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Tel. +514-4893242 - Fax +514-4854513 - E-mail: canlux@mgroun-online.com - www.irog.net

Editorial Office (M. Critelli):

Galleria Storione, 2/A - 35123 Padua (Italy) - Tel. (39) 049 8756900 - Fax (39) 049 8752018

CLINICAL AND EXPERIMENTAL OBSTETRICS AND GYNECOLOGY (ISSN 0390-6663) publishes original work, preferably brief reports, in the fields of Gynecology, Obstetrics, Fetal Medicine, Gynecological Endocrinology and related subjects. (Fertility and Sterility, Menopause, Uro-gynecology, Ultrasound in Obstetrics and Gynecology, Sexually Transmitted Diseases, Reproductive Biological Section). The Journal is covered by **INDEX MEDICUS, MEDLINE, EMBASE/Excerpta Medica**.

CLINICAL AND EXPERIMENTAL OBSTETRICS AND GYNECOLOGY is issued every three months in one volume per year by IROG CANADA Inc. Montréal. Printed in Italy by "La Garangola", Tipografia Editrice - Via E. Dalla Costa, 6 - 35129 Padova (Italy).

EDITORIAL ARTICLES

- Advances in oocyte cryopreservation - Part I: slow cool rapid thaw technique** 237
 J.H. Check - *Camden, NJ (USA)*
 Fertilization by intracytoplasmic sperm injection and use of sodium depleted choline substitute culture media has allowed successful pregnancies from the fertilization of frozen oocytes using slow-cool methodology.
- Regulatory mechanism of *Bcl-2* in uterine leiomyomas** 240
 N. Ohara - *Kobe, JAPAN*
 The regulatory mechanism of *Bcl-2* in uterine leiomyomas is described.

REVIEW ARTICLES

- High-grade cervical intraepithelial neoplasia, human papillomavirus and factors connected with recurrence following surgical treatment** 242
 M.C. Ramos, B.H. Pizarro De Lorenzo, M.A. Michelin, E.F.C. Murta - *Uberaba, MG, BRAZIL*
 Surgical margins, mitoses, methylated genes, HPV18 and local CD3 T lymphocytes are related with recurrence of CIN after surgical treatment.
- Human papilloma virus in adolescence** 248
 P. Christopoulos, K. Papadias, K. Panoulis, E. Deligeoroglou - *Athens, GREECE*
 Basic and advanced knowledge of HPV and guidelines need to be focused on adolescents, as they form a group of special interest with different needs.

ORIGINAL ARTICLES**Reproductive Biology Section**

- Correction of failed fertilization despite intracytoplasmic sperm injection with oligoasthenoteratozoospermia but with acrosomes present by oocyte activation with calcium ionophore - case report** 252
 J. Pinto, J.H. Check - *Camden, NJ (USA)*
 Artificial oocyte activation by calcium ionophore can result in fertilization and embryo transfer using sperm with acrosomes present previously but failing to fertilize eggs despite intracytoplasmic sperm injection.
- Early endometrial changes following successful implantation: 2 and 3-dimensional ultrasound study** 255
 E. Zohav, I. Bar-Hava, S. Meltzer, J. Rabinson, E.Y. Anteby, R. Orvieto - *Beer Sheva, ISRAEL*
 During an IVF cycle the dynamic changes in endometrial thickness and volume between day of ET and two weeks later may predict IVF treatment outcome.

General Section

- Increased expression of GRP94 protein is associated with decreased sensitivity to adriamycin in ovarian carcinoma cell lines** 257
 L.Y. Zhang, X.C. Zhang, L.D. Wang, Z.F. Zhang, P.L. Li - *Harbin, CHINA*
 High basal level of GRP94 is correlated with an obvious decrease in sensitivity to adriamycin in the HO-8910PM cell line.
- Role of dietary habits on fetal anomaly development: Review of 315 consecutive fetal anomaly cases** 264
 F.B. Cebesoy, O. Balat, E. Dikensoy, M.G. Ugur, I. Kutlar, H. Kalayci - *Gaziantep, TURKEY*
 Dietary habits such as excess tea, chili peppers, and barbecued meat consumption may have an important role in development of fetal anomaly.
- Non classical congenital adrenal hyperplasia due to 21-hydroxylase deficiency in families from a Greek island with a closed society** 267
 E. Trakakis, A. Chryssikopoulos, G. Basios, P. Trompoukis, A. Sarandakou, M. Spyropoulou, E. Salamalekis, P. Karanikolopoulos, G. Salamalekis, P. Skarpas, D. Kassanos - *Athens, GREECE*
 Young members of a closed Greek island society presented hormonal findings of 21-hydroxylase deficiency. The proposed Marina index aims to classify the disease severity.

Lipid peroxidation and antioxidant status in hypertensive pregnancies	272
S.B. Patil, M.V. Kodliwadmath, S.M. Kodliwadmath - <i>Karnataka, INDIA</i>	
Decrease in antioxidant probably provoked by increased peroxide load in preeclamptic and eclamptic patients may be an index of severity of disease.	
Incidence of cervical cancer in Montenegro	275
B. Ćolaković, V. Ćolaković-Popovic - <i>Montenegro, ROMANIA</i>	
Cervical cancer is the most frequent disease of the female genital organs in Montenegro (with around 45%) and shows constant growth.	
Abortion rates and the role of family planning: A presentation of the Greek reality	279
N. Salakos, K. Bakalianou, O. Gregoriou, C. Iavazzo, G. Paltoglou, G. Creatsas - <i>Athens, GREECE</i>	
The role of family planning programmes in the reduction of abortion rates in Greece is presented.	
CASE REPORTS	
Serous cystadenoma with omental caking and ovarian torsion: an unusual case presentation	284
P. Brezina, J. Woelk, D. Brezina, J. Devente - <i>Greenville, NC (USA)</i>	
A 21-year-old was found with radiological findings suspicious for malignancy; postsurgical pathology showed benign disease and ovarian torsion.	
Spontaneous uterine rupture during preterm labor in the second trimester of a twin IVF pregnancy without any apparent risk factor	287
C. Ficicioglu, G. Yildirim, F. Arioglu, N. Cetinkaya - <i>Istanbul, TURKEY</i>	
A case of a twin pregnancy with IVF treated successfully after spontaneous uterine rupture is described.	
Successful methotrexate treatment of an abdominal pregnancy in the pouch of Douglas	289
R. Crespo, J.M. Campillos, A. Villacampa, B. Madani, R. Navarro, J.J. Tobajas - <i>Zaragoza, SPAIN</i>	
A case of successful combined systemic and transvaginal sonographic-guided methotrexate treatment of an abdominal pregnancy is reported.	
Xanthogranulomatous salpingitis: report of three cases and comparison with a case of pseudoxanthomatous salpingitis	291
E. Kostopoulou, A. Daponte, A. Kallitsaris, R. Papamichali, G. Kalodimos, I.E. Messinis, G. Koukoulis - <i>Larissa, GREECE</i>	
Three cases of xanthogranulomatous salpingitis are presented and their clinicopathological features are compared to those of a case of pseudoxanthomatous salpingitis.	
Bowel obstruction due to endometriosis in the rectovaginal septum	295
N. Takai, T. Ueda, M. Nishida, K. Nasu, H. Narahara - <i>Oita, JAPAN</i>	
A very rare case of a patient whose endometriotic lesions in the rectovaginal septum caused ileus obstruction is presented.	
Conservative management of cervical ectopic pregnancy: case report and review of literature	297
A. Corticelli, M. Grimaldi, E. Caporale - <i>Lavagna, GE, ITALY</i>	
A case of a patient with a cervical ectopic pregnancy at six weeks and subsequent treatment by conservative medical and surgical procedures is presented.	
Infertility in a new 46, XX male with positive SRY confirmed by fluorescence in situ hybridization: a case report	299
C.E. Pepene, I. Coman, D. Mihiu, M. Militaru, I. Duncea - <i>Cluj-Napoca, ROMANIA</i>	
A case of 46, XX male syndrome with hypo-testosterone and infertility is presented.	
A rare case of uneventful pregnancy in a woman with Cogan's syndrome	301
K. Bakalianou, N. Salakos, C. Iavazzo, K. Danilidou, K. Papadias, A. Kondi-Pafiti - <i>Athens, GREECE</i>	
The clinical manifestations, pathologic findings of the placental examination and treatment of a woman with Cogan's syndrome during pregnancy are described.	
Pelvic packing method (after two laparotomies): a salvage procedure to control intractable pelvic hemorrhage after vaginal hysterectomy: a case report	303
A. Kale, U. Kuyumcuoğlu - <i>Diyarbakir, TURKEY</i>	
A case of a patient treated successfully with pelvic packing after two failed surgical procedures for intractable hemorrhage is described.	

Fetal ovarian cysts: report of two cases and literature review	306
D. Grapsa, C. Salakos, V. Tsapanos, N. Salakos, K. Bakalianou, A. Kondi-Pafiti - <i>Athens, GREECE</i> Two cases of fetal ovarian cysts with emphasis on their pathologic features are presented.	
Unusual presentation of a dermoid cyst that derived from the bladder dome presenting as subserosal leiomyoma uteri	309
U. Kuyumcuoğlu, A. Kale - <i>Diyarbakir, TURKEY</i> The first case of a dermoid cyst deriving from the bladder dome presenting as a subserosal leiomyoma is described.	
BRIEF COMUNICATION	
Anti-Lewis alloimmunization: report of seven cases	311
L.M.M. Nardoza, G.R. Lobo, A.F. Moron, L. Camano, E. Araujo Jr., H.A. Guimarães Filho - <i>São Paulo, BRAZIL</i> Perinatal outcomes of seven cases of pregnant women affected by Anti-Lewis alloimmunization are reported.	
Contents index vol. XXXV, 2008	313
Authors index vol. XXXV, 2008	317

Advances in oocyte cryopreservation - Part I: slow cool rapid thaw technique

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Summary

Purpose: The need for freezing oocytes has been established for females undergoing potential therapy that could damage their ovarian egg reserve, ethical or religious reasons (not having excess embryos frozen) or women nearing the age of lower fecundity but not married and not ready to use donor sperm. Applying the cryopreservation techniques for oocytes as used for embryos resulted in very poor pregnancy results. *Methods:* Changes in methodology including fertilization by intracytoplasmic sperm injection because of zona hardness and using a sodium-deplete choline substitute freezing media are some of the changes made for the slow cool rapid thaw method. *Results:* These modifications have led to significant improved survival rates of frozen oocytes not to mention fertilization rates and subsequent pregnancy rates. *Conclusions:* Since some in vitro fertilization (IVF) centers do poorly with frozen embryo transfer pregnancy rates despite good pregnancy rates following fresh embryo transfer, there is suspicion that the culpability may lie in the programmable freezer used in the slow cool technique. A simplified slow cool freezing technique using a bio-cool freezer instead of a programmable freezer has been described which has resulted in consistently good results with embryos. It would be interesting to see if this technique would work well with oocytes with the new changes to the freezing method.

Key words: Cryopreservation; Oocytes; Slow cooling; Choline.

Oocyte Freezing

Part 1: Slow cool - fast thaw technique

Though there may be a trend toward decreasing the number of oocytes stimulated for the purpose of in vitro fertilization (IVF), most protocols create multiple follicles and thus a lot of eggs are retrieved. If one fertilizes all of these eggs this leads to extra embryos which are then cryopreserved for future use. If the couple conceives on the fresh transfer then they can transfer frozen-thawed embryos in the future without having to go through controlled ovarian hyperstimulation and oocyte retrieval. Frozen embryos still function quite well when transferred even when they have been frozen more than ten years. If the couple failed to conceive the first time with fresh embryos they can immediately proceed with frozen embryo transfer without going through the expense of IVF-ET.

However, sometimes this present delivery is all the children that the couple wants. The question is what to do with the extra embryos? Options are to keep them frozen in case the couple changes their mind or transfer them and just hope that the transfer does not work. Other options include anonymously donating the embryos to another couple or destroying them. Some couples feel better about donating them to research, e.g., stem cell research. Generally most IVF centers charge some fee for the storage of embryos.

There are some couples also for religious or ethical reasons who cannot choose the option of destroying the extra embryos or donating them to research. Though donating them to another couple would circumvent the problem of destroying life, many couples are not comfortable with the fact that this could result in siblings of their children and they cannot deal with that emotionally.

One strategy used by some couples is to limit the number of retrieved eggs that are to be inseminated by sperm. However, if the embryo transfer does not result in a pregnancy then the couple regrets having to go through another controlled ovarian hyperstimulation cycle and not having frozen embryos to transfer. Sometimes a couple has fulfilled their dreams of a sufficient family only to have some tragedy occur to one of their children and then the couple desperately wants another. Possibly the woman is now at an age when achieving a pregnancy would be difficult with her own eggs. However because the uterus in humans ages more slowly than the ovaries, had she had frozen embryos left over from a younger age, another pregnancy would be feasible. In this case she might wish that she had embryos left.

The ethical and moral dilemmas about what to do with remaining embryos which are considered "life" would be obviated if the extraneous oocytes were not fertilized but frozen as eggs. If a divorcee or widow remarries at the age of 45

to a man who has no children and they desire children including his and her genetic makeup there is very little chance at this age that she can conceive with her own eggs. Thus she would have to consider donor eggs. If there were embryos from a younger age fertilized by her previous male partner that would not solve the problem. However, if there were frozen eggs available from a younger age that would provide the opportunity to fulfill their dreams if the state of the art for egg freezing would not only provide a good chance of surviving the thaw but a good chance of fertilization and pregnancy after embryo transfer.

If technology improved to the point where egg freezing was successful there are many other scenarios where the preference would be to freeze eggs rather than embryos. These include, but are not limited to, young women about to undergo potential treatment that would damage the ovaries for the future, e.g., radiation therapy, chemotherapy, surgery for cancer, career women who have not married yet but have reached their mid 30's wanting to preserve their ability to conceive in the future, or a sister who is undergoing IVF-ET who wants to leave eggs not embryos for possible use by her older sister who is struggling to conceive.

The problem was that the freezing technique used to freeze embryos was not very successful for freezing eggs. The first pregnancy from fertilization of frozen-thawed oocytes using the slow cool rapid thaw method used for embryo freezing was reported in 1986 by Chen [1]. However it was not viable. More than a decade passed before the first live delivery from the fertilization of an oocyte frozen by the slow cool rapid thaw technique was reported by Porcu *et al.* in 1997 [2].

When one freezes oocytes the zona pellucida becomes hardened [3-5]. This hardening (possibly related to premature release of cortical granules) when freezing oocytes inhibited fertilization of the oocytes. Furthermore the fertilization rate of cryopreserved-thawed oocytes was poor (or more than one sperm fertilized the egg, i.e., polyspermy, related to premature cortical granule release). The advent of intracytoplasmic sperm injection (ICSI) in 1992 provided a significant advancement in the oocyte cryopreservation problem.

One of the main challenges in freezing any live material is the formation of intracellular ice crystals [6-8]. Cryoprotectants are needed to prevent ice crystal formation. Permeating cryoprotectants are small molecules that readily permeate the membranes of cells. They form hydrogen bonds with water molecules and thus prevent ice crystallization. At high enough concentrations, these cryoprotectants inhibit the formation of the characteristic ice crystal and lead to the development of a solid glasslike, so-called vitrified state in which water is solidified but not expanded.

