the use of a individual questionnaire. We recorded information for 3 generations (brothers and sisters, parents, grandparents, grandparents' sisters and brothers and their children, and all nephews and granddaughters). The questionnaire was sent to all patients. If answers were not clear, patients were contacted directly. Group D was not interviewed, for ethical reasons. The study was approved by an institutional review board.

In group A, we were able to contact 20 (87%) patients. All women from this group were interviewed in person. The patients from groups B and C were given a genetic questionnaire. We received 65% of the questionnaires from group B, and 55% from group C. The incidence of MZT in families was analyzed using a generalized linear model for binary responses (presence or absence). Only proven cases of MZT were subjected to analysis.

For statistical analysis of the data, we used only the first records from patients who were pregnant >1 time (n = 1,876). Of such patients, 1,715 (91%) couples had complete records for all parameters tested and were used in the statistical analysis of the data. We characterized the groups based on the mean and standard error (SE) values for all relevant predictors tested within the framework of generalized linear mixed models. We assumed a normal error distribution and used the year of data collection as a random effect on both the intercept and slope. Where appropriate (consumption of FSH per oocyte), variables were log-transformed before the analysis, to stabilize variance. The number of oocytes retrieved and the number of embryos transferred were assumed to follow a Poisson distribution.

The GLIMMIX Procedure of SAS, version 9.2 (SAS Institute, Inc.) was used to fit the generalized linear mixed models. Because we approximated the marginal log-likelihood with an adaptive Gauss-Hermite quadrature, we selected the best model according to the lowest Akaike information criterion (AICc). The difference in AICc (ΔAICc) between the best model and the second-best model >2 was considered evidence for the effect. Tukey–Kramer adjustments were applied for multiple comparisons between groups. A genetic survey on multiple pregnancies in families (up to 3 generations) was conducted for groups A, B, and C. Group D was not interviewed, for ethical reasons. The differences in percentages of MZT among groups A, B, and C were compared with the use of a generalized linear model for binary responses (presence or absence), and ΔAICc value >2 was used to identify the best model.

RESULTS

Of the data for 1,876 couples, data from 1,715 (91%) couples were used for statistical evaluation in which all evaluated parameters were properly recorded and available. Among the 1,715 couples investigated, we identified 23 couples in the MZT group (A). Sixteen (69.5%) MZT pregnancies arose from the division of 1 of the transferred embryos. In 6 (26.1%) patients, both of the transferred embryos divided into MZT embryos and resulted in 4 embryos. In 1 (4.3%)

patient, 1 transferred embryo divided into 3 monozygotic embryos. Three (13%) pregnancies ended during weeks 24–26 of pregnancy after embryo reduction. Group B had 423 (24.66%) patients; group C had 880 (51.31%) patients; and group D had 389 (22.68%) patients.

With the exception of basal FSH, all parameters of ovarian function (E_2 level, number of oocytes, total consumption of gonadotropins, and consumption of gonadotropins needed for recovery of 1 oocyte) and the implantation rate were better in group A (Table 1); however, statistical support was obtained for only the E_2 level (ΔAICc = 102.49; significant multiple group comparisons: AB, AC, AD, BC, BD, and CD). The amount of FSH needed to recover 1 oocyte (ΔAICc = 3.94; significant multiple comparisons: AC), and the implantation rate, were significantly different (ΔAICc = 76.45; significant multiple comparisons: AB, AC, and AD). The age of patients differed among the 4 groups (ΔAICc = 18.51; significant multiple-pair comparisons were for pairs AD, BC, BD, and CD).

When analyzing differences between response groups in terms of the micromanipulation technique used (ICSI: A, 100%; B, 98%; C, 97.8 %; and D, 96%), we could not decide which of the 2 models (models with and without effect) was better (ΔAICc = 0.28). The same was true with the percentage of MZT in cycles with (n = 1,754) or without (n = 122) assisted hatching (1.20% vs. 1.64%; ΔAICc = 1.84). No differences were found, owing to the length of embryo cultivation (1.34% after 48 hours of cultivation, n = 283 IVF cycles; 0.96% after 72 hours, n = 840 IVF cycles; 1.40% after 96 hours, n = 753 IVF cycles; and 1.81% after 120 hours, n = 110 IVF cycles; ΔAICc = 3.66). For the effect of type of cultivation medium, no difference was observed among the 4 groups (ΔAICc = 3.39). The incidence of MZT in the 3 media was as follows: QA cleavage medium (Sage), 1.55% MZT (n = 513); sequential cleavage medium (Origio), 1.08% MZT (n = 185); and Vitrolife (Vitrolife), 1.10% MZT (n = 1,178). The number of transferred embryos did not differ among the 4 groups (A: 2.28; B: 2.72; C: 2.58; D: 2.70), nor did FSH consumption (A: 1,987; B: 2,237; C: 2,257; D: 2,263).