The most commonly used permeating cryoprotectant for embryos and eggs is 1,2 propanediol. When used in a concentration high enough to prevent ice crystal formation they are very toxic. To reduce this toxicity either the cell must be exposed to the cryoprotectant for very short time periods (as with the vitrification technique which will be described later) or at a time when the metabolic rate of the cell is low, e.g., very low temperatures.

The use of more than one cryoprotectant can maximize inhibition of ice crystal formation with lower toxicity by not requiring a high concentration of any one cryoprotectant. This is best accomplished by a step-wise addition of cryoprotectants. Generally 1,2 propanediol is added first then a non-permeable cryoprotectant sucrose is added later.

At first the general concept was that the reason why embryos were resistant to ice crystal damage was the difference in the state of the chromosomes between embryos and eggs. The DNA of mature unfertilized oocytes is compacted into chromosomes that are aligned on a metaphase plate. In contrast the majority of DNA in embryos exists as decondensed chromatin at interphase. The thought process was that the meiotic spindle of the oocyte was more susceptible to cryo-damage and that the spindle becomes deorganized leading to altered DNA. There is evidence however that with the use of cryoprotectants the meiotic spindle can reorganize in the oocyte and that this is not the main reason for oocytes being less cryo-hearty [9].

As mentioned two of the improvements in oocyte cryopreservation technology were the use of ICSI for fertilization of frozen thawed oocytes with exposure to toxic cryoprotectants at lower temperatures and the use of multiple cryoprotectants to minimize the toxicity of any one specific cryoprotectant. Certain changes in the cryoprotectant when introduced along with using ICSI for fertilization and making certain changes in the freezing media, e.g., substituting choline in the cryopreservation media for sodium also resulted in marked improvement in pregnancy rates with the slow cool rapid thaw technique [10]. Choline may have beneficial effects on the membrane [11, 12].

Thus the slow-cool freezing method relies on low initial cryoprotectant concentrations which are associated with lower toxicity. This is important because the oocyte is still at a temperature at which it is metabolizing. Thus an important strategy is not to increase the concentration of cryoprotectants and other solutes until the oocyte has been cooled down to a temperature at which metabolism is slow. The initiation of adding cryoprotectants begins at room temperature which is gradually lowered about 2°/minute to the seeding temperature of -6°C. Seeding is the initiation of ice formation in a controlled manner. It involves the minimal introduction of a stable ice crystal to the freeze solution which acts as a template for additional ice formation.

Following seeding at -6°C the solutions are held for 10-30 minutes to allow equilibration. The growing ice crystals result in squeezing out of the oocytes the cryoprotectants and other solution which results in a gradually rising concentration of the cryoprotectants in the remaining solution.

The temperature is slowly decreased to -32°C which allows gradual diffusion into the oocyte of additional permeating cryoprotectants. Ice crystal formation has increased in size in the intracellular medium thus increasing the concen-

tration of the cryoprotectant in the intracellular space. Further dehydration of the intracellular space is accomplished through the use of nonpermeating cryoprotectants, e.g., sucrose.

At the final temperature of -32°C the freezing temperature for 1,2 propanediol has not been reached but its concentration has markedly increased. The metabolic rate of the oocyte is now quite slow further limiting the toxicity of the increasing concentration of the cryoprotectants. The freezing vessel is now plunged into liquid nitrogen.

Substitution of sodium by choline in the cryopreservation media enhances cryopreservation outcome. In addition to solution effects sodium in the culture medium is thought to accumulate intracellularly as a result of impairment of the plasma membrane Na^+K^+ pump during cryopreservation [13, 14]. Unlike the sodium ion, choline is not thought to cross the cell membrane and thus not contribute to intracellular osmolarity or toxicity.

The largest experience with human oocyte cryopreservation using the slow cool technique has come from an Italian group [10, 15]. The success rate after transfer of embryos derived from the thawed oocytes has approached the pregnancy rates for transfer of frozen thawed embryos [15].

The basic methodology that was modified for oocyte freezing was the embryo cryopreservation technique described by Lassalle *et al.*, in 1985 [10, 15, 16]. Though some IVF centers show good pregnancy rates with frozen embryo transfer other IVF centers do not seem to fare so well despite good success with fresh embryo transfer. It has been my belief that the weak and variable part of this cryopreservation procedure is the programmable freezer. My colleagues and I have had good pregnancy rates following frozen embryo transfer with a simplified freezing protocol with a one-step removal of the cryoprotectant 1,2 propanediol upon thawing that avoids the programmable freezer [17-19]. Possibly pregnancy rates following fertilization of cryopreserved-thawed eggs can be increased using the slow cool methodology that avoids the programmable freezer.

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Regulatory mechanism of *Bcl-2* in uterine leiomyomas

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Summary

Bcl-2 has been thought to play a vital role in the growth of uterine leiomyomas. However, it remains to be fully understood how *Bcl-2* expression is regulated in uterine leiomyomas. Several factors have been speculated to affect the induction of *Bcl-2* in these cells, including progesterone, endoplasmic reticulum stress, and microRNAs. The elucidation of the regulatory mechanism of *Bcl-2* will contribute to a better understanding of the molecular biology of uterine leiomyomas.

Key words: *Bcl-2*; Leiomyoma; Progesterone; Endoplasmic reticulum stress; MicroRNAs.

The *bcl-2* protooncogene was discovered in lymphoma tumors composed of B cells [1]. *Bcl-2* is localized to mitochondrial and perinuclear membranes. *Bcl-2* acts to prevent the apoptotic cell death in a variety of cells induced by tropic factor deprivation or other stimuli without altering proliferation [2, 3]. In addition to extending the life span of certain cells, *Bcl-2* protein can promote cell replication by reducing the requirements for growth factors [4, 5].

Uterine leiomyoma is the most common benign smooth muscle cell tumor of the myometrium. *Bcl-2* protein has been thought to play an important role in the growth of uterine leiomyomas, however, it remains to be fully understood how its expression is regulated in uterine leiomyoma cells. Nonetheless, accumulating data provide evidence that *Bcl-2* may be regulated by sex steroid hormones and a pro-apoptotic factor in uterine leiomyoma cells. Furthermore, recent studies raise the possibility that microRNA may be involved in the regulation of *Bcl-2* in these cells.

Our laboratory demonstrated that *Bcl-2* protein was abundantly present in uterine leiomyoma tissue extracts compared with normal myometrial tissue extracts [6]. This suggests that the greater abundance of *Bcl-2* protein in uterine leiomyoma cells may be responsible, at least in part, for the growth of leiomyomas by preventing apoptotic cell death. Moreover, the addition of progesterone resulted in a striking increase in *Bcl-2* protein content in cultured leiomyoma cells compared with control cultures, whereas the addition of 17 β -estradiol attenuated *Bcl-2* protein content in these cells [6]. Thus, it is speculated that the abundant expression of *Bcl-2* protein in uterine leiomyoma cells may be one of the molecular basis characteristic of uterine leiomyomas and that progesterone may play a vital role in the enhanced expression of *Bcl-2* protein in these cells.

Other collaborators and I investigated the effects of progesterone receptor modulators on *Bcl-2* protein content in cultured uterine leiomyoma cells [7, 8]. We found that progesterone receptor modulators inhibited the cell proliferation of cultured leiomyoma cells and induced apoptosis by up-regulating cleaved caspase-3 and down-regulating *Bcl-2* protein content. These data reinforce the progesterone-induced stimulatory action on *Bcl-2* protein expression. A recent study has demonstrated that progesterone via progesterone receptor interacts with the *bcl-2* promoter to induce its expression in leiomyoma tissue, suggesting the progesterone-dependent enhancement of growth in uterine leiomyoma [9].

We have recently demonstrated that the induction of endoplasmic reticulum (ER) stress modulates *Bcl-2* protein content in cultured leiomyoma cells [10]. The ER is the site of synthesis and folding of secreted, membrane-bound, and organelle-targeted proteins [11]. The conditions that interfere with ER function are referred to as ER stress [11], including the perturbations in calcium homeostasis or redox status, elevated secretory protein synthesis, expression of misfolded proteins, and glucose deprivation [12]. The ER triggers the unfolded protein response to cope with accumulated unfolded or misfolded proteins [12]. However, if the ER stress is prolonged, apoptotic cell death ensues [13]. A pro-apoptotic transcription factor, growth-arrest- and DNA-damage-inducible gene 153 (GADD153), is activated during ER stress [13]. We found that progesterone receptor modulator elicited the ER stress and increased GADD153 protein levels in cultured leiomyoma cells [10]. RNA interference of GADD153 augmented *Bcl-2* protein content in cultured leiomyoma cells [10]. This provides further insight into the action of GADD153 in the regulation of *Bcl-2* protein in cultured leiomyoma cells.

Lastly, it is postulated that microRNAs (miRNAs) may be involved in the pathogenesis of uterine leiomyomas. MiRNAs are a recently discovered group of small RNA molecules involved in the regulation of gene expression and play a crucial role in diverse functions, including cell proliferation, cell differentiation, stress response, apoptosis, immunity, and transcriptional regulation [14, 15]. MiRNAs function as tumor suppressors (tumor suppressor miRNAs)

and oncogenes (oncogenic RNAs; oncomiRs). OncomiRs are frequently up-regulated in cancers and show proliferative and/or anti-apoptotic activity [15].

Recent studies have demonstrated that miRNAs are differently expressed in uterine leiomyomas compared with normal myometrium [16-19] and that ovarian steroids modulate the expression of miRNAs [18]. Among deregulated miRNAs examined in these investigations, it is noteworthy that oncomiR, microRNA-21 (mir-21), is consistently up-regulated in uterine leiomyomas [16-18]. Knockdown of mir-21 was reported to activate caspases and induce apoptosis in cultured glioblastoma cells [20]. Si *et al.* [21] reported that anti-miR-21 inhibited tumor growth and induced apoptosis in breast cancer MCF-7 cells in association with down-regulation of Bcl-2, suggesting the regulation of Bcl-2 by mir-21. However, the biological significance of mir-21 in the growth of uterine leiomyomas remains unknown. Further studies will be necessary to elucidate the function of miRNAs in uterine leiomyomas.

In conclusion, Bcl-2 is strongly suggested to participate in the growth of uterine leiomyomas by preventing apoptosis. Several factors are thought to regulate the expression of Bcl-2 protein, including progesterone, GADD153, and possibly miRNAs. The elucidation of the regulatory mechanism of Bcl-2 could contribute to a better understanding of the molecular biology of uterine leiomyomas.

Acknowledgement

This work was supported by Grants-in-Aid for Scientific Research 18390449 from the Japanese Ministry of Education, Science and Culture.

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High-grade cervical intraepithelial neoplasia, human papillomavirus and factors connected with recurrence following surgical treatment

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Summary

Although effective strategies for preventing cancer of the uterine cervix exist, this disease continues to be a serious health problem worldwide, especially in developing countries. Today, the role of human papillomavirus (HPV) as a causal factor for the emergence of cervical cancer and its precursor lesions is well established, and prevention programs against cervical cancer are based on detecting cervical intraepithelial neoplasia (CIN). HPV present immunological evasion mechanisms that inhibit detection of the virus by the host, which may result in persistent chronic infection and irrevocably compromise the host defenses. Conization is the surgical technique most used for treating high-grade CIN, since it makes it possible to exclude invasive neoplasia, evaluate resection margins and preserve fertility. However, several factors have been considered to be indicators for residual disease. This review had the aim of covering some factors relating to persistence and recurrence of high-grade CIN following conization.

Key words: Cervical intraepithelial neoplasia; Human papillomavirus; Conization; Recurrence; Immunological response.

Introduction

Human papillomavirus (HPV) is an epitheliotropic deoxyribonucleic acid (DNA) virus belonging to the family *Papillomaviridae*. It is mostly transmitted sexually and is considered to be the principal factor in the pathogenesis of cervical cancer and its precursor lesions. Today, more than 100 types of HPV have been detected, of which 40 infect the human genital tract. Of these, 15 are associated with cervical carcinogenesis [1, 2]

Prevention of cervical cancer is potentially effective since several forms of intervention for combating the multiple manifestations of the disease now exist. Nonetheless, estimates indicate that this is a disease with high prevalence, incidence and mortality, especially in developing countries [2].

In Brazil, the National Cancer Institute (INCA) states that cancer of the uterine cervix is the third most common malignant neoplasia among women, only surpassed by skin cancer (non-melanoma) and breast cancer, and it constitutes the fourth most important cause of death due to cancer among women. It is also forecast that there will be around 18,680 new cases of this disease in Brazil in the year 2008, with a risk estimated as 19 cases for every 100,000 women [3].

HPV infects the basal layer of the epithelium in the metaplastic region of the transformation zone of the uterine cervix, where the cells present the greatest vulner-

ability [4]. The relationship between HPV and carcinogenesis depends fundamentally on the type of virus (high or low oncological risk), viral load, persistence and integration of the virus with the host cell, genetic constitution and the individual's immunological response [5].

In the host, according to the type of virus, HPV may remain in the episomal form or be incorporated into the DNA. In the episomal form, its activity accompanies cell differentiation by the layers of the epithelium, thus resulting in surface cells with copies of the HPV that are ready for transmission. When incorporated into the DNA, the viral DNA forms part of the host's cell genome, thereby inducing the production of certain proteins that modify cell activity through a sequence of reactions that result, after a varying period of time, in cell proliferation and inhibition of apoptosis [6, 7].

Cervical intraepithelial neoplasia (CIN) can be divided into mild dysplasia (CIN I), moderate dysplasia (CIN II) and severe dysplasia or in-situ carcinoma (CIN III), and this may progress to invasive cancer if it is not treated [8].

The low-risk types of HPV that only induce benign genital warts include HPV 6 and 11. The high-risk group includes HPV 16, 18, 31, 33, 45 and 56, and these types are associated with the development of anogenital cancer and can be detected in 99% of cervical cancer cases [9, 10].

The viral oncoproteins E6 and E7 interact with the tumor-suppressor proteins (p53 and pRB) to alter their cycles and functions [11-13]. However, there is evidence suggesting that, in cases of HPV infection, other addi-

tional factors are involved in the progression of precursor lesions, such as starting sexual activity at an early age, use of oral hormonal contraceptives, parity, smoking, multiple sexual partners and immune system deficiency. Thus, although virtually all cases of CIN III or cervical cancer contain HPV of high oncogenic risk, it seems that for this virus to cause lesions, various cofactors are involved [14, 15].

The incidence of cervical cancer and precancerous lesions is greatly reduced by screening for precursor lesions that can be treated by exfoliative cytological procedures, even in women who undergo total hysterectomy because of the risk of vaginal intraepithelial neoplasia [16]. The diagnostic methods for evaluating morphological abnormalities in lesions induced by HPV include clinical examination, colposcopy, oncological colposcycytological analysis and histological analysis [17].

Molecular biology techniques have modified the knowledge of the epidemiological profile of HPV infection through recognizing the different subtypes of the virus. These techniques are considered to be an essential prerequisite for clinical studies that associate HPV with carcinogenesis. The set of techniques for detecting the genetic material (DNA) of HPV in cervical samples consists of molecular hybridization of nucleic acids of Southern blot type, hybrid capture, in situ hybridization and the polymerase chain reaction (PCR). HPV DNA analysis may improve clinical sensitivity in detecting precancerous lesions in cervical cancer cases. It is also used for monitoring after surgical procedures to treat CIN III [18].