We interviewed all women in group A, but were able to contact only 20 (87%) regarding the incidence of MZT in their families. In 7 families, no twins were identified. In 9 families, monozygotic twins were previously born without question; 2 families reported twins without an understanding of monozygosity, and in the other 2 families, the occurrence of dizygotic twins was reported. One man had monozygotic twins twice in his life. The first time, he and his former wife had monozygotic twins after IVF with ICSI in our center, and subsequently, he had MZT spontaneously with his then wife. In 9 group-A couples with positive MZT anamnesis, we reported 51 families with a total of 19 (37%) monozygotic twins. Using a generalized linear model for binary responses, we revealed among-group differences in the percentages of MZT in patient families (ΔAICc = 21.04). The group-A patient families had 45% proven cases of MZT up to the third generation. We received 65% of the genetic questionnaires for group B, and 55% for group C. In groups B and C, the occurrences of MZT in families were 13.4% and 9.5%, respectively. Genes for monozygosity were more often transferred through women (8) than through men (4).

DISCUSSION

Although the increased incidence of MZT after assisted reproduction is widely recognized, general mechanisms underlying this phenomenon are poorly understood (23). Using an extensive data set involving the performance of IVF patients, we focused on the effects of ovarian function, micromanipulation, and genetic factors. We found that the incidence of MZT is associated with elevated levels of E_2 and low FSH consumption per oocyte. In contrast, micromanipulation techniques or the type of cultivation media did not have an effect on the frequency of MZT. A detailed inquiry into the hereditary components in families of patients revealed evidence for a genetic background underlying MZT.

A questionnaire given to all patients is unlikely to be absolutely correct, but the failures are expected to be equally common in all interviewed groups. For the patients aged 30–35 years, ultrasound was not available to all pregnant women when they were born, and even now, ultrasound detection of monozygotic and dizygotic twins may

TABLE 1

Several variables of ovarian function estimated for 4 response groups of patients by fitting the generalized linear mixed model.

Variable	Monozygotic twins (A)	Dizygotic twins (B)	Singletons (C)	Abortions (D)
No. of patients	23.0 (1.34)	423.0 (24.66)	880.0 (51.31)	389.0 (22.68)
Age (y)	29.3 (0.9) NS	29.6 (0.3)	30.2 (0.3)	31.1 (0.3)
Basal FSH (IU/l)	6.97 (0.50) NS	6.56 (0.14)	6.79 (0.11)	6.86 (0.15)
E_2 (pmol/l)	4,192.0 (123.0) S	1,168.0 (38.0)	1,520.0 (49.0)	1,308.0 (42.0)
Oocytes retrieved	14.4 (1.0) NS	12.8 (0.5)	12.3 (0.4)	12.4 (0.5)
Total FSH consumption (IU)	1,987.0 (154.0) NS	2,237.0 (43.0)	2,257.0 (34.0)	2,263.0 (45.0)
FSH consumption/oocyte (IU)[a]	180.69 (5) NS	223.39 (2.0)	258.54 (2.0)	269.63 (2.5)
Implantation rate (%)	126.0 (0.05) S	84.0 (0.03)	46.0 (0.02)	49.0 (0.03)
No. of embryos transferred	2.28 (0.32) NS	2.72 (0.11)	2.58 (0.09)	2.70 (0.11)
Positive MZT family history (%)	45.0	13.4	9.5	NA

Note: Values are mean (SE). Based on the ΔAICc, the age, E2, FSH consumption per oocyte and implantation rate affected the means among patients with monozygotic twins, dizygotic twins, singleton pregnancies, and abortions. The parameters were estimated by fitting the generalized linear mixed models containing age of patients to account for age difference among groups. NA = not applicable.

[a] The variable was log-transformed before parameter estimation.

Sobek. Genetic basis of monozygotic twinning. Fertil Steril 2015.

be in error. A deceased grandmother who was born as a twin and survived could not be confirmed. We believe that biases of this kind were equal in all groups. In addition, we are aware of the fact that twinning resulting from a single embryo transfer is not necessarily monozygotic. The twinning could be instead a spontaneous conception superimposed on the pregnancy that resulted from IVF, as previously described by Mains et al. (28); the risk of this phenomenon is probably low.

The genetic background of MZT has been described in the general population (24, 29), but the genetic background of MZT has rarely been discussed in the context of ART (30). The division of embryos in MZT follows an autosomal dominant pattern of inheritance with low expression and transfer by males or females. In agreement with the higher incidence of gonosomal mosaicism after ICSI, which is not related to ART procedures but correlates with genetic information in the oocyte (31–35), we found that the incidence of MZT does not increase with the use of ART. Instead, the main cause of this phenomenon is, we believe, a characteristic of the patient. Autosomal dominant genetic information "to produce MZT" is enhanced in ART with the use of stimulation, a higher number of oocytes with good quality, a higher implantation rate, and a low abortion rate.

A close link between genes for embryo division, zona pellucida architecture, and abnormal mucopolysaccharide synthesis in the zona could account for female-only participation in MZT (7); however, the genes for MZT are transferred through males as well, and we have to seek an explanation for this phenomenon in the embryo. The embryo can divide into 2 and sometimes 3 parts (36). This phenomenon has been observed in animals (37). The genes for MZT were expressed in approximately 37% of all families in our group of 20 MZT pregnancies for which a response on the survey was obtained.

The same expression was reported by Harvey et al. (24) in one Indian family. The low penetrance of this attribute can be explained by the Menezo theory (16) of linear polarization of embryos by apoptosis (38, 39). Apoptosis can be regulated by genes (38). We hypothesize that embryos that divide 50:50 undergo MZT. In embryos dividing unequally (30:70), only the larger embryo survives. The smaller embryo grows for a limited time and eventually vanishes before clinical detection. Such unequal division and separation of blastoceles was first described by Malter and Cohen (40), and documented by Aston et al. (23). Further biological MZT reduction can be expected during implantation (37). A higher incidence of MZT in the group of women with high fecundity was likely caused by the high implantation rate of embryos of high quality (37).

The autosomal dominant transfer of genes for MZT can be documented by the fact that 22% of all couples had >1 embryo divide. The same experience has been described by Abusheika et al. (6). In one case, a single embryo divided into 3 fetuses. Salat-Baroux et al. (30) reported 3 monozygotic embryos in 1 gestational sac. The incidence of monozygotic twins in women treated for infertility was threefold higher than that in women who conceived spontaneously. This finding can be explained by a higher number of embryos per embryo transfer, as previously described by Sills et al. (3) (3.6 embryos per ET), and others: 2.5–3.9 per ET (10); 4 per ET (30); 2–3 per ET (6); 2.8 per ET (4); 3.2 per ET (7); 1–6 per ET (12), and/or by a higher quality of embryos generated by stimulation (2).

The group of patients with MZT was characterized by lower age and excellent ovarian function (high E_2 level, high number of oocytes recovered, low consumption of FSH needed for stimulation of 1 oocyte, and high implantation rate). The E_2 level was significantly higher in the MZT group, which is an expression of good ovarian function, and we believe, not the trigger for embryo division. Indeed, embryo division is possible in natural cycles with average E_2 levels.

High-quality oocytes produced in the MZT group resulted in premium-quality embryos with a high implantation rate (in 7 couples, >1 embryo divided, and in 1 patient, a single embryo split into 3 fetuses). We assume that such embryos have a better chance of surviving MZT reduction than nonselected embryos from a natural cycle. If those embryos are "genetically" prone to produce monozygotic twins, and if we implant more such embryos, then more monozygotic twins may be born after IVF. In the past 2 years, we most often performed single-embryo transfers in our center, and we did not detect MZT during this period, which provides support for this possibility. The high implantation rate of embryos in highly fertile women in our group of MZT patients was in agreement with the rates found by Blickstein and Keith (37) and Bortolus et al. (41).

The results suggest that the incidence of MZT is under the control of hereditary factors and is enhanced in ART by excellent ovarian function. To reduce the incidence of MZT, collecting information on the family history of MZT before the actual stimulation process is advisable, so that elective single-embryo transfer can be performed in women with excellent ovarian function and a positive family history of MZT. Consideration on an individual basis of whether to recruit such women as oocyte donors is appropriate, and proper counseling is advisable.

In conclusion, micromanipulation techniques, length of cultivation, and type of cultivation media did not influence the incidence of MZT. We believe that the incidence of MZT is under the control of hereditary factors and enhanced in ART by excellent ovarian function. The MZT gene is inherited autosomally dominantly, and the gene is transferred from both parents. Collecting information on the family history of MZT before the actual stimulation is important, as is making this information part of the decision-making process regarding stimulation policy. To reduce the incidence of MZT, performing elective single-embryo transfer in women with excellent ovarian function and a positive family history of MZT is advisable.

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12.4. Appendix 4

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High incidence of monozygotic twinning in infertility treatment

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Background. Monozygotic twinning is associated with increased perinatal morbidity and mortality. There is evidence that the number of monozygotic twins increases after assisted reproductive techniques.
Methods. We searched PUBMED, MEDLINE, and Scopus from 1987 to 2015 for studies analyzing the incidence and possible etiology of monozygotic twinning in infertility patients and critically reviewed the current state of knowledge.
Results and Conclusions. Monozygotic twinning is a rare in natural conception but occurs around twice the normal rate after assisted reproduction. Factors associated with this phenomenon remain speculative, though there is some evidence that micromanipulation techniques, prolonged culture, and genetics are involved. In view of the possible complications, adequate pre-conception counselling is advocated.

Key words: monozygotic twins, infertility, incidence, risk factors

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INTRODUCTION

It is well known that multiple births occur more often after assisted reproductive technologies (ART) than after spontaneous conceptions. The higher incidence of dizygotic twins arising from the transfer of two embryos is an understandable consequence of ART but the mechanism of monozygotic twinning after ART is still unclear. Monozygotic twins occur when the fertilization of one oocyte by one sperm produces genetically identical twins. Monozygotic twinning (MZT) is a relatively rare phenomenon, with an incidence of about 1% of natural conceptions. In assisted conceptions, the risk of monozygotic twinning has been estimated to be about twice as high[1]. Multiple births are generally associated with many maternal and fetal complications, which are more severe in MZT that share a single placenta. Therefore, understanding the mechanism of increased monozygotic twinning in ART is important and could minimize the incidence of such high-risk pregnancies. We reviewed the scientific literature on the etiology, frequency, risk factors, and complications associated with MZT in patients treated with ART.