In cases of high-grade CIN, the choice of therapeutic method depends on various factors, such as lesion severity, age, patient's clinical state, patient's reproductive desires, extent of the colposcopic image (grade, location and number of lesions), type of HPV and the technological capacity of the clinic. Although regression of CIN may occur [19, 20], excision methods and particularly classical cold conization, high frequency loop surgery (LEEP, loop electrosurgical excision procedure) and laser surgery are the techniques most used for treating high-grade CIN. These techniques make it possible to exclude invasive neoplasia, evaluate resection margins and preserve fertility. Clinical follow-up for women who undergo conization has the aim of detecting persistence or recurrence of CIN [21].

Factors relating to persistence and recurrence of high-grade cervical intraepithelial neoplasia following conization

Lesions classified as high-grade CIN are lesions with a greater likelihood of persistence and progression. Patients with high-grade CIN need to be divided into two treatment groups: those among whom the presence of an invasive lesion has been securely ruled out, and those among whom doubts persist. The latter group is related to recurrences and persistence of the disease [22].

Various factors have been considered to be indicators for residual disease, such as age, skin color, CIN grade,

lesion severity, presence of lesions in several quadrants (extensive or multifocal lesions), smoking, margin involvement, gland involvement, number of mitoses, state of endocervical curettage, pregnancy and delivery, socioeconomic status, marital status, immunosuppression, type of therapy and oncogenicity of the HPV involved [23].

The presence of remaining dysplastic cells after the apparent complete excision of the lesion can, in the first instance, be explained by supposing that the histopathological result was incorrect because of an insufficient number of thin sections cut from the surgical specimen. A second hypothesis would be that the dysplastic epithelium was friable and could easily be separated from the stroma, such that damage occurred while removing the surgical specimen. Alternatively, there is the possibility that the intraepithelial lesions might be multifocal within the transformation zone. A further hypothesis that can always be borne in mind is that the appearance of a new lesion is unrelated to the one diagnosed previously [24].

Even if HPV infection is directly connected to CIN, it may spontaneously regress without leaving any histological mark [25]. The great majority of women present transitory infections due to this virus that are eliminated naturally without lesion development. CIN, and especially low-grade CIN, also regresses spontaneously in most women, and particularly in younger women [26]. These characteristics of HPV infection and the natural history of the neoplasia are complicating factors for clinicians following-up women who have undergone conization. Tests performed after the procedure that may be positive for HPV theoretically may not represent unfavorable evolution but, rather, transitory reinfection without clinical repercussions. In the following, some factors relating to persistence and recurrence of CIN following conization are presented.

Human papillomavirus

Certain risk factors predisposing towards persistence of HPV in CIN cases following conization have been suggested. However, variable and sometimes controversial results have been reported. Age, preventive Papanicolaou smears, HPV type, lesion grade from colposcopy-guided biopsy, lesion grade from the cone, state of the resection margin and conization with positive margins have been described as risk factors for persistence of HPV [27].

It is rare to detect HPV in morphologically normal squamous tissue surrounding the sites where CIN is present. This does not rule out occult infection within the natural history of CIN, but it indicates that when HPV-induced cervical lesions occur, the occult infection does not spread into the surrounding normal epithelium [28].

The viral load may be influenced by various factors, among which the sample quality, site from which it was collected (endocervix, ectocervix or pouch base), size of the histological lesion and, especially, the number of epithelial cells obtained [29].

Persistence of HPV infection after conization indicates

that the cervix is still exposed to a major oncogenic factor, with increased risk of recurrence and progression [30, 31]. After CIN excision, the HPV DNA detection rate is lower and, in women with HPV still detectable, the viral load tends to be lower than what was found before conization. This can be explained through the observation that abnormal epithelial cells of a preneoplastic or clearly invasive nature are rich in HPV genetic material. This material is integrated with the nucleus and promotes anarchical proliferation. Nevertheless, complete removal of the HPV-induced lesions does not ensure virus eradication [32].

Persistence of the virus following CIN excision, especially in cases of high viral load, may represent the existence of residual high-grade lesion or even recurrence, including in cases in which no abnormalities are found in oncological colposcycological tests. Infection with new types of HPV after conization may also be associated with recent exposure to risk through sexual relations with different partners, and thus constitutes new infection [33, 34]. Persistence of high viral loads of HPV of high oncogenic risk after conization, especially types 16 and 18, is strongly indicative of a risk of recurrence and even progression of CIN to invasive carcinoma or recurrence of CIN III [35]. In contrast, women in whom the viral load decreases to undetectable levels after conization present very low risk of developing cancer over the coming years.

Surgical margins

The frequency of findings of compromised margins in the surgical specimen from conization depends on several factors, such as the degree of CIN severity, involvement of the endocervix, dimensions of the cone and conization technique. Thus, there is a correlation between compromised surgical margins in the cone and the residual disease rate [36].

After conization, compromised surgical resection margins due to CIN or carcinoma may occur in 10 to 45% of the cases. The variability in this percentage is mainly due to the surgeon's level of experience and inability to view the endocervical resection margin. This latter is of importance for patient follow-up [37].

Anatomopathological findings of involvement of the surgical margins due to CIN are frequently used to clinically predict the presence of residual lesion. When this occurs, one of the treatments proposed is total hysterectomy. Some studies have shown a direct correlation between involvement of the cone margins and the presence of residual CIN in the hysterectomy specimen [37].

Studies have shown that there is no difference in the recurrence rate between cases using classical conization and LEEP. In both situations, more than 95% of the recurrences occur within the first five years. Extensive endocervical involvement of the cone margin following LEEP for treating CIN II and III strongly predisposes towards residual disease [38].

Recurrence or persistence of the disease may not be

directly related to the state of the cone margins. More than 70% of the women with compromised margins are found not to present residual disease when undergoing hysterectomy. These women may present compromised margins because of the surgical trauma, which through the regenerative process may give rise to an immunological response. Nevertheless, women with cones presenting free margins may present recurrence due to multifocal disease, inadequate evaluation of the surgical specimen or persistence of HPV [39].

Extension to glands

In addition to positive margins, gland involvement has also been considered valuable as a prognostic factor predisposing towards lesion recurrence. Dysplastic cells may remain in the endocervical glands that are covered with normal epithelium, and they may progress to more advanced degrees of dysplasia or even to invasion of the cervical stroma, without altering the cytological and colposcopic findings. This phenomenon may explain cases of detection of invasive carcinoma in patients who previously underwent conization and who, during postoperative follow-up, presented normal results from cytological tests [40]. However, results obtained by our research group have not shown that extension to glands is associated with recurrence of CIN III [41].

Number of mitoses

In cases of high-grade CIN, the cell organization is altered over almost the whole thickness of the epithelium and the cells present a high degree of nuclear and cytoplasmic abnormalities, with typical and atypical mitoses. In atypical mitoses, it is common for parts of chromosomes or whole chromosomes to be missing, thereby leading to polyploidy, aneuploidy or other anomalies of greater complexity. This may be lethal to daughter cells, thus inducing apoptosis, but it may also cause the appearance of cell clones that are more aggressive, because of the deletion of other cell growth-regulating genes [42].

The presence of tripolar atypical mitoses in compromised margins can be considered to be a morphological criterion for progression of CIN I to CIN III. Greater numbers of mitoses are related to a greater CIN recurrence rate [43].

Human immunodeficiency virus

The incidence of CIN recurrence recorded among individuals with the human immunodeficiency virus (HIV) is very high and the literature indicates that, in such cases, conization should be performed for all grades of CIN. Clinical and laboratory control for HIV-infected patients, by means of CD4⁺ cell counts and quantification of the viral load, seems to be closely related to persistence and/or recurrence of the lesion. High recurrence

rates have been observed after all types of treatment implemented among immunosuppressed patients, even after hysterectomy [44, 45].

Genetic abnormalities

When HPV infects cells, there may be an interaction between the HPV genome and that of immature host cells, thereby impeding cell differentiation and maturation. Thus, transformed cells contain viral DNA and infections persisting for 10-20 years enable the development of additional genetic abnormalities, with progression of low, moderate and high-grade lesions to invasive cancer [46].

Infection with HPV of high oncogenic risk seems to be important with regard to the occurrence of high-grade CIN. However, recurrence of CIN has only been observed in cases with hypermethylation of three or more of the eight genes studied (p16, RARBeta, GSTP1, MGMT, p14, TIMP3, E cad and DAPk), thus showing the importance of genetic abnormalities in cases of cervical carcinogenesis [47].

Viruses, together with chemical substances and radiation, appear to be causes of cancer. Viral carcinogenic action is associated with genetic abnormalities in control processes for the cell cycle and cell differentiation. In cancerous cells, the genetic control is faulty and these cells reproduce in an uncontrolled manner to form tumors instead of the normal cells that, over the course of the natural process of the life cycle, replicate, differentiate into different types and then die [48].

Smoking is considered to be one of the principal factors associated with persistence of viral activity, thereby increasing the risk of progression or recurrence of lesions in patients with CIN associated with HPV infection [49].

The two main mechanisms through which the smoking habit contributes towards cervical oncogenesis include direct exposure of the DNA of cervical epithelial cells to nicotine and cotidine. These have been found at high concentrations in the cervical mucosa and may induce mutations and abnormalities during gene activity. Indirectly, these substances inhibit the cell response of the immunological system and enable viral replication and infection of adjacent cells, thus increasing the possibility of incorporating the virus into the cell genome [49].

Follow-up for high-grade cervical intraepithelial neoplasia after conization

Independent of the surgical margins, the follow-up for CIN subsequent to conization should include oncological colposcycytological tests, colposcopy and also the test to detect HPV DNA. The latter test has high predictive value for post-treatment follow-up, since HPV acts as a marker for undetected residual disease. It is observed that women with cytological abnormalities or persistence of HPV following conization are at greater risk of presenting such recurrence, although the positive predictive value of these tests is low. On the other hand, the HPV

test has high negative predictive value, i.e., when HPV is not detected six months to one year after conization due to CIN II or III, it very unlikely that the woman will suffer recurrence [50, 51].

The potential for recurrence depends not only on whether the dysplasia is completely removed, but also on the individual's sexual habits and immune response and the oncogenicity of the HPV involved [52].

Local and systemic inflammatory response in patients with CIN III

Each individual's immunological response seems to be the principal determinant for occurrence, progression and recurrence of HPV infection. However, the exact mechanisms that trigger an efficient immune response against HPV-related lesions may relate to activation of the immunological system or the host's genetic composition [53].

Data on the natural history of HPV infection suggest that, soon after the initial infection, the viral DNA is transiently detectable (within two years), with spontaneous resolution in up to 80% of such cases. Most women are able to develop an affective immune response that avoids the development of cervical carcinoma. However, it is not known whether carcinomas appear only in women who maintain detectable levels of viral DNA over the course of decades, or whether some carcinomas develop from late-stage reactivation of transitory infections after a long period of time [54].

HPV efficiently evades the innate immunological response and delays the activation of the adaptive immunological response. The host dendritic cells are exposed to low levels of protein in a non-inflammatory environment for a period of time and, as a result, an absence of local immunological response may become established in the infected mucosa. In this environment that is operationally tolerant to the HPV antigen, the host defenses become irrevocably compromised and HPV or non-HPV antigen-specific effector cells are recruited to the infected area, or their activity is under-regulated. Thus, if there is a lack of regulation of high-risk HPV E6 and E7 with high protein expression, in the presence of persistent HPV infection, and this does not result in an immunological response mediated by activated effector cells, evolution to high-grade intraepithelial squamous lesions and invasive carcinoma mediated by HPV will be unimpeded [55].

There is controversy regarding studies on inflammatory responses in CIN cases. Decreased numbers of CD4-positive T cells and an inverted CD4/CD8 ratio have been reported in cases of cervical infection due to HPV, at all grades of CIN. Conversely, increased total numbers of lymphocytes have been reported in 18 cases of CIN II and III. In addition to this, studies using different markers have shown decreases in numbers of macrophages, in comparison with normal controls [56, 57].

There are changes in the numbers and functional capacity of neutrophils circulating in patients with neoplasia of the uterine cervix, and these changes are asso-

ciated with invasive or preinvasive staging. This suggests that even CIN III is a systemic disease, thus indicating that these cells participate in the immune response to the tumor [58].

In patients with neoplasia of the uterine cervix of different staging, changes in the number and function of circulating mononuclear cells have been observed. Following surgical treatment, there are changes in the number and functional capacity of mononuclear cells only in cases with advanced staging, thus suggesting that circulating factors are produced by the neoplastic cells [59].

In CIN III patients presenting recurrence following conization, greater positivity (expression) of CD3 T lymphocytes has been found, in comparison with patients without recurrence. This suggests that strong positivity for this antibody is a factor indicative of worse evolution [60].

Conclusion

HPV presents evasion mechanisms that block efficient immunological responses and cause persistent infection to become established. This may evolve to high-grade CIN or invasive carcinoma if not treated.

In this review, some factors that contribute towards persistence and recurrence of high-grade CIN following surgical treatment have been discussed. It is essential for such patients to be aware of the importance of continuing with medical care for the remainder of their lives, since it is possible for the disease to occur or reoccur even several years after conization. In this respect, it is emphasized that there is a greater chance of recurrence in cases with compromised surgical margins, greater numbers of mitoses in the lesion, presence of HPV 18, greater numbers of methylated genes and presence of local CD3 T lymphocytes.

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Human papilloma virus in adolescence

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Summary

Human papilloma virus (HPV) is one of the most widespread sexually transmitted diseases especially in adolescence. The majority of the infections are self-sustained by the immune system. However, HPV may lead to genital warts, cervical dysplasia and cervical cancer. Sexually active adolescent females should be encouraged to obtain gynecologic screening for HPV and be well informed about HPV and the risks associated with this infection. All the efforts are now focused on the vaccines that are being developed to reduce the morbidity and mortality associated with HPV infection if administered in time.

Key words: HPV infection; Adolescence; Teenager; Cervical lesions; Prevention; Treatment.

Introduction

Human papilloma virus (HPV), one of the most widespread sexually transmitted diseases, particularly in adolescence, has a prevalence of 30% to 50% among sexually active young women [1]. Although the majority of the infections self-sustain automatically, it may conduct to clinical sequelae such as anogenital condylomata and cervical squamous cell carcinomas with high morbidity and mortality rates. A lot of initiatives have been conducted in order to inform young people about HPV, its implications and how they can protect themselves from acquiring and transmitting the disease.

Biology, epidemiology and transmission of HPV

Human papilloma virus is a small non-enveloped DNA virus, with icosahedra structure. Its genome contains two regions, one early E region and one late L region. More than 80 types of HPV have been identified, 30 of which are known to affect the genital tract [2]. They are divided into low-risk types including HPV 6,11,40,42,44,54, associated with genital warts and into high-risk types including 16-like (16,31,33,35,52,58,67), 18-like (18,26,39,45,59,68,70,69,51) and 56-like (53,56,66) associated with high risk for oncogenesis [3]. HPV infects basal epithelial cells [1]. After the viral infection, the epithelial cells in most samples from adolescents are characterized by large, hyperchromatic, often bizarre shaped nuclei. The most important cytopathic effect on the cervical squamous epithelium caused by HPV infection is the presence of koilocytes in cervical biopsies,

which are mostly seen in the lower epithelial layers in young women compared to the common presence of koilocytes in the upper layers in older females [4].