MECHANISM OF MONOZYGOTIC TWINNING

Monozygotic twins arise from one zygote which divides into two separate individuals. The time at which the embryo divides is a critical factor in subsequent placental development, and the risk of complications is related to placental sharing[2]. Division on days 1–4 (morula), before

Days after fertilization	1–4	4–8	8–12
Twins produced	dichorionic–diamniotic	monochorionic–diamniotic	monochorionic–monoamniotic

Fig. 1. Type of monozygotic twins depends on the time of division.

the chorion has differentiated, results in dichoriotic–diamniotic twins (20%), which are indistinguishable from dizygotic twins resulting from two embryos. Division on 4–8 days (blastocyst) results in monochoriotic–diamniotic twins (75%), and division on 1–2 weeks results in monochoriotic–monoamniotic twins, which share one placenta and one amniotic sac. Conjoined twins result from division after day 12.

Monozygotic twinning has been induced experimentally in animal models. In 1921, Stockard[3] demonstrated an increased incidence of MZT in fish after either the available oxygen or the incubation temperature was reduced. Similarly, increased MZT has been identified in mice under in vitro conditions, although mice do not normally show MZ twinning in natural cycles[4]. MZT were also induced by delayed fertilization in rabbits[5]. In vitro conditions also seem to be related to increased monozygotic twinning in humans. The reason for this phenomenon has been discussed for almost three decades and several potential mechanisms have been proposed. Earlier studies examined the effect of ovulation induction, whereas later research concentrated on the effects of micromanipulation techniques and the length of culture. Recent studies have identified a possible genetic etiology[6]. The rate of monozygotic twinning after natural conception varies only slightly in the literature, from 0.4% (ref.[1]) to 1.6% (ref.[7]). In contrast, studies of assisted conceptions show wider variance, ranging from 0.72% (ref.[8]) to 12.5% (ref.[9]). Generally, the incidence of monozygotic twinning after assisted reproductive techniques is about twice as high as after natural conceptions, although some large studies have detected no association between MZ twinning and infertility treatment[10].

Micromanipulation techniques

The routine application of intracytoplasmic sperm injection (ICSI) and assisted hatching (AH) has prompted discussion of a possible connection between micromanipulation techniques and increased MZT. To successfully implant into the uterine wall, an embryo must hatch out of the zona pellucida (ZP), a protein layer covering the embryo in the initial stages of development, and attach to the inner lining of the uterus. Both techniques (ICSI and AH) manipulate the ZP in different ways. ICSI involves direct injection of a spermatozoon into the oocyte. The technique requires a very small number of sperm and allows the use of sperm with limited motility. The other technique, AH, involves mechanical, chemical, or laser incision of the ZP of a fertilized embryo. AH has developed based on the observation that embryos with a thin ZP have a higher rate of implantation after *in vitro* fertilization (IVF). Both of these techniques (ICSI and AH) leave small defects in the ZP, which may complicate the natural process of embryo hatching. The embryo may bypass its own mechanism of ZP lysis and herniate through these defects, thus resulting in MZT (ref.[11]). If so, the size of the defect will probably affect the rate of MZT. There is a distinct difference in the size of the artificial breach in the ZP when ICSI and AH are used. The zona opening formed by AH is 25–30 µm in diameter, whereas the puncture site following ICSI is much smaller (7–8 µm in diameter) (ref.[12]). In theory, a smaller hole would not allow the embryo to hatch appropriately and MZT would be more common in ICSI than AH patients. This finding is in agreement with the results of Vitthala[11], who collected data from 27 studies in a meta-analysis, and concluded that couples who underwent ICSI had a higher MZT rate than couples who underwent conventional IVF or AH. In contrast, Luke et al.[2] reported a greater effect after AH, although only in 2–3 day embryos (cleavage stage). The same observation was recently reported by Kanter et al.[13], who showed that early stage embryos may be more vulnerable to the effect of AH than blastocysts. Unlike the previous results, there are also reports, although involving a smaller number of patients, which show that micromanipulation techniques have no effect on MZT (ref.[14,15]). It is apparent that larger studies with statistical power are needed to determine whether or not micromanipulation of the ZP increases the rate of monozygotic pregnancies in IVF patients.