Human papilloma virus is one of the most common sexually transmitted diseases among adolescents. In the United States it is estimated that 20 million Americans have HPV infection and 5.5 million new cases are diagnosed every year [5]. Epidemiologic studies suggest that the cumulative risk for someone to acquire HPV infection during his lifetime is approximately 79% [6]. Moscicki *et al.*, found in a study of adolescents who were initially HPV negative that 55% of them acquired HPV within three years [7]. Fifty percent of all the reported sexually transmitted diseases occur among adolescents, although they represent only 25% of the sexually active population [8].

HPV is transmitted by skin to skin contact. For full infection it is important that the virus gets access to basal cells through tears, in the squamous or mucosal epithelium, that are often produced by sexual activity. Cervical infection, almost in all cases, requires sexual intercourse but HPV can be transmitted without sexual intercourse by skin to skin contact, using fingers, sex toys or even tampons [9]. Indeed, different forms of sexual behavior may lead sexually inactive adolescents to HPV infection in other parts of the anogenital area [10]. HPV can also be transmitted to neonates from their mothers during vaginal delivery resulting in recurrent respiratory papillomatosis (RRP) which may be fatal for the neonate [8].

Risk factors for acquiring HPV

It is reported that HPV infection is more common in adolescents than in adult women [11]. A lot of reasons can explain this difference. First of all, the cervix in adolescents is more vulnerable to HPV infection due to the physiological immaturity [1, 12]. It is covered by columnar epithelium [13] giving the cervix a scarlet, velvet appearance [6]. Adolescent menarche is characterized by anovulatory menstrual cycles due to a lack of cyclic progesterone secretion leading to inadequate production of

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cervical mucus which acts as a protective barrier against infectious agents [1]. In addition their cervix is characterized by a large area of immature columnar and metaplastic cells on the ectocervix, referred to as ectopy. This area is very vulnerable to sexually transmitted infections such as HPV, Chlamydia trachomatis and HIV. The immaturity of adolescent cervixes leads to HPV infection by two other mechanisms such as the rapid rate of metaplastic change and the incompletely developed immune responses [1]. There are also cervical cell membrane differences that allow enhanced interaction between infecting virus and target cells. Thus, it is very easy to understand why HPV is most common in sexually active women younger than 25 years old.

There are not only the biological mechanisms that make adolescents more vulnerable to HPV infection but also their sexual behavior plays an important role too. Epidemiologic studies have supported the link between sexual activity and acquisition of HPV infection. They underscore the role of early age of first sexual intercourse and the number of sexual partners [14]. Higher risks of HPV infection also affect young women who practice risky sexual behaviors under the influence of alcohol and illicit drugs [1]. The history of other sexually transmitted infections, such as genital warts or herpes simplex virus (HSV), plays an important role as well, because these factors cause inflammation and breaks in the epithelium barrier allowing HPV direct access to basic epithelial cells [8, 15]. Unlike Chlamydia trachomatis or other sexually transmitted diseases, use of condoms is not highly effective in reducing HPV, because when the virus is present it involves almost the whole genital area [6].

Clinical implications of HPV infection

The majority of HPV infections in adolescents are occult or asymptomatic [16]. It has been reported that over 90% of them are cleared by the immune system [8]. However, the majority of chronic infections are associated with cancer in women. Low-risk HPV types are the cause of benign lesions, including anogenital condylomata and low-grade genital abnormalities, but are not found in genital cancers. High-risk HPV types cause precancerous and cancerous lesions of the cervix. HPV is also a very important etiologic factor for vaginal, vulvar, penile and anal cancers, and has been associated with pharyngeal and skin cancer [8].

Cervical cancer is the second most common cancer in women worldwide, following breast cancer [5]. It is reported that, more than 500,000 new cases are contracted each year, most commonly found in middle-aged or older women [6, 8]. Cervical cancer has proven to be the result of a chronic, progressive cellular transformation and HPV is the primary causative factor in this process [6]. Epidemiologic studies have shown that HPV is a necessary but not sufficient factor in initiating the transformation that leads to cervical intraepithelial neoplasia (CIN) and to cancer [6]. There are a lot of co-factors in this process. The virus needs to be a persistent high-risk HPV type [8]. Most likely to persist is HPV 16

which is considered to be responsible for 40% to 60% of invasive cancers worldwide. It has been shown that HGSIL after a persistent HPV infection leads to cervical cancer within two years and that the incubation period from initial HPV infection to carcinoma in situ is approximately seven to 12 years [8].

Prospective studies demonstrate that HPV infected women do not all progress to cervical cancer, but that the lesions persist or regress [6]. It is important to identify all the biological co-factors that increase cell transformation and begin the oncogenic process because HPV is easily acquired. Early age of initiating sexual activity, high number of sexual partners and early age of parity lead to increased risk of acquiring HPV [6]. Apart from the risky sexual behavior, immunosuppression seems to be a significant co-factor for cervical cancer. This is proven by the high rate of HPV and cervical carcinoma in transplant patients or patients on chemotherapy [6]. In HIV-infected girls HPV not only is more common but is also persistent due to CD4 immunosuppression (the lower CD4 cell count, the more likely HPV persists) [3] and as it is known, persistent infection is characterized by a greater risk of developing a high-grade squamous intraepithelial lesion (HGSIL) and cervical cancer. The condition of the immune system is very important when talking about adolescents concerning acquisition of HPV and its persistence because their general health is often compromised by behaviors impacting the immune system, such as poor diet and smoking.

Smoking is an important risk factor for invasive cervical cancer [17]. It has a negative affect on the immune system and as a fact, smokers become more vulnerable to infections such as HPV [6, 8, 11, 14, 18]. Several studies have shown that increased risk for precancerous and cancerous lesions also have been noticed in women with high parity (more than three pregnancies) [6], prolonged use of oral contraceptives [19], remarkable alcohol consumption, uncircumcised male partner and history of infection with HSV or Chlamydia trachomatis [8].

Screening and treatment guidelines for adolescents

A 70% reduction in the incidence of cervical cancer has been reported in the United States over the last five decades as a result of the introduction of the Papanicolaou (Pap) smear as a routine screening program [16]. According to the American Cancer Society women should begin cervical screening three years after the initiation of sexual activity or at 21 years of age, whichever comes first [20]. Screening should take place every year with traditional cytology (Papanicolaou smears). After three normal tests, screening should take place every two years. At the age of 30, women can reduce cytologies to one every two or three years provided they do not have abnormal pap tests or other risk factors such as immunosuppression. Women over 30 are now able to have a HPV DNA test as a primary screening test but its utility in adolescents has not been proven yet because at this age a lot of girls are HPV positive [8, 17].

The American College of Obstetricians and Gynecolo-

gists (ACOG) and the American Cancer Society (ACS) have recently published evidence-based guidelines that cover a variety of issues regarding cervical cancer screening especially in adolescence. Among adolescents, cytology is usually positive for HPV and the majority of pathology is a low-grade (LG) SIL. Few adolescents have HGSIL or cervical cancer [9]. These new guidelines allow cytological LGSIL to be followed by repeat cytology in six months or by a HPV DNA test in 12 months. If the repeat cytology is normal a second cytology should be done in six months. If a diagnosis of ASCUS or greater is the result of the repeat cytology, colposcopy is recommended. On the other hand, HPV DNA testing can be performed 12 months after the LGSIL diagnosis. If the test is positive for high-risk HPV type the adolescent should be referred for colposcopy. Adolescent progression from LGSIL to HGSIL is really low, thus using repeated cytologies or HPV DNA tests for monitoring LGSIL is more preferred than colposcopy. Consequently, for CIN 1, observation is the preferred method of treatment. When cervical dysplasia is suspected in an adolescent, colposcopy is recommended. Adolescents with HGSIL and possibly persistent LGSIL need more aggressive procedures such as cryotherapy and the loop electrical excision procedure (LEEP). Physicians should be very careful when they decide to do laser cone treatments or LEEP because these have been found to increase the risk of preterm delivery [21]. All these strategies are referred to non-immunosuppressed adolescents. Teenagers with impaired cellular immunities need closer monitoring for the development of HPV-related lesions. Further studies are needed to define the most appropriate screening strategies for adolescents [8, 9, 11, 16, 22-25].

Prevention of HPV infection

Many courses of action can be taken to prevent adolescents from acquiring HPV. Primary prevention is more efficient and a cost-effective strategy. Adolescents should be more aware of HPV. They must be encouraged to postpone the initiation of their sexual activity, in order to allow the cervix to mature, before exposure to HPV [6]. Sexual abstinence and mutually monogamous sexual relationships although recommended are not realistic or feasible [14]. Efforts should be focused on "selective sex" rather than on "safe sex" behaviors such as delaying first coitus, limiting the number of sexual partners and choosing partners who have been selective [6]. It has already been stated that condom use is not very efficient in preventing HPV transmission. However, it is highly effective in reducing other sexually transmitted diseases, like Chlamydia trachomatis or HSV which are important risk factors for HPV and cervical cancer. Changes in lifestyle should also be made, including abstinence from alcohol and tobacco use, and appropriate amelioration of the diet which will have a positive effect on the condition of their immune system [6].

It is accepted that the most beneficial impact on human health was obtained by vaccinations during childhood. Large-scale vaccination programs have controlled dis-

eases with great morbidity and mortality [26]. During the last 20 years many researches have focused on the development of anti-HPV vaccines. Till now much progress has been made. This effort is hindered by the main strains of the virus and therefore little knowledge is known about which strains are oncogenic and which may stimulate the immune system without transforming the cells [6]. There are two categories of vaccines in development. Prophylactic vaccines, whose targets are L1 and L2 proteins of the viral capsid, are designed to prevent primary HPV infection by including virus-neutralizing antibodies. The ideal age of vaccination has proven to be nine to 13 years old, in order to protect the most vulnerable young adolescents and to confer immunity before initiation of sexual activity [27, 28]. Vaccination programs should include both girls and boys because males are an important vector in the transmission of the virus, and they can develop both genital warts and anogenital cancer [27]. An ideal HPV vaccine should be characterized by safety, efficacy and cost effectiveness. In addition, vaccines should be feasible for low resource settings; that is the reason why they would be inexpensive, they would not require refrigeration and they would be effective after one single dose providing long-lasting immunity [26].

The success of HPV vaccination programs depends on individuals' willingness to accept it, on parents' willingness to have their children vaccinated and on healthcare providers' willingness to recommend HPV vaccinations [27]. HPV educational efforts need to be made in order to increase vaccine acceptance [29]. There are a lot of parents afraid to have their children vaccinated because they think it would lead to risky sexual behavior [30]. However, education does not imply that HPV vaccine will be widely accepted because HPV is a sexually transmitted disease where there is still a stigma [27]. Despite all these factors it is widely accepted that large-scale HPV vaccination programs will reduce HPV infection, genital warts and cervical cancer both in developed and developing countries where screening programs (Papanicolaou test) have principally failed.

The second category includes therapeutic vaccines, designed to prevent progression of HPV infection to LG or HGSIL, to regress CIN or condylomata or to extirpate remnant cervical cancer. The aim of therapeutic vaccines is the elimination of E6 and E7 proteins, which are associated with cancer by eliciting a cell-mediated cytotoxic t-cell response.

Summary

Adolescents who are sexually active are at high risk of acquiring HPV and other sexually transmitted diseases, the majority of which are self-sustained by the immune system. However, HPV may lead to genital warts, cervical dysplasia and cervical cancer. Cervical cancer is the result of a chronic and persistent infection with high-risk HPV types. Other risk factors such as risky sexual behavior (early age of first intercourse, great number of sexual partners), smoking, immunosuppression and infections with other sexually transmitted diseases play an impor-

tant role in the progression of cervical cancer. Sexually active adolescent females should be encouraged to obtain gynecologic screening for HPV and cervical cancer. In addition all adolescents should be well informed about HPV and the risks associated with this infection. LGSIL in adolescents is better to be observed than treated by using repeat cytology or HPV DNA testing because it is usually transient in this population. All efforts are now focused on developing vaccines that are expected to reduce morbidity and mortality associated with HPV infection if administered before initiation of sexual activity.

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Correction of failed fertilization despite intracytoplasmic sperm injection with oligoasthenoteratozoospermia but with acrosomes present by oocyte activation with calcium ionophore - case report

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Summary

Purpose: To determine if fertilization and embryo cleavage can be achieved by artificial oocyte activation in circumstances of repeated failed fertilization with sperm that have an acrosome. **Methods:** A woman with three IVF cycles with intracytoplasmic sperm injection (ICSI) failed to fertilize any eggs. The sperm had severe oligoasthenoteratozoospermia with no sperm with normal morphology. In the fourth IVF cycle fertilization was evaluated by performing ICSI with the husband's sperm and egg activation with calcium ionophore, ICSI with the husband's sperm without artificial oocyte activation, and ICSI with donor sperm. **Results:** Five mature oocytes were retrieved. Of the four eggs having ICSI with the husband's sperm only one of the two activated by calcium ionophore fertilized and resulted in a cleaved day 3 embryo. Interestingly, the one egg fertilized by donor sperm did not fertilize. **Conclusions:** The data could be consistent with conclusions that in some cases the failure to fertilize may be related to an oocyte activation factor/receptor problem in the oocyte that can be overcome by the use of calcium ionophore.

Key words: Artificial oocyte activation; Failed fertilization; Calcium ionophore; Acrosomes.

Introduction

Successful fertilization depends on sperm and oocyte interactions: sperm-zona binding, zona-induced acrosome reaction, sperm-zona penetration and sperm-oolemma binding [1]. Of these interactions, the acrosome reaction has been shown to be a requirement for normal fertilization. Sperm that lack acrosomal vesicles and are unable to bind to or penetrate the zona pellucida, commonly result in fertilization failure following in vitro fertilization (IVF) [1]. The physiological stimuli for the acrosome reaction involve the zona pellucida protein 3 (ZP3) [2]. Thus, when sperm are unable to reach the zona pellucida, either an external activating factor or another pathway towards activation must be utilized.

In the past, men who had this type of male factor defect were considered irreversibly infertile. The advent of intracytoplasmic sperm injection (ICSI) circumvented capitation and the acrosome reaction. Intracytoplasmic sperm injection involves the direct injection of spermatozoa into the oocyte, increasing fertilization rates to approximately 70% as reported by one study [3]. However, there is still a 2-3% failure rate with ICSI [4]. Such a failure rate has been explained by a lack of oocyte activation [5].

In normal conception, oocyte activation occurs by a two-signal process. The acrosome reaction stimulates the initial signal of Ca^{2+} release. In ICSI, injecting the spermatozoa into the oocyte stimulates signal 1. The interaction between fertilizing spermatozoa and oocyte then triggers the release of Ca^{2+} (signal 2) in an oscillatory fashion [6-8]. It is not known how the sperm activates the fertilization calcium wave. Two theories have arisen: sperm either activates a signal transduction receptor on the oocyte surface or transports an active messenger, in the form of a soluble sperm factor (SSF) [8, 9]. In failed ICSI cycles, the lack of adequate SSF may be overcome by using a calcium ionophore booster [8-11]. Calcium ionophore serves as nonphysiologic stimuli of the acrosome reaction, thus affecting signal 1 of oocyte activation. Furthermore, it increases the oocyte calcium load, allowing for the development of Ca^{2+} oscillations (signal 2) [11]. Activation of human oocytes using calcium ionophore improves fertilization rates of cases with poor ICSI fertilization [8-11]. In failed ICSI cycles with calcium ionophore, it may be inferred that while there are adequate stores of calcium, the oocyte calcium receptor is defective.

In this case report, we present a couple with prolonged unexplained infertility with repeated IVF and ICSI fertilization failure and compare the effect of using calcium ionophore on fertilization and cleavage on oocytes inseminated.

Revised manuscript accepted for publication September 20, 2007

inated by ICSI versus two controls: donor sperm with ICSI and the husband's sperm with ICSI without calcium ionophore.

Case Report

A gravida 0, para 0, 32-year-old woman and her 33-year-old husband were referred to our IVF center after three failed IVF cycles with ICSI at another center. A total of 17 eggs with seven mature eggs were produced in the three cycles. No viable embryos were produced from the three cycles and thus no embryo transfers. One oocyte had two pronuclei but failed to cleave. The couple had been married for five years with four years of primary infertility. The wife's physical and gynecological examinations were within normal limits, including sonohystogram, hysterosalpingogram, ovarian egg reserve tests, and thyroid function tests. Her menses occurred at regular intervals approximately 27 to 29 days apart. The semen analysis showed a volume of 1.1 ml; count of 0.4 mill/ml; motility of 0%; and sperm viability of 75%. Morphology studies showed that the sperm were 100% abnormal but were not round-headed sperm in that they retained an acrosome. The husband underwent further hormonal testing which all came back as normal with the following values – FSH of 7.3 mIU/ml; LH of 4.6 mIU/ml; estradiol of < 30 pg/ml; testosterone of 348 ng/dl and free testosterone of 67.7 pg/ml. Given that previous cycles of IVF had failed and the low numbers in the semen analysis, we suggested the use of ICSI with calcium ionophore and/or donor sperm with ICSI, following minimal stimulation.

One consideration for the previous failures was that the controlled ovarian hyperstimulation regimen may have adversely affected the fertilization process. Previous data did find that the longer use of leuprolide acetate could inhibit conventional fertilization of eggs [12]. Though there is evidence that the use of gonadotropins can be associated with diminished fertility it is usually considered to be related to an adverse effect on the uterine environment especially in women with normal egg reserves [13, 14]. The mid-luteal phase leuprolide acetate protocol was used in the three previous failed IVF with ICSI cycles in the other center. Thus she was placed on the minimal stimulation protocol starting with 75 IU of FSH on day 3 and used a total of 975 IU of gonadotropins. The gonadotropin releasing hormone antagonist cetrorelix (250 mcg/day) was initiated when the follicle reached 12 mm on day 7 of her cycle. Five oocytes were retrieved. All oocytes were used. Four oocytes were injected with her husband's sperm through ICSI. Two of the four oocytes received calcium ionophore. The fifth oocyte was injected with donor sperm with ICSI and without calcium ionophore. The only viable embryo produced resulted from injection of the oocyte with her husband's sperm and supplementation with calcium ionophore. The embryo was then transferred on day 3 at the 6-cell stage but failed to achieve a pregnancy.

Discussion

The failure to fertilize any eggs that appeared morphologically normal after three IVF with ICSI attempts using sperm with severe oligoasthenozoospermia would normally lead to the conclusion that a sperm defect was responsible for the failed fertilization. Even though ICSI has resulted in successful fertilization and pregnancies with extremely poor sperm, e.g., non-motile stick-like

sperm, obtained from testicular aspiration posthumously from a man who had gone almost 24 hours without life support, the ICSI process may not be a panacea for all male factor problems [15].

Some round headed sperm without apparent acrosomes have been able to fertilize eggs with ICSI though some can only do so with artificial egg activation, e.g., with calcium ionophore [16-20] and some completely fail [21]. This difference may be related to whether there is complete absence of the acrosome or at least a small portion of a critical area of the acrosome still present. Theoretically, however, sperm with perfectly normal appearance of the acrosomes could fail to fertilize even with ICSI implying a defect in the oocyte activating factor (OAF) in the sperm or a receptor defect in the oocytes.

The present case could still be interpreted as a male with severe oligoasthenoteratozoospermia who lacks the OAF and is thus helped by the use of calcium ionophore. The failure to fertilize the egg by donor sperm could be explained possibly by the fortuitous selection of one poor egg or some inefficiency in the mechanism of the ICSI process on that one egg. One hundred percent fertilization by ICSI even with perfectly normal sperm is not common.

However, these data could also be interpreted as to the possibility that there could be some women with defective oocytes that are insensitive to sperm triggering signal 2 of calcium release. However, fertilization is possible with a more potent stimulus, e.g., calcium ionophore.

The continued failure to fertilize any eggs with the male partner's sperm or donor sperm is sufficiently convincing that the previous three failed IVF-ICSI cycles were not related to the controlled ovarian hyperstimulation (COH) regimen and that the successful fertilization of the two oocytes was related to the addition of the calcium ionophore rather than the use of a minimal stimulation regimen. The plan is for her next IVF cycle to use traditional COH but to use a gonadotropin-releasing hormone antagonist rather than an agonist to make eggs available for ICSI. The plan is to attempt to fertilize 75% of the eggs with the male partner's sperm with ICSI and artificial egg activation with calcium ionophore and 25% with donor sperm and ICSI but no artificial egg activation. If once again the male partner's sperm with the help of calcium ionophore fertilizes the eggs but donor sperm again fail to fertilize then it can be concluded that in some cases an oocyte defect exists where there is relative insensitivity to the normal sperm in the oscillation phase of calcium release. If on the other hand the donor sperm does fertilize, then this would evidence that some sperm with an acrosome present may still lack the ability to activate the oocyte. However, fertilization under these circumstances with cleavage to normal appearing embryos is still possible using artificial egg activation.

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Early endometrial changes following successful implantation: 2 and 3-dimensional ultrasound study

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Summary

Objective: To study of the possible role of ultrasound (US) measurements of the endometrium in the prediction of IVF outcome. **Patients and Methods:** 28 infertile women underwent US measurements of endometrial thickness and volume on day of ET and two weeks later. US measurements were compared between day of ET and two weeks later, and between those who conceived and those who did not. **Results:** While in the group of patients who conceived ($n = 7$) endometrial thickness and volume rose significantly between day of hCG and two weeks later, no differences were observed in patients ($n = 21$) who did not. **Conclusion:** The dynamic changes in endometrial volume and thickness between day of ET and two weeks later may predict IVF treatment outcome.

Key words: Endometrial volume; 3-D ultrasonography, Prediction; ET; IVF.

Introduction

Pelvic ultrasound (US) imaging [1] and serum hCG measurements [2, 3] are part of the routine follow-up after in-vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatments. While hCG measurements are reliable as early as 11 or 12 days after embryo transfer (ET), pelvic US may visualize a gestational sac only 17-21 days after ET.

The recent advent of computerized three-dimensional (3D) US systems has led to improvement in the quality and precision of US examination. Moreover, this tool allowed endometrial volume estimation with a high degree of reproducibility [4]. While, few studies have examined the role of 3D endometrial volume on the day of hCG administration [5], oocyte pick-up (OPU) [6], embryo transfer (ET) [7] or one week later (mid-luteal phase) [8] in the prediction of IVF outcome, only one study has assessed its role during early pregnancy [9].

We therefore aimed to evaluate whether US measurements of endometrial thickness and volume differ between day of ET and two weeks later and whether this difference may predict IVF outcome.

Patients and Methods

The study population included 28 infertile women who have been treated in our IVF unit. During the routine follow-up patients underwent 2D and 3D US (VDW5-8B Probe, Voluson 530D MT, Medison-Kretz) transvaginal measurements of endometrial thickness and volume on day of ET and two weeks later. The study was approved by the institutional Clinical Research Committee.

Patients were classified according to hCG results into two further groups, those who conceived and those who did not. US measurements were compared between day of ET and two weeks later, and between those who conceived and those who did not.

The results are expressed as means and standard deviations. The statistical analysis was performed with the Student's *t*-test; $p < 0.05$ was considered significant.

Results

Twenty-eight patients were included in the study. In the whole study group no differences were observed in endometrial volume and thickness between day of ET and two weeks later.

Of the 28 patients, seven conceived. While in the group of patients who conceived endometrial volume and thickness rose significantly ($p < 0.02$, for both) between day of ET and two weeks later, no differences were observed in patients who did not (Table 1). Moreover, while comparing the mean differences in endometrial volume and thickness between day of ET and two weeks later, patients who conceived showed a significantly ($p < 0.002$) higher mean difference as compared to those who did not (Table 1).

In addition, while endometrial volume and thickness measurements two weeks following ET, were significantly higher in those who conceived as compared to those who did not ($p < 0.01$ for all), no between-group differences were observed on day of ET (Table 1).

Discussion

In the present study, in patients undergoing IVF, US measurements of the endometrium between day of ET and two weeks later, showed dynamic changes, which were unique only to patients who conceived. These changes reflect successful implantation, and the subse-

Table 1. — US measurements of endometrial thickness and volume (mean \pm SD) in patients who conceived and those who did not.

	Patients who conceived (n = 7)			Patients who did not conceive (n = 21)		
	Day of ET	2 weeks following ET	Mean differences	Day of ET	2 weeks following ET	Mean differences
US endometrial thickness (mm) (range)	11.7 \pm 1.2 (10-13)	15.7 \pm 3.7 ^a (9.5-20)	4.5 \pm 3.08 ^c (-0.5-7)	10.2 \pm 3.01 (5.1-13.5)	8.7 \pm 3.5 ^b (3-13.3)	-1.31 \pm 3.9 (-7.5-3.6)
US endometrial volume (ml) (range)	4.5 \pm 2.9 (2.5-10.1)	8.9 \pm 2.9 ^a (4.25-12.2)	5.7 \pm 3.09 ^c (1.66-8.5)	3.0 \pm 2.0 (0.83-5.5)	2.7 \pm 2.0 ^b (0.27-8.4)	-0.09 \pm 2.4 (-2.6-3.5)

^ap < 0.02 when compared to day of ET; ^bNon significant difference, when compared to day of ET; ^cp < 0.002 when compared to patients who did not conceive.

quent induced changes at the endometrial level. Although endometrial thickness rose as well, the most significant change was found in endometrial volume, which doubled from an average of 4.7 ml on the day of ET to 8.9 ml, two weeks later.

Rabinowitz *et al.* [10] observed a slower linear growth of the endometrium through the luteal phase of an IVF cycle, with a subsequent accelerated growth in conception cycles as compared with non-conception cycles. However, in their excellent review of the literature, Friedler *et al.* [11] have comprehensively described the controversy between studies comparing the mean endometrial thickness in conception and non-conception cycles and concluded that at present, insufficient data exist describing a linear correlation between endometrial thickness and the probability of conception.

To the best of our knowledge, we present the first report demonstrating dynamic changes in US measurement of endometrial thickness and volume between day of ET and two weeks later and before the appearance of a visible gestational sac. Moreover, these changes could predict IVF outcome. Further studies are needed to evaluate the role of US measurements of endometrium at different stages of IVF treatment and their role in the prediction of treatment outcome.

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Increased expression of GRP94 protein is associated with decreased sensitivity to adriamycin in ovarian carcinoma cell lines

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Summary

Purpose: This study examined the involvement of glucose regulated protein 94 (GRP94) in chemotherapy-resistance in human ovarian cancer cells. **Methods:** Three human ovarian cancer cells were examined for basal levels of GRP94 mRNA by RT-PCR and protein by Western blotting. Sensitivities to adriamycin of these cell lines were determined by means of MTT assay. The suppression of GRP94 expression was performed using specific siRNA in HO-8910PM cells, and cell apoptosis was assessed by flow cytometry. One-way ANOVA and the Student-Newman-Keuls test were used to determine which were significantly different. **Results:** HO-8910PM cells, with the highest basal levels of GRP94, exhibited the lowest sensitivity to adriamycin. In HO-8910PM cells, the sensitivity to adriamycin was increased when the GRP94 gene was disturbed by specific siRNA transfection. **Conclusions:** High GRP94 expression might be one of the molecular mechanisms causing resistance to adriamycin, and therefore GRP94 siRNA maybe useful in tumor-specific gene therapy in ovarian cancer.

Key words: Glucose regulated protein 94; Adriamycin-resistance; Human ovarian cancer cells; siRNA.

Introduction

Chemotherapy plays a very important role in the treatment of epithelial ovarian cancer. However drug-resistance seriously affects the effect of treatment and survival rate. Current approaches to the treatment of ovarian cancer are limited because of the development of the resistance to chemotherapy [1]. GRP94 is a possible candidate protein that contributes to development of drug resistance, which could be targeted in neoplastic cells [2]. Silencing of GRP94 by small interfering RNA (siRNA) could induce cell apoptosis [2]. Our study was designed to determine the role of GRP94 in regulating cellular apoptosis in ovarian cancer cells. Furthermore, silencing of GRP94 gene expression may prove to be a valuable therapeutic approach for chemoresistant ovarian cancer by increasing sensitivity of cancer cells to apoptosis.

The glucose regulated proteins (GRPs) were first described as a set of proteins whose synthesis was enhanced when mammalian cells were deprived of glucose [3]. These proteins are ubiquitously expressed and are located in the endoplasmic reticulum (ER). Because of their ability to assist in protein folding and assembly, the GRPs are referred to as molecular chaperones [4]. GRP94 has a molecular weight of 94 kDa and is one of the most common members of the GRP family [5, 6]. Its expression is up-regulated by so-called ER stress conditions, such as low glucose levels, low pH, viral infection, hypoxia and the expression of mutated

proteins [7]. Elevated levels of GRPs appear to protect tissue culture cells against adverse physiological conditions [8]. Through multiple actions, many of which remain unidentified, GRPs contribute to tumor proliferation and confer resistance to anti-cancer treatment [9]. From these reports, we speculated that GRP94 might be involved in drug-resistance in ovarian cancer.

In this study we used ovarian cancer cell lines to determine the levels of GRP94 and their relationship to adriamycin-resistance. Furthermore, we also examined whether decrease in GRP94 protein level by the specific small-interfering RNA (siRNA) would lead to a reduction in adriamycin-resistance.

Materials and Methods

Cell line and culture

HO-8910PM, SKOV₃ and 3AO cell lines were obtained from Scientific Research Foundation of the Second Affiliated Hospital of Harbin Medical University. The three cell lines were epithelial ovarian cancer cell lines, in which HO-8910PM is a highly metastasizing human ovarian cancer cell line and malignant degree is higher than the other two cell lines; SKOV₃ is a serous cystadenocarcinoma cell line and 3AO is a mucinous cystadenocarcinoma cell line. These cell lines were maintained in DMEM (Gibco, Rockville, MD) supplemented with 10% fetal bovine serum containing 100 u/ml penicillin and 100 u/ml streptomycin.

RT-PCR

We detected the basal levels of GRP94 mRNA of the three cell lines by RT-PCR. Total RNA was isolated from the three

Figure 1A shows that the expression of GRP94 mRNA in the HO-8910PM cell line was significantly higher compared to the other two cell lines (SKOV₃ and 3AO). The relative GRP94 mRNA level in the HO-8910PM cell line was 0.67 ± 0.019 , while it were 0.33 ± 0.023 and 0.33 ± 0.026 , respectively in SKOV₃ and 3AO cell lines. There was statistical significance between the HO-8910PM cell line and SKOV₃, 3AO cell lines ($p < 0.001$), while there was no statistically significant differences in SKOV₃ and 3AO cell lines ($p > 0.05$).