Length of culture

It has previously been reported that culture to the blastocyst stage (day 5) in women with good-quality embryos may facilitate embryo selection, reduce aneuploidy embryos[16] and improve live birth rates[17]. Rijnders[18] and Peramo[19] were the first to report an association between the incidence of MZT and blastocyst transfer. They observed a significant difference between the MZT rate after embryo transfer (ET) on day 3 (0.68%) and transfer on day 5 (2.7%). Da Costa[20] reported that 3.9% of pregnancies generated by blastocyst transfer were complicated by MZT, and Behr[21] detected an incidence of 5%. Wrigth et al.[22] examined the 1999–2000 data from the American Society of Assisted Reproductive Technologies (SART) for 39,198 pregnancies. The incidence of MZT was four-fold higher after embryo transfer on day 5 than after embryo transfer on day 3. Similarly, a more recent and larger study that examined data from the SART showed that MZT was more likely to develop from embryos transferred on days 5–6 than from cleavage embryos[2].

Conversely, Franasiak et al.[23] recently reported that transfer in the blastocyst stage is not associated with increased MZT rates when controlling for embryo quality based on a cohort of 342 monozygotic pregnancies.

To explain the possible mechanism by which prolonged cultivation affects the MZT rate, most theories center on hardening of the ZP (ref.[20]). Zona hardening may squeeze the inner cell mass (ICM) and induce embryo splitting during hatching[24]. Increased MZT might also correlate with the transfer of high-quality embryos, which are more often transferred after prolonged culture. These embryos are more sensitive to the effects of mechanical manipulation in the laboratory or to changes in temperature and pH during monitoring[2], which might result in higher rates of MZT after blastocyst transfer.

Culture medium conditions

Studies of animals have shown that mice blastocysts duplicate their ICM more frequently in vitro than in

vivo[25]. A similar phenomenon can be expected in humans. Steinman[26] speculated that the prolonged exposure of blastocysts to lower calcium levels in the culture medium could enhance ICM division because the intercellular bonds are destabilized. Others believe that changes in the culture medium, including an absence of growth factors and a higher glucose content, could produce free radicals, which induce apoptosis, leading to the disruption of the ICM and presumably zygotic splitting[22]. Similarly, Behr[21] suggested that the current culture media cause perturbations of the cell-to-cell adhesions, facilitating the splitting of the ICM. Several researchers also detected a possible association between temperature changes and a higher incidence of MZT in animal models[3]. However, no association has been demonstrated between the transfer of thawed embryos and monozygotic twinning in humans[27].

Genetics

Autosomal dominant inheritance with reduced penetrance has been proposed as the possible etiology for familial MZT. Inheritance has been reported to occur through the maternal and paternal lines[28]. Hamamy[29] reported a high incidence of MZT (n=13) in an extended Jordanese family. Shapiro[30] investigated 10 families with multiple pairs of monozygotic twins born to parents who were also born as monozygotic twins. In a recent study, Sobek et al.[6] documented that 45% of the group of women who had monozygotic twins also had a family history of MZT. The results of those studies suggested that the incidence of MZT might be under the control of hereditary factors with genes transferred similarly by both the male and female parents. In the past, the transfer of an increased number of embryos was common in an effort to increase fertility rates. In studies reporting a higher incidence of monozygotic twins, the average rate was 2–4 embryos/ET (3.6 embryos/ET[12], 3.2/ET[24], and 2.8/ET[8]). Theoretically, if some of the embryos were "genetically" prone to produce MZT, and more of such embryos were implanted, MZT might have been increased after IVF in those studies than the normal population.

Ovulation induction

Derom et al.[1] reported an increased incidence of MZT in patients after the induction of ovulation with gonadotrophins or clomifen citrate. They believed that medication can alter the structure of the ZP, making the embryo more vulnerable to ICM splitting.

Age

Later maternal age is considered by many as the only factor that increases the frequency of monozygotic twinning in natural cycles[31] A gradual reduction in the thickness of the ZP with increasing age in women has been reported[32]. A thinner ZP could be more vulnerable to inner cell protrusion at multiple sites during zona lysis, facilitating the division of the ICM. In contrast, a recent study by Knopman et al.[27] found increased MZT rates in women < 35 years old. However, it must be noted that the rate of blastocyst transfer, which is believed to increase monozygotic twinning, is higher in younger women with good-quality embryos than in women over 35 years old, which could confound these results.

Preimplantation genetic diagnosis (PGD)

Verpoest et al.[33] were the first to report the incidence of MZT after preimplantation genetic diagnosis (PGD). They assumed that the incidence of MZT was increased by breaks in the ZP associated with blastomere biopsy, similar to those formed by micromanipulation techniques. They found higher a incidence in the group treated with PGD than in the group treated without it, but the difference was not significant (2.1% vs 1.5%, respectively).

Complications of monozygotic twins

Multiple pregnancies are associated with an increased risk of maternal and fetal complications. Women carrying twins more frequently suffer from nausea, hypertension, and pre-eclampsia. Multiple pregnancies incur a higher risk of perinatal morbidity, mortality, prematurity, and growth restrictions. MZT pregnancies are associated with a perinatal mortality rate at least three-fold higher than that associated with dizygotic twin pregnancies[34], and the twins are also at greater risk of perinatal morbidity associated with prematurity than are dizygotic twins. They display higher rates of fetal abnormalities, including neural-tube defects, congenital heart diseases, limb reduction defects, and deformities. The risk of congenital anomalies in MZT is about 10% (ref.[5]). The placental arrangement in monozygotic monochorionic twins poses additional risks. Monochorionic twins share one placenta and tend to suffer hemodynamic complications. These include twin-to-twin transfusion syndrome (TTTS), twin embolization syndrome (0.1%), reversed arterial perfusion syndrome, umbilical knots, and thrombosis. TTTS is the commonest disorder and occurs in response to unbalanced vascular communication in the placenta. Vascular anastomosis are present in more than 90% of monochorionic pregnancies[35], but do not usually cause problems. When the placenta is unequally shared, blood can be transfused disproportionately from one twin (the donor) to the other twin (the recipient). This transfusion causes the donor twin to lose blood volume, whereas the recipient twin must deal with an overload of blood. The excess blood exerts abnormal strain on the heart of the recipient fetus, causing the development of polyhydramnion and eventual heart failure. In the donor twin, the loss of blood leads to slower growth, and poor urinary output, causing oligo- or anhydramnion. TTTS is a severe complication in MZT, accounting for 10%–16% of perinatal mortality[36].

DISCUSSION

The major limitation in the research into MZT is the extremely low incidence of monozygotic twinning, even in patients treated for infertility. Monozygotic twins are a rare phenomenon and very large studies would be required to obtain satisfactory statistical power. Studies of the effects of culture conditions are limited because the media used, the sources of mineral oil, and the plastic-

ware used change over time. They can also be obtained from different suppliers, which makes any comparison even more difficult. Another factor limiting the accuracy of the studies is the actual identification of monozygotic twins. The only reliable identification of monozygotic twins is by ultrasound after single-embryo transfer or by DNA analysis of the twins born when more than one embryo was transferred. Most studies identify MZT from first-trimester ultrasound data based on the number of fetuses present relative to the number of embryos transferred, which identifies approximately two thirds of MZT (ref.[11]). The reasons for the higher incidence of MZT after ART most commonly discussed in the literature are micromanipulation techniques, blastocyst transfer, genetics, maternal age, and culture medium and conditions. Most of the larger studies and meta-analyses agree on a possible association between prolonged culture or micromanipulation techniques and the higher incidence of monozygotic twinning in women treated for infertility. There is also growing evidence of genetic background of this phenomenon.

CONCLUSION

MZT is a rare phenomenon that occurs more often after assisted conception than natural conception. The reasons for the increased MZT in IVF patients have been widely discussed but no general consensus has been achieved. The major limitation of studies on MZT is the very low incidence of MZT in natural and IVF cycles. Thus, only large sample size studies provide sufficient statistical power. The current data show that several factors might contribute to the overall increased rate of MZT using assisted reproductive techniques. The majority of larger studies agree on the possible impact of micromanipulation techniques and prolonged culture, some studies suggest genetic influences but the results differ. In view of the complications arising from MZT pregnancies, proper counselling of patients before infertility treatment and examination of chorionicity in the early weeks of gestation are essential.

ABBREVIATIONS

AH: Assisted hatching; ART: Artificial reproductive technology; ET: Embryo-transfer; ICSI: Intracytoplasmic sperm injection; ICM: Inner cell mass; IVF: In vitro fertilization; MZT: Monozygotic twinning; TTTS: Twin-to-twin transfusion syndrome; PGD: Preimplantation genetic diagnosis; ZP: Zona pellucida.

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12.5. Appendix 5

Triple pregnancy with mixed chorionicity following *in vitro* fertilization (IVF): is fetal reduction neccesary ?

Trojčetné těhotenství se smíšenou chorionicitou po léčbě IVF: je fetoredukce vhodná ?
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Abstract

Objective: The increasing incidence and management of monozygotic twinning in patients undergoing *in vitro* fertilization (IVF) has been the subject of much debate. Here, we describe the management and outcome of two triple pregnancies with mixed chorionicity with a monochorionic-diamniotic twin pair and a singleton following the transfer of two embryos during IVF treatment.

Design: Case report

Setting: Department of Obstetrics and Gynaecology, Palacký University Hospital, I. P. Pavlova 6, 77520 Olomouc, Czech Republic. Prof. MUDr.Radovan Pilka, PhD.

Methods: This study involved Patient A (30 years of age; 0 Para) and Patient B (32 years of age; I Para), both with triplets of mixed chorionicity following the transfer of two embryos during IVF treatment, and treated in The Fetal Medicine Centre, Palacky University Olomouc. Detailed counselling led to the deployment of different management strategies for each case.

Results: The monochorionic twin component of Patient A was terminated by fetal reduction in the 15th week of gestation, while the remaining single pregnancy was delivered at term without complication. Patient B opted for expectant management. However, the pregnancy was complicated by severe maternal morbidity and was terminated in the 28th week of gestation following the death of one fetus.

Conclusion: Fetal reduction should be offered as a management tool to patients carrying triplets in order to improve perinatal survival. In triplets with mixed chorionicity, the reduction of monochorionic twins is particularly advisable in preventing the additional risk posed by a shared placenta.