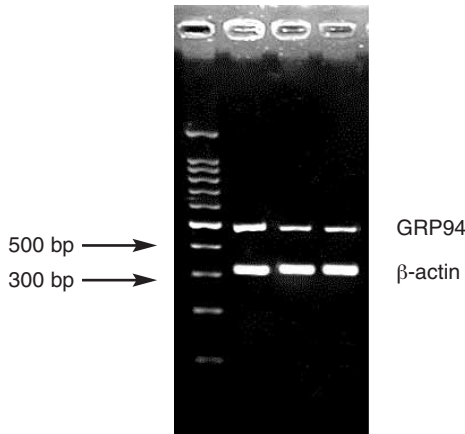


Figure 1A. — Basal levels of GRP94 mRNA in human ovarian cancer cell lines.

Lane 1: 100 bp DNA maker; Lane 2: HO-8910PM; Lane 3: SKOV₃, Lane 4: 3AO

Basal levels of GRP94 protein in human ovarian cancer cell lines

The semi-quantified Western blot technique was used to detect the basal levels of GRP94 protein in three ovarian cancer cell lines. During the experiments, β -actin was found to be suitable as standard proteins for the normalization of total protein because the expression was steady in different cell lines (Figure 1B). Figure 1B shows that the expression of GRP94 protein in the HO-8910PM cell line was significantly higher than the other two cell lines (SKOV₃ and 3AO). The relative GRP94 protein levels of HO-8910PM, SKOV₃ and 3AO were 0.774 ± 0.108 , 0.387 ± 0.085 , 0.369 ± 0.120 , respectively. From this, we can see that the expression of GRP94 in SKOV₃ and 3AO cell lines was nearly half compared with that of the HO-8910PM cell line.

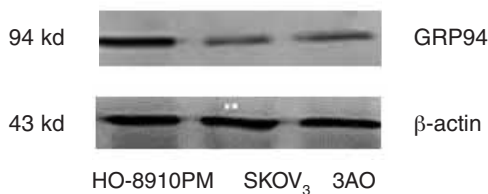


Figure 1B. — Basal levels of GRP94 protein in human ovarian cancer cell lines.

The sensitivity to adriamycin of HO-8910PM, SKOV₃ and 3AO

The sensitivity to adriamycin of HO-8910PM, SKOV₃ and 3AO was analyzed by methyl thiazolyl tetrazolium (MTT) assay. The results of MTT indicated that the survival rates of the three cell lines were gradually decreased with the gradually increased adriamycin concentration (Table 1). In the same adriamycin concentration, the survival rate of HO-8910PM cell line was significantly higher than SKOV₃ and 3AO ($p < 0.05$), which indicated HO-8910PM had lower sensitivity to adriamycin than the other two cell lines. From the above results, we can hypothesize that the low sensitivity to adriamycin is relative to the high expression of GRP94.

Table 1. — Comparison of cell viability (%) among HO-8910PM, SKOV₃ and 3AO cells exposed to ADM till 72 hr.

Group	Adriamycin concentration (ug/ml)						
	0	0.01	0.25	1.0	2.0	4.0	8.0
HO-8910PM	100	99.5*	92.4*	87.9*	82.4*	77.3*	71.8*
SKOV ₃	100	75.4	70.9	66.3	61.8	58.4	52.9
3AO	100	73.8	69.6	66.5	62.9	56.8	50.3

Cell viability (%) = $A_{546}(\text{experimental group})/A_{546}(\text{blank group}) \times 100\%$; * $p < 0.05$.

Recombination vector assessment

PsiSTRIKE™U6 vector contains a pst enzyme site and the recombination vector will form two pst enzyme sites. After the digestion of restriction pst incision enzyme, the successful recombination vector will form two DNA fragments, while the vector without oligonucleotide insertion will have only one linearity fragment in the agarose gel electrophoresis. In the agarose gel electrophoresis (Figure 2), two DNA fragments presented, which indicated the vector construction was successful.

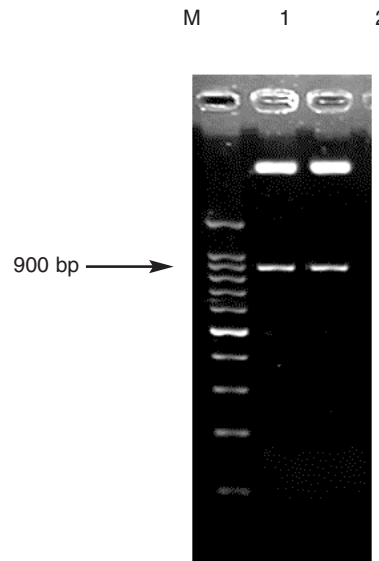


Figure 2. — GRP94 recombination vector assessment. M: 100 bp marker; 1: psiSTRIKE™/GRP94 recombination vector; 2: psiSTRIKE™/NC-GRP94 recombination vector.

GRP94 mRNA expression in HO-8910PM cells and siRNA-transfected HO-8910PM cells

To deregulate the basal GRP94 mRNA expression, siRNA targeted to GRP94 was designed and transfected into HO-8910PM cells (psiSTRIKE™/GRP94) and non-specific siRNA was also designed (psiSTRIKE™/NC-GRP94) and used for observing the non-specific effect of siRNA transfection. As Figure 3A shows, we can see that GRP94 mRNA expression was significantly degraded after siRNA-transfection, especially at 48 hr and 72 hr, while the expression of GRP94 mRNA was not degraded significantly with the treatment of NC siRNA. The specific value is shown in Table 2. β-actin mRNA expression was invariable before and after siRNA-transfection.

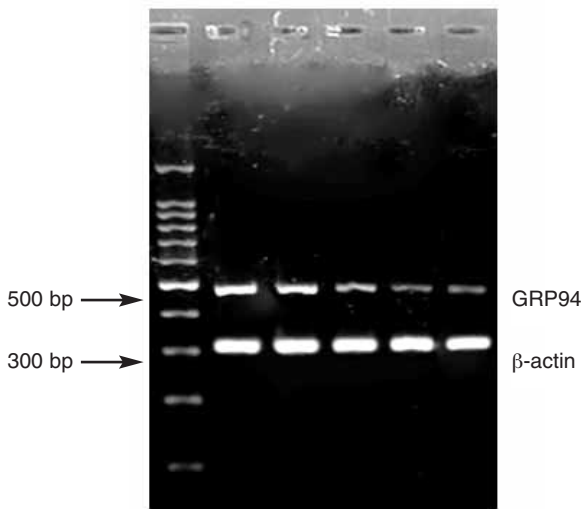


Figure 3A. — GRP94 mRNA expression in HO-8910PM cells and siRNA-transfected HO-8910PM cells.

Lane 1: 100bp DNA maker; Lane 2: HO-8910PM; Lane 3: NC siRNA-transfected HO-8910PM cells; Lane 4: siRNA-transfected HO-8910PM cells after transfection 24 hr; Lane 5: siRNA-transfected HO-8910PM cells after transfection 48 hr; Lane 6: siRNA-transfected HO-8910PM cells after transfection 72 hr.

Table 2. — *The value of GRP94 mRNA expression in HO-8910PM cells and siRNA-transfected HO-8910PM cells.*

Group	Comparative expression of mRNA	p
I HO-8910PM cell	0.61 ± 0.16	*p < 0.001
II NC- HO-8910PM cell	0.55 ± 0.40	*p > 0.05
III siRNA-transfected 24 hr	0.27 ± 0.13	*p < 0.001
IV siRNA-transfected 48 hr	0.13 ± 0.08	*p < 0.001
V siRNA-transfected 72 hr	0.12 ± 0.03	*p > 0.05

a: total comparison; b: comparison between I and II; c: comparison between II and III; d: comparison between III and IV; e: comparison between IV and V.

GRP94 protein expression in HO-8910PM cells and siRNA-transfected HO-8910PM cells

The results of protein expression were similar to that of mRNA expression. We can see in Figure 3B that the expressions of HO-8910PM cells and psiSTRIKE™/NC-GRP94 HO-8910PM cells were high and the expression

was gradually decreased at 24 hr, 48 hr and 72 hr after siRNA-transfection, while β-actin protein expression was invariable. The difference was significant (Table 3).

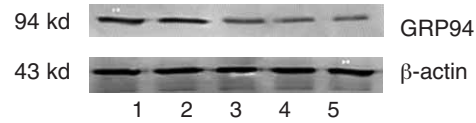


Figure 3B. — GRP94 protein expression in HO-8910PM cells and siRNA-transfected HO-8910PM cells

1: HO-8910PM cells; 2: NC siRNA-transfected HO-8910PM cells; 3: siRNA-transfected HO-8910PM cells after transfection 24 hr; 4: siRNA-transfected HO-8910PM cells after transfection 48 hr; 5: siRNA-transfected HO-8910PM cells after transfection 72 hr;

Table 3. — *The value of GRP94 protein expression in HO-8910PM cells and siRNA-transfected HO-8910PM cells.*

Group	Comparative expression of protein	p
I HO-8910PM cell	0.765 ± 0.0151	*p < 0.001
II NC- HO-8910PM cell	0.759 ± 0.0162	*p > 0.05
III siRNA-transfected 24 hr	0.396 ± 0.094	*p < 0.001
IV siRNA-transfected 48 hr	0.213 ± 0.0111	*p < 0.001
V siRNA-transfected 72 hr	0.207 ± 0.090	*p > 0.05

a: total comparison; b: comparison between I and II; c: comparison between II and III; d: comparison between III and IV; e: comparison between IV and V.

Increased sensitivity to adriamycin in GRP94 of siRNA-transfected HO-8910PM cells

The sensitivities to adriamycin of HO-8910PM cells, psiSTRIKE™/GRP94 HO-8910PM cell and psiSTRIKE™/NC-GRP94 HO-8910PM cells were analyzed by methyl thiazolyl tetrazolium (MTT) assay. The results of MTT indicated that survival rates of the three groups were gradually decreased with the gradually increased adriamycin concentration, but the degree of decrease was different; the psiSTRIKE™/GRP94 HO-8910PM cells decreased significantly (Table 4). With the same adriamycin concentration, the survival rate of psiSTRIKE™/GRP94 HO-8910PM cells was significantly lower than HO-8910PM cells and psiSTRIKE™/NC-GRP94 HO-8910PM cells (p < 0.05). The results indicated that psiSTRIKE™/GRP94 HO-8910PM cells had higher sensitivity to adriamycin than the other two groups. The sensitivity to adriamycin of HO-8910PM cells before and after siRNA transfection was changed dramatically.

Table 4. — *Comparison of cell viability (%) among HO-8910PM, NC-HO-8910PM and Si-HO-8910PM cells exposed to ADM till 72 hr.*

Group	Adriamycin concentration (ug/ml)						
	0	0.01	0.25	1.0	2.0	4.0	8.0
HO-8910PM	100	99.4	93.1	87.4	81.9	75.6	71.3
NC-HO-8910PM	100	98.7	93.2	86.3	81.5	74.8	69.4
Si-HO-8910PM	100	60.6*	52.2*	43.9*	34.7*	27.5*	21.9*

Cell viability (%) = A₅₄₆ (experimental group)/A₅₄₆ (blank group) x 100%; *p < 0.05.

Cell apoptosis assess in GRP94 of siRNA-transfected HO-8910PM cells

In the psiSTRIKE™/GRP94 HO-8910PM cells, the apoptosis ratio was gradually increased with transfection and the apoptosis ratio at 72 hr after transfection was the highest reaching 22.72 ± 0.94 . The change of apoptosis ratio in the psiSTRIKE™/NC-GRP94 group and blank group was not obvious. The difference between the psiSTRIKE™/GRP94 HO-8910PM group and psiSTRIKE™/NC-GRP94 group, and the blank group had statistical significance, respectively ($p < 0.01$), while the difference between the psiSTRIKE™/NC-GRP94 group and blank group had no statistical significance ($p > 0.05$). GRP94 siRNA can obviously induce apoptosis (Figure 4, Table 5).

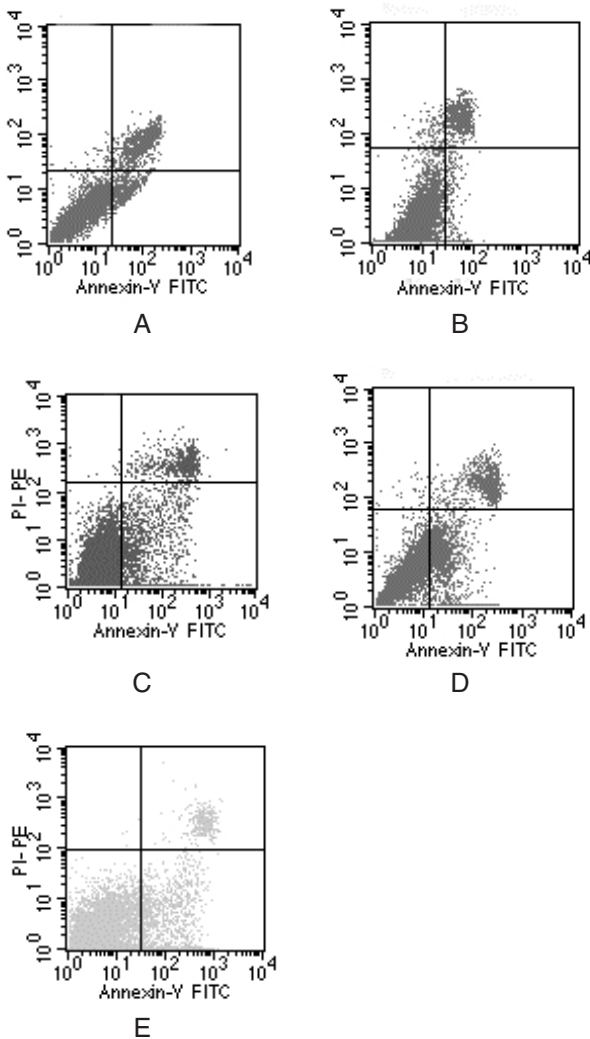


Figure 4. — Cell apoptosis.
 A: HO-8910PM cells;
 B: NC- HO-8910PM cells;
 C: siRNA-transfected HO-8910PM cells after transfection 24 hr;
 D: siRNA-transfected HO-8910PM cells after transfection 48 hr;
 E: siRNA-transfected HO-8910PM cells after transfection 72 hr.

Table 5. — Cell apoptosis ratio.

Group	Cell apoptosis ratio	p
I HO-8910PM cell	6.12 ± 0.095	^a $p < 0.001$
II NC- HO-8910PM cell	6.19 ± 0.085	^b $p > 0.05$
III siRNA-transfected 24 hr	13.59 ± 0.065	^c $p < 0.001$
IV siRNA-transfected 48 hr	21.77 ± 0.935	^d $p < 0.001$
V siRNA-transfected 72 hr	22.72 ± 0.94	^e $p > 0.05$

a: total comparison; b: comparison between I and II; c: comparison between II and III; d: comparison between III and IV; e: comparison between IV and V.

Discussion

Mammalian stress response is an evolutionarily conserved mechanism, which can induce cells to respond to stimulates from adverse environmental or metabolic conditions [10]. This response is represented at the molecular level by the induced synthesis of specific sets of cellular proteins with protective functions. The most functional proteins are glucose-regulated proteins (GRPs) and the most important place in cells to produce such proteins is the endoplasmic reticulum (ER) [11]. GRP94 is one of the most important members of GRPs. In a variety of cancer cell lines, solid tumors and human cancer biopsies, the levels of GRP94 are elevated, correlating with malignancy.

The glucose-regulated protein (GRP) system in mammalian cells is induced by glucose deprivation, anoxia, calcium ionophore A23187, and 2-deoxyglucose [12]. Since a glucose-regulated response is produced by hypoxia and nutrient deprivation that occurs naturally in solid tumors, the resistance observed here can occur in some solid tumors and can be an obstacle to chemotherapy [13].

GRP94 expression is known to be up-regulated by viral infections and other ER stresses [14]. It has also been reported that GRP94 was an effective protein in vaccination against cancer cell proliferation [15, 16]. GRP94 has also been shown to be over-expressed in some cancer cell lines and human tumor samples, and to be correlated with malignancy [17-19]. In addition, GRP94 overexpression could be closely related to increased tumor cell survival resulting in resistance to cancer treatment. Some reports suggested that resistance to hyperoxgen - or thermo-therapy and chemotherapy might be related to GRP94.

The induction of glucose-regulated proteins by a variety of specific inducers leads to an increase in resistance to adriamycin [12].

For example, GRP94 inhibited etoposide-induced apoptosis, which is dependent on Ca^{2+} , in a human T cell leukemia line, Jurkat, and a hamster fibroblast line, K12 [20], while the induction of GRP94 inhibited apoptosis of esophageal cancer cells induced by reactive oxygen stress [19].

According to recent researches, overexpression of GRP94 was associated with many malignant tumors, such as lung cancer, esophagus cancer and so on [21, 22]. Wang *et al's* research indicated that GRP94 was related to the differentiation and progression of lung cancer, and the expression in mRNA and protein level may be valuable in evaluating the grade of differentiation and clinical stage of human lung cancer [23]. Another research also

showed that the majority of esophageal cancer expressed GRP94, which differed significantly from that in adjacent normal tissue [24]. A recent study suggests that higher GRP94 protein expression is one of the molecular mechanisms causing resistance to radiation, and therefore GRP94 siRNA might be useful in tumor-specific gene therapy by reversing radio-resistance prior to radiation in cervical cancer [25]. As to ovarian cancer, there is no systemic research on the expression of GRP94 and the relationship of drug-resistance thus far.

In our study, we have found that basal expression of GRP94 mRNA and protein existed in three human ovarian cell lines (HO-8910PM, SKOV₃, 3AO), and the expression in the HO-8910PM cell line was obviously higher than the other two cell lines. This result was associated with the malignant degree of cells. The malignant degree of HO-8910PM was higher than the other two cell lines and is a kind of high metastasis cell line. And high basal levels of GRP94 expression were related with an obvious decrease in sensitivity to adriamycin chemotherapy. In addition, when GRP94 expression was inhibited specifically by siRNA in the HO-8910PM cell line, sensitivity to adriamycin chemotherapy was increased dramatically. This result suggests a strong correlation between GRP94 expression levels and resistance to adriamycin-chemotherapy. This abnormal elevation could be attributed to glucose deprivation, oxidative stress, and hypoxia in ovarian cancer cells. Such situation may induce ER stress, and GRP94 may be produced.

As a therapy target, GRP94 has been used to treat many cancers in past years. In one study on breast carcinomas, GRP94 has been shown to associate with and stabilize p185/erbB2, which is commonly overexpressed and associated with a poor prognosis. Disrupting the GRP94-p185 complex by using an antiproliferative agent, called geldanamycin, enables degradation of p185 in breast cancer cells [24]. Antisense knockdown strategies could suppress the expression levels of GRP94, which results in enhanced sensitivity to etoposide-induced cell death [27, 28]. The enhanced sensitivity could trigger proteolytic cleavage of GRP94 by calpain, which also cleaves Bcl-xl during apoptosis, therefore turning an anti-apoptotic protein into a proapoptotic molecule [29]. GRP94 could also be used in immunotherapy [30]. In a recent study, Liu *et al.*'s results indicated that combined GRP94-based immunotherapy and radiation therapy may be a potentially effective strategy for cancer treatment [31]. Our results showed that the siRNA of the GRP94 reversed chemotherapy sensitivity in vitro thus providing further evidence for the notion that GRP94 specific RNA interference might be a viable approach to ovarian cancer chemotherapy treatment. Moreover, ovarian cancers with higher levels of GRP94 mRNA and protein, treated with siRNA, could be treated with a lower chemotherapy dose to reduce the adverse reaction of drugs. We further suggest that it may be a possible therapeutic agent to improve clinical outcome of ovarian cancer. The success of such an approach, however, still awaits the development of an efficient delivery system that will affect most tumor cells in vitro.

Acknowledgements

This work was supported in part by grants from the Scientific Research Foundation, Second Affiliated Hospital of Harbin Medical University.

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Role of dietary habits on fetal anomaly development: Review of 315 consecutive fetal anomaly cases

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Summary

Objective: This study aimed to investigate and speculate on the dietary habits and certain environmental factors of the Southeast Anatolia region which are thought to be related with fetal anomaly development. **Materials and Methods:** Patients admitted to Gaziantep University Faculty of Medicine Obstetrics and Gynecology Department with fetal anomalies between January 2003 and June 2007 were evaluated. Three hundred and fifteen patients with intrauterine fetal anomaly were detected. The number of total deliveries during this period were 7,554. Twenty-eight of the patients were above the age of 35. According to the history of patient, mean gravidity was 6.6 (1-13), consanguineous marriage rate was 25%, previous abnormal rate was 15%, and previous missed abortion was 35%. Eighty-eight percent of patients were of low sociocultural status. The frequency of daily dietary habits, especially tea consumption, red chili pepper consumption and eating barbecued meat were questioned with the anamnesis. **Results:** Total fetal anomaly incidence was found to be 4.17%, neural tube defects 1.37%, renal anomalies 0.54%, non-immune fetal hydrops 0.46%, cystic hygroma 0.39%, central nervous system anomalies 0.36%, chromosomal anomalies 0.17%, gastrointestinal system anomalies 0.147%, sacrococcygeal teratoma 0.12%, cardiac anomalies 0.09%, and respiratory system anomalies 0.049%. Mean daily tea consumption during pregnancy was 8 cups/day. Mean frequency of eating barbecued meat was 4 times/week. Mean chili pepper consumption was 10 g/day. **Conclusion:** In this study, four times higher overall fetal anomaly incidence appeared to be strongly correlated with Southeast Anatolia region's dietary habits. In the future monitoring women's dietary habits should have an important role in the prevention of fetal anomaly development.

Key words: Fetal anomaly; High incidence; Dietary habits; Environmental factors.

Introduction

Intrauterine fetal anomalies especially affecting the central nervous system (CNS) include neural tube defects, and cardiovascular, gastrointestinal and urinary abnormalities cause a wide spectrum of social and medical problems. There are many factors and agents that are known or suspected to be teratogenic and considered responsible in etiology [1]. There are also many compounds that are unidentified and effects are not well known.

Recently widespread use of fetal ultrasonography (US) in the first and second trimester screening resulted in early detection of fetal anomalies, and the possible termination before viable gestational age [2, 3].

In this study, 315 fetal anomalies in the Southeast Anatolia region were classified and the prevalence identified. Additionally, dietary habits, certain environmental features, and sociocultural status were investigated in addition to demographic factors. Based on the literature, compounds that were thought to be causative were discussed and speculated on.

Material and Method

The study was performed on the patients admitted to Gaziantep University Faculty of Medicine Obstetrics and Gynecology Department with fetal anomalies between January 2003 and June 2007. Three hundred and fifteen patients with

intrauterine fetal anomalies were detected. The patients were in the 12th to 40th gestational week. The number of total deliveries during this period totaled 7,554. All of the patients admitted for delivery were living in the Southeast Anatolia region (Gaziantep, Sanliurfa, Adiyaman, Kilis, Diyarbakir).

Maternal mean age of the women with a diagnosed intrauterine fetal anomaly was 31.5 (17-46). Twenty-eight patients were above the age of 35. According to their histories mean gravidity was 6.6 (1-13), consanguineous marriage rate was 25%, previous abnormal neonate rate was 15%, and previous missed abortion rate was 35%. Eighty-eight percent of patients were of low sociocultural status.

The frequency of daily dietary habits, especially tea consumption, chili pepper consumption and eating barbecued meat were questioned with the anamnesis.

Results

In the review of the patients between January 2003 and June 2007 total fetal anomaly incidence was found to be 4.17%. Detected anomalies included:

- 1) 105 patients with neural tube defects (1.37%):
 - 30 with spina bifida;
 - 30 with anencephaly;
 - 28 with meningomyelocele and hydrocephalus;
 - 17 with encephalocele.
- 2) 42 patients with renal anomalies (0.54%):
 - 15 with renal agenesis;
 - 27 with polycystic kidney.
- 3) 35 with non-immune fetal hydrops (0.46%).
- 4) 33 with multiple anomalies (especially CNS and extremity anomalies) (0.44%).

Revised manuscript accepted for publication March 19, 2008

- 5) 29 with cystic hygroma (0.39%).
- 6) 27 with CNS anomalies (0.36%):
three with Dandy-Walker syndrome;
24 with hydrocephalus.
- 7) 13 with chromosomal anomalies (trisomy 21) (0.17%).
- 8) 11 with gastrointestinal system anomalies (0.147%):
eight with omphalocele;
three with gastroschisis.
- 9) nine with sacrococcygeal teratoma (0.12%) (Figure 5).
- 10) seven with cardiac anomalies (0.09%).
- 11) four with respiratory system anomalies (0.049%).

It has been documented that the neural tube defect is the most common fetal anomaly with an incidence of 1.37%. It is followed with renal anomalies and non-immune fetal hydrops.

Mean daily tea consumption during pregnancy was eight cups/day (7-9 cups/day) in the fetal anomaly group. Mean frequency of eating barbecued meat was four times/week (3-5 times/week). Mean chili pepper consumption was 10 g/day (9-12 g/day).

Discussion

Congenital anomalies are serious problems especially in developing and underdeveloped countries. Many factors are considered to be responsible in the etiology and numerous agents are still to be evaluated.

Fetal anomaly types and rates have been reported in case series from different areas of the world with similar results. Especially changes in neural tube defect (NTD) incidence have been widely studied in many countries and areas of the world. The prevalence rates of NTD was found to decrease in the early seventies to late nineties from 0.33% to 0.08% [4-6].

There is also a report from the middle Anatolia region, in which the overall congenital anomaly incidence was 1.11% and neural tube defect incidence was 0.27% [7]. Urogenital system anomalies were found to be the second most common type of malformation with an incidence of 0.21% [7]. Facial and musculoskeletal system abnormalities were the third and fourth most common malformations. Omphalocele incidence was five in 9,160 births and gastroschisis was one in 9,160 [7]. These data are similar to other reports [6, 8].

In our study, overall congenital anomaly incidence in the Southeast Anatolia region was found to be 4.17%. The most commonly encountered anomaly was NTD with a rate of 1.37%, followed by renal anomalies and non-immune fetal hydrops. The rate of NTD was about six times higher than some other series reported from Turkey [7]. Also the overall anomaly incidence in the Southeast Anatolia region was four times higher compared to other series reported from Turkey and worldwide [4, 5, 7].

Many chemicals, pharmaceuticals used in medical therapy, environmental effects, pollution, infectious agents and solvents are considered to have role in the etiology of fetal anomalies. Moreover, numerous studies have reported the association of neural tube defects and

folic acid deficiency, and emphasized its importance in prophylaxis [5, 9, 10].

In our study, dietary habits were questioned and certain environmental factors were evaluated. Excess consumption of tea, chili peppers and barbecued meat were obvious in the dietary habits of the Southeast Anatolia region compared with other parts of the country. Thus we supposed such consumption could be related with fetal anomaly development.

In the Southeast Anatolia region, tea is an important part of dietary intake. Already in our patient group, mean tea consumption was eight cups/day. In some studies, especially including the English and Irish populations, teratogenic effects of the tea were evaluated and the results suggested increased NTD incidence [11].

Another characteristic of the Southeast Anatolia region is the tendency of eating barbecued meat. Compared to other regions of the country, it is a very common part of the culture and frequency of barbecue was found to be four times/week in the patient group. Although data in the literature indicate that barbecue and carbon exposure is carcinogenic, there are no clear reports suggesting teratogenicity. In our study, a 4-times higher anomaly incidence may suggest a teratogenic effect of barbecued meat.

Chili peppers, a common used spice in the region, can also contain some compounds that can be teratogenic due to inappropriate production, preservation conditions and use of color additives during production. In our study mean chili pepper consumption was documented as 10 g/day. There are reports in the literature pointing to a teratogenic effect of conserved foods containing nitrites, nitrosamines and fumonisin [12, 13]. Inappropriate preservation and preparation of chili peppers may produce nitrites and nitrosamines that cause fetal anomalies.

When demographic factors are evaluated, high gravidity and associated increase in advanced maternal age, and high incidence of consanguineous marriages may be factors in the increase of overall anomaly incidence. In some studies, congenital anomaly incidence is reported to be two-times higher in consanguineous marriages compared to non-relatives [14-16].

Low sociocultural status is another characteristic of the Southeast Anatolia region. Studies have shown that in low sociocultural populations, risk of NTD is especially increased, and this is related to poor nutrition and vitamin intake of women during pregnancy. Especially in reports from large series on Mexican people, association between increased fetal anomaly and low socioeconomic and sociocultural status was prominent [17-19]. Also studies on Mexican people evaluating the effect of maternal hyperthermia and high environmental temperature on fetal anomaly incidence have shown significant results [20]. The Southeast Anatolia region is one of the parts of Anatolia with a high average temperature. It has been thought that high environmental temperature may also be related to the increase of anomaly incidence in this region.

However how many cups of tea/daily, how frequent

consumption of barbecued meat, and what amount of chili pepper may cause fetal anomaly have not been reported clearly in the current literature. In our study, a 4-times higher overall fetal anomaly incidence appeared to be strongly correlated with the Southeast Anatolia region dietary habits: 1) 10 g/day chili pepper consumption, 2) eight cups/day tea consumption, and 3) four times/week barbecued meat consumption.

In conclusion, it should be mentioned that dietary habits of regions could contribute to fetal anomaly development. Perhaps in the future, preconceptional, antenatal evaluation, and monitoring of women's dietary habits could have an important role in prevention of fetal anomalies.

Undoubtedly, large prospective studies evaluating these factors all around the world are needed.

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Non classical congenital adrenal hyperplasia due to 21-hydroxylase deficiency in families from a Greek island with a closed society

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Summary

In young members of a large family from a Greek island with a closed society, clinical and hormonal symptoms of 21-OH deficiency (CAH) were present. To discriminate those affected from those unaffected, we measured the basal and ACTH stimulated 30 values of 17-hydroxyprogesterone (17-OHP) progesterone (P) and cortisol (F) in combination with HLA-phenotypes in 25 out of 40 members of this family. The indices of the Gutai_{30-min} assessment (17-OHP+P response to ACTH testing at 30 min), GF (F response at 30 min) and the ratio GF₃₀/Guai₃₀ named the Marina index were evaluated. The Marina index showed a very statistically significant difference among the three groups ($p < 0.001$). HLA phenotypes of the members of groups A and B showed a powerful association with B₁₄, DR₁, B₇, and B₃₅ phenotypes that were related with 21-OH/CAH. In conclusion, in our study population, a high incidence of a clinically asymptomatic form of 21-OHdef was found only after the ACTH stimulation test. The Marina index seems to be of high diagnostic value in classifying disease severity.

Key words: Congenital adrenal hyperplasia, Greek island.