Keywords: Embryo transfer, *in vitro* fertilization, triple pregnancy, embryo reduction

Souhrn: Cíl studie: Zvýšený výskyt monozygotních vícečetných těhotenství po léčbě asistované reprodukce je tématem mnoha studií. V této práci popisujeme průběh dvou trojčetných těhotenství po transferu dvou embryí v rámci léčby metodami asistované reprodukce, u kterých byla zvolena různá strategie.

Typ studie: Case report

Název a sídlo pracoviště: Gynekologicko-Porodnická klinika, FNOL, I. P. Pavlova 6, 77520 Olomouc, Prof. MUDr.Radovan Pilka, PhD.

Metodika: Pacientka A byla 30 letá 0 Para a pacientka B byla 32 letá I Para. U obou bylo v I.trimestru diagnostikováno trojčetné těhotenství se smíšenou chorionicitou-biamniální monochoriální dvojčata a jednočetné těhotenství. Po podrobné konzultaci se obě rozhodly pro odlišnou strategii vedení těhotenství.

Výsledky: U pacientky A byla monozygotní komponenta ukončena fetocidou v 15.týdnu gravidity a pacientka porodila zbylý plod v termínu bez dalších komplikací. Pacientka B se rozhodla pro observaci těhotenství bez další intervence. Těhotenství bylo komplikováno rozvojem těžké mateřské morbidity a ukončeno císařským řezem ve 28.týdnu gravidity po úmrtí jednoho z plodů.

Závěr: Párům s troj-a vicečetným těhotenstvím byla měla být nabídnuta možnost fetoredukce. U trojčat se smíšenou chorionicitou je vhodné zvážit redukci monochoriální komponenty a předejít tak riziku, které navíc představuje sdílená placenta.

Klíčová slova: Embryo transfer, *in vitro* fertilizace, trojčetná gravidita, fetoredukce

Introduction

Assisted reproduction is associated with an increased incidence of multiple pregnancies. [15] Furthermore, recent data show that the incidence of monozygotic twins in this group of patients with multiple pregnancies is higher compared to spontaneous conception. [13, 17] Monozygotic twins have an 80% chance of being monochorionic and have a 6-fold higher abortion rate before 24 weeks of gestation and a 3-fold greater risk of stillbirth and early neonatal death than dichorionic twins. These problems predominantly arise because the placenta is not always shared equally between such twins and by the presence of arterio-venous shunts. [7] In triple pregnancies, monochorionic placentation (MC) poses additional risk factors; in fact, triplets with MC placentation have the highest risk for adverse pregnancy outcome compared with trichorionic pregnancies [6] increasing the risk of fetal growth restriction (FGR), intrauterine fetal death, preterm delivery and weight discordance [2,3,6]. Consequently, the clinical management of such cases is complex and is frequently debated. However, it is generally accepted that selective reduction in higher-order multiple pregnancies has a positive impact upon the duration of gestation but may increase the risk of intrauterine fetal death. [19]. An expectant management strategy, on the other hand, might increase the risk of preterm birth and long-term morbidity of the fetus. [1] The purpose of the present study was to compare the outcome of two triple pregnancies, each with a monochorionic twin component, and discuss how these cases were treated by differing clinical management strategies.

Materials and Methods

In 2016, two patients with dichorionic triamniotic (DCTA) pregnancies were referred to The Fetal Medicine Center at Palacky University during the 12 th week of gestation following IVF treatment. In both cases, embryo-transfer (ET) was carried out at the blastocyst stage and two embryos were transferred to each patient. For both patients, the embryonic disk split following ET, resulting in triamnial triplets with mixed chorionicity. Patient A (0 Para) was 30 years of age while Patient B (I Para) was 32 years of age. Both patients were examined and carefully informed about the risk and

prognosis of triple pregnancies with a monochorionic component. In both cases, we offered fetal reduction.

Patient A opted for selective fetal reduction of the monochorionic twins. The procedure was, however, delayed due to the presence of a sub-placetal hematoma which led to the procedure being postponed until the 15th week of gestation. The procedure was subsequently carried out without complication via the intra-cardiac injection of 1 ml of 7.5% potassium chloride (KCl) using a transabdominal needle guided by ultrasound. Afterwards, serial ultrasound scans were used to screen the patient for potential aberrations in fetal growth and disturbances in the volume of amniotic fluid.

Patient B, however, opted to continue the pregnancy without intervention. Ultrasound diagnostic screening was performed on a weekly basis. We paid particular attention to a range of Doppler parameters, including the pulsatility index and peak systolic velocity of the middle cerebral artery, the pulsatility index of the umbilical artery and ductus venosus, the cerebroplacental ratio and amniotic fluid disturbances. In addition, we analysed the biometry of all foetuses on a bi-weekly basis.