Introduction

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21-OH) is the most common enzymatic disorder in the metabolism of steroids in the adrenals [1]. The deficiency is inherited under the autosomal recessive pattern and is located on the short arm of chromosome 6, between the histocompatibility antigens (HLA), B-cell and drug resistant phenotypes (DR) [2]. More specifically, the genetic disorder is located in the gene CYP21B which controls the function of cytochrome P450 C21 [3]. Due to that enzymatic deficiency a smaller quantity of cortisol is produced (final product), while an unacceptable quantity of androgens (precursor substances) are found in the patient's blood. The negative feedback effect of cortisol/adrenocorticotropin (F/ACTH) results in stimulation of the pituitary gland and increased release of ACTH, leading to stimulation of the adrenals and adrenal hyperplasia, which is responsible for the clinical manifestations of CAH [4, 5].

The severity and onset of clinical symptoms depend on the percentage of 21-hydroxylase deficiency enzyme (21-OH CAH) [6]. When expressed with its most severe type (classic CAH), the symptoms are masculinization of the female external genitalia during intrauterine life or loss of

sodium chloride in the neonate leading to hydration and finally death, supposing that the pregnancy is not diagnosed early (prenatal diagnosis not only by measurement of 17-hydroxyprogesterone (17-OHP) in the amniotic fluid but also by detecting genetic changes of the gene CYP21B and given the appropriate corticosteroid treatment [7].

When expressed with its mild types, the symptoms due to blood androgen excess appear in puberty, therefore these forms are called "late onset" (late onset CAH, LO-CAH or non-classic types, NC-CAH). The symptoms are amenorrhea or oligomenorrhea, hirsutism and acne, oily skin, possibly a mild enlargement of the clitoris or masculine body image, short stature in girls, short stature and possibly sperm disturbances in boys, in the severe form of NC-CAH, due to hyperandrogenemia, which lead to penile enlargement but small testes, because the androgens are adrenal in origin. The outcome in both sexes is common; hyperandrogenemia, infertility and short adult height [8].

Each CAH form is linked to different human leucocyte antigen (HLA) phenotypes [9]. Based on literature data Bw₄₇ phenotype is linked to the severe form and B₁₄, DR₁, and B₃₅ phenotypes to the mild forms [7, 10]. Lately there have been reported cases of a non-symptomatic form of the 21-OH CAH (cryptic), in which despite their abnormal genotype and pathological response in the ACTH

Revised manuscript accepted for publication February 25, 2008

stimulation test, the patients appear with normal phenotype (regular menstruation and no clinical symptoms of hyperandrogenemia) [11].

The frequency of the classic type is between 1/5,000 and 1/25,000 among living infants in North America and Europe while that of the non-classic type is 0.2% among the white population, especially high in Eskenazi Jews and in some mediterranean countries (Spain, Italy). The frequency of the heterozygous types of 21-OH CAH is estimated to be 1/60 in the general Caucasian population and extremely high in Ashkenazi Jews (about 1/3) [1, 6, 7]. In our country several studies have been done in the Greek population [12-15]. Our aim is to contribute to the map making of the disease in the Hellenic area. Therefore we have launched two research programs approved and funded by the Ministry of Science and Technology and the Prefecture of Piraeus, respectively, to study the syndrome in the families of an island in the Saronic Gulf (with a closed society), in which young people with severe symptoms of hyperandrogenemia have been detected. The target of this study was to examine the diagnostic value of hormonal parameters and to associate the results of these parameters with HLA phenotypes, in order to discriminate between unaffected/affected people and to apply the appropriate medical treatment, especially to young people as well as prenatal care for young couples.

Materials and Methods

Patients

The subjects in our study belong to a numerous family of 40 living members formed by nine smaller families (3 generations) (Figure 1). The target person who induced us to create this study belongs to the central family, the originating family, and then the study expands to the rest of the families in which the two branches of the smaller families meet. This young woman (26 years old) presented with severe hyperandrogenemia, abnormal menstruation and infertility. She was treated with oral contraceptives as was thought to have polycystic ovarian syndrome (PCOS). After three-months of prednisolone treatment, she had regular menstruation, became pregnant, and finally after a hormonal controlled pregnancy, gave birth to a healthy female infant with normal external genitalia.

Study design

The methodology in the diagnosis of our patient population in relation to 21-OHdef and the differential diagnosis from other endocrinological disorders consisted of: a) detailed personal and family history, b) clinical examination, c) complete evaluation of hormonal levels before and after an ACTH stimulation test, and d) evaluation of the HLA phenotypes genetically linked to the disease. Patients with Cushing syndrome, adrenal or ovarian virilizing tumors, hyperprolactinemia or thyroid dysfunction were excluded from the study. None of the patients received hormonal therapy for at least 12 weeks before testing. All patients underwent an acute adrenal stimulation test to rule out or confirm 21-hydroxylase deficiency, as described below. The study was approved and funded by the Ministry of Science and Technology and the Prefecture of Piraeus and the Institutional Review Board of Athens University.

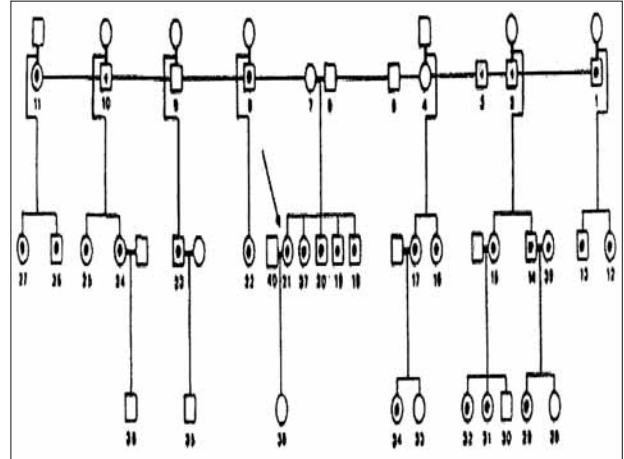


Figure 1. — Family tree of population with 21-OH deficiency. The target-patient is marked with \rightarrow and the patients that were stimulated with ACTH are marked as: male: dotted square; female: dotted circle.

We present the results of the hormonal evaluation, applied to the 25 younger members of the families. We evaluated the basal levels of 17-OHP, progesterone (P) and cortisol (F) and response to the ACTH stimulation test by measuring the levels of 17-OHP, P and F, 30 min after the ACTH stimulation test ($GF_{30} = F_{30} - F_0/30$, $Gutai_{30} = 17-OHP_{30} - 17-OHP_0 + P_{30} - P_0/30$). Thereby we measured and evaluated the speed of cortisol production (GF, 30 min) and the ratio of these two parameters, an index suggested for the first time, called Marina (named for the target member in our study): $GF_{30}/Gutai_{30}$ expressing the ratio of the final product to the precursor substances.

ACTH stimulation test

All patients were informed about and accepted the diagnostic procedure. After overnight fasting, 0.25 mg of ACTH (1-24) (Synacthen, Ciba-Geigy, Basel, Switzerland) were injected as an intravenous bolus with the patient in the supine position, in the morning, between 08:00 and 10:00 a.m. No dexamethasone was administered before testing. The test was performed in the early follicular phase (days 3-7 of the menstrual cycle) if the person was a woman, while in cases with secondary amenorrhea, menstrual bleeding was produced after progesterone administration for ten days. Blood samples were obtained before (0 min) and 60 min following ACTH administration. Serum was separated, aliquoted and stored at -20°C until assay.

Hormonal assays

Hormone measurement of F was performed by polarization fluoroimmunoassay using commercially available kits (Medgenix, Fleurus, Belgium; TDX Abbott Laboratories, IL, USA). Sensitivities, intra- and interassay coefficient of variation (CV %) were found to be 12.41 nmol/l, 5.1 and 7.0 for F; 17-OHP measurements were performed with RIA kits provided by Diagnostic Systems Laboratories (Webster, TX USA), with CV 6.3%. Progesterone measurements were performed by fluoroimmunoassay and the CV was found to be 0.25 nmol/l, 5.0 and 7.0.

Human leukocyte antigen (HLA) typing

HLA A and B phenotypes were determined on peripheral blood leukocytes using the standard National Institutes of Health (NIH) two-stage microtoxicity test [16]. HLA DR typing was performed using the PCR-SSP (sequence specific primer) technique [17, 18].

Table 1. — Classification of the study population and levels of hormonal parameters ($x \pm SEM$) in the three groups.

Group	No.	Basal levels of 17-OHP (ng/ml)	Gutai 30 min test (ng/dl/min) (17-OHP ₃₀ -17-OHP ₀)+(P ₃₀ -P ₀) 30 min	GF30 E30-F0 30 min	Marina index GE ₃₀ Gutai ₃₀ *
1	3	> 100 (105-172)	107 ± 25	76.7 ± 18.5*	0.85 ± 0.3*
2	16	≤ 3 (0.7-3)	21.6 ± 3.5	601.5 ± 42.2*	35.8 ± 4.2*
3	6	< 3 (0.5-2.8)	4.8 ± 0.9	385.0 ± 29.6*	96.8 ± 19.5*

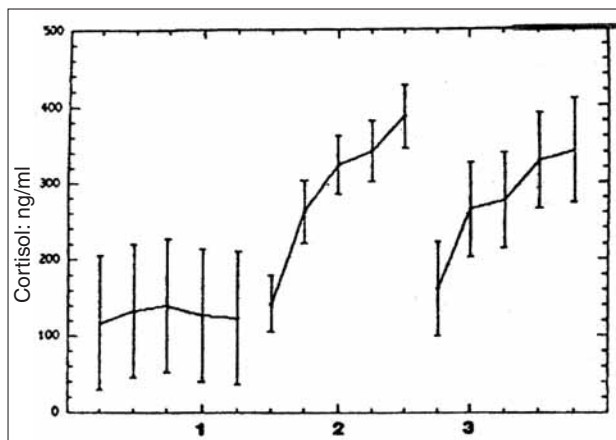
* = $p < 0.0001$: One-way ANOVA analysis.

Figure 2. — Levels of cortisol (ng/ml) after the ACTH stimulation test at 0, 15, 30, 45, and 60 min of the groups in our study.

Statistical analysis

One-way ANOVA analysis was used for the statistical analysis of the hormonal parameters; a p value less than or equal to 0.05 was considered significant.

Results

By using as a criterion the basic values of 17-OHP (3 ng/ml) and the criteria of Gutai, 30 min (7 ng/dl/min) result, the subjects of our study were divided in three groups with numerous important differences concerning clinical manifestations of 21-OH CAH and also the hormonal parameters (Table 1, Figure 2).

I. Three members of the central family, the target person and two of her brothers, with an obvious clinical picture of the 21-OH CAH (the woman with secondary amenorrhea, short stature, muscular body image, hirsutism, acne, infertility, mild enlargement of the clitoris but not masculinized external genitalia and the brothers with short stature, acne and sperm disturbances), resulted in very high basic levels of 17-OHP (105-172 ng/ml) and of P (4-8.8 ng/ml), and a very fast production of the same hormones after the ACTH stimulation test (Gutai₃₀ = 76-156 ng/dl/min). The speed of cortisol production was statistically significantly smaller compared to the other two groups, a fact that can be seen in the very low Marina ratio (Table 1).

II. Sixteen subjects from both branches of the other families, mainly without obvious clinical manifestations of 21-OH CAH and with very low basic values of 17-OHP < 3 ng/ml, resulted to have a pathological response to the ACTH stimulation test (Gutai₃₀ = 8.40-62 ng/dl/min), and the highest speed of cortisol production. As a result the

Marina index was significantly improved in comparison to group I (Table 1).

III. Six other subjects with the same low levels of 17-OHP < 3 ng/ml, and with a normal clinical picture, resulted to have a normal response to the ACTH stimulation test (Gutai₃₀ = 2-7 ng/dl/min) and a moderate production of cortisol, and as a result, the highest Marina index compared to the other two groups (Table 1).

Identification of the HLA phenotypes which are genetically linked to 21-OH CAH, has shown a very frequent appearance of B₁₄, DR₁ B₇, and B₃₅ phenotypes with localization of the previously reported phenotypes to the central family and to the middle of the right branch where the members of group B mainly belong (Figure 1).

Discussion

Our findings allowed us to make some comparisons of our diagnostic methodology with the international standards. Therefore, we suppose that the cutoff limit of basic values of 17-OHP > 3 ng/ml, or according to others the limit of basal values of 17-OHP > 2 ng/ml, to establish suspicion of the 21-OH CAH, especially in high-risk members of suspect families, should not always be fixed in order to perform a check of the differential diagnosis with the ACTH stimulation test. It is evident from our study, but as other researchers also propose [11, 12], there are clinically asymptomatic forms of 21-OH CAH which can be exposed only after the stimulation of the adrenals with ACTH [19, 20].

Accordingly, it is particularly important for the study of families with 21-OH CAH that all members of the families be examined according to the protocol of our study. On the other hand, very high basic values of 17-OHP > 8 ng/ml and a dramatic increase after an ACTH stimulation test have to be recognized as safe signs for the diagnosis of NC-CAH, as can also be seen from group I.

Also it should be noted that the criteria of Gutai₃₀ > 6.5 ng/dl/min or > 7 ng/dl/min, which is internationally accepted as a clean-cut diagnosis of the disease, can clearly detect severe cases of NC-CAH and give a degree of enzymatic insufficiency with the calculation of the precursor substances, however cannot clearly differentiate asymptomatic forms from normal cases [21]. We also believe that since there is not a calculation of the speed of production of the final product of cortisol, we do not obtain an integrated picture of the enzymatic reserve of each person.

Moreover, in the histogram proposed by New *et al.* at the two axons where the basic values of 17-OHP and the values of 17-OHP₆₀ min stand after the ACTH stimulation test, there is an important overlap between heterozygous carriers (asymptomatic forms) and normal persons [22]. For this reason we have tried to evaluate some other indices that can reflect the speed of cortisol production. We suppose that the GF index confirms the arrangement of clinically asymptomatic forms into a separate group based on the highest production of cortisol compared to the other groups. The good basic values of cortisol

together with the capability of production of sufficient quantities in cases of stress, is of particular importance for the normal clinical and biochemical profile of a person, even if there is a pathological genotype. In these cases there is no activation of the hypophyseal/adrenal axis, there is no permanent hyperfunction of the adrenals and the concentration of androgens in blood, and as a result there are no final clinical features of CAH presenting with various degrees of hyperandrogenemia. Accordingly, the ratio of cortisol/precursor substances, the Marina index, which for the first time is proposed because it includes the GF parameter, we assume that it is more indicative for the precise, clinical characterization of both healthy and affected persons, since it better approaches the enzymatic adequacy of the person and measures the person's ability to react to stress.

As a conclusion, we assume that a) the ratio of the clinically asymptomatic forms in the subjects of our study is big enough and testifies to our diagnostic methodology for their exposure and identification; b) the Marina index has an important diagnostic value for the categorization and the classification of patients since it seems to better approach the enzymatic adequacy of the person which is importantly relevant to his/her genetic defect; c) with the identification of HLA phenotypes that are genetically linked to the disease and the comparison with the hormonal data, the subjects of group I should genetically present the more severe form of NC-CAH, the genotype severe/mild; the persons of group II should belong to the milder forms of the 21-OHdef: mild/mild, severe/normal or mild/normal; and the persons of group III, the phenotypically normal, probably belong to other categories of enzyme defects and/or present PCOS or a very small number of these are heterozygous carriers, or they have a completely normal genotype. With