Results

Fetal reduction (FR) was performed upon Patient A in the 15th week of gestation without complications. Subsequently, serial ultrasound scans detected no pathology and the remaining fetal material was absorbed. The remaining pregnancy was terminated at 40+1 weeks of gestation via the induction of labour owing to a suspicion of placental insufficiency, as indicated by abnormal Doppler parameters. A healthy child, weighing 3220 g, with an APGAR score of 10-10-10, was born without complication and postnatal adaptation was excellent.

The pregnancy of patient B was complicated by hypertension from the 16th week of gestation and by the development of gestational diabetes mellitus from the 24th week. Growth restriction stage I and anhydramnion were detected in the fetus with its own placenta and the patient was hospitalized in the 25th week due to signs indicative of

preeclampsia. We administered two intra-muscular doses of Betamethasone (14 mg) to help the fetal lungs mature in accordance with the standard protocol. Following extensive counselling, Patient B opted for the expectant management of her pregnancy. During the 28th week, we confirmed the intrauterine death of the fetus with its own placenta, and diagnosed growth restriction stage I of the remaining monochorionic fetuses. Ophthalmological examination of Patient B revealed hypertonic retinopathy grade II. After consultation with a gynaecologist, neonatologist and psychologist, Patient B decided to terminate her pregnancy and Caesarean section was performed during the 28th week. Monochorionic twins were delivered, with birth weights of 940g and 780g, both with an APGAR score of 8-8-10. Afterwards, Patient B was monitored for preeclampsia and diabetes; medication was adjusted repeatedly before the patient was discharged from hospital care.

Discussion

The prevalence of monochorionic twins in triplet pregnancies following spontaneous conception is approximately 40% [1] and 10% after assisted conception [11]. Dichorionic triplets have a perinatal mortality rate which is eight times higher than trichorionic pregnancies [3] and thus represent a specific subgroup for triplets which is associated with the highest complication rate.

This case report focuses upon the clinical management and outcome of two patient cases, each with triple pregnancies with a monochorionic-diamniotic twin pair and a singleton following the transfer of two embryos during IVF treatment. When couples are faced with the dilemma of dichorionic triamniotic (DCTA) pregnancy, the first option is to adopt a conservative approach. Attempting to continue a pregnancy with all fetuses is associated with a high risk of perinatal morbidity and mortality caused mainly due to premature delivery [1]. Abel et al. [1] evaluated data from 47 triplet pregnancies with monochorionic twin pairs and found that the risk of premature delivery <30 weeks was significantly higher in non-reduced pregnancies compared to pregnancies which had been reduced (25% versus 0%) and in deliveries <34 weeks (88% versus 3%). A high proportion of triplets (28 %), managed in an expectant manner, were also shown to

be complicated by twin to twin transfusion syndrome. [1] In contrast, FR has been associated with a rate of miscarriage approximately two times higher than that seen in pregnancies which are managed expectantly. [14] A slightly higher early miscarriage rate was found in cases involving selective fetal reduction from 3 to 1 than in cases involving 3 to 2 reductions (22% versus 17%). [1] There are two predominant mechanisms underlying miscarriage following FR. The first of these relates to procedure-related trauma or infection, in which miscarriage would be expected within 2 weeks of FR.

The second relates to the consequence of the mother resorbing dead feto-placental tissue, which is likely to result in miscarriage several weeks, or even months, after FR. [10]

A variety of techniques have been proposed for the reduction of multifetal pregnancies, including the transvaginal [8] or transabdominal [4] administration of KCl or NaCl directly into the fetal heart [20], the aspiration of embryos during the early weeks of gestation [5,11] or umbilical cord coagulation. [8] Pregnancy loss from such procedures is known to vary from 5% to 30%. [8] In dichorionic triplets the most common approach is the reduction of a monochorionic pair to prevent the negative impact of a shared placenta. Reduction of the singleton, and maintenance of the monochorionic pair, is a less common option, as this procedure is associated with the higher rate of complications. Furthermore, Rong et al. [16] investigated 35 pregnancies and showed that monochorionic twins were associated with a higher rate of late miscarriage than retained singletons (18.5% versus 0 %); retained twins also suffered from a premature birth rate (11%) and lower birth weight in comparison to a retained singleton.

The first visit was planned in the 12 th week to meet criteria for the first trimester screening. After the results were obtained, we planned the strategy for ongoing pregnancy. The policy in our own department is to offer fetal reduction of a monochorionic pair to patients with DCTA. In each case, however, the final decision rests with the patient. In cases of trichorionic triplets, we offer fetal reduction for twin

pregnancies, which is in line with the recommendations of Wimalasundera and Van de Mheen, [18,19] who indicated that the reduction of triplets to twins resulted in a significant reduction of risk associated with preterm delivery and intrauterine fetal death.

Conclusions

We suggest that fetal reduction should be offered to patients with triplets as a clinical management tool to improve perinatal survival. In triplets with mixed chorionicity, which are associated with the highest rate of complications, we emphasize the reduction of monochorionic twins to prevent the additional risks posed by the shared placenta. While previous studies have reported higher miscarriage rates for pregnancies undergoing multifetal reduction compared to an expectant management plan, it is important that these concerns are balanced against the lower risk of prematurity and fetal morbidity.

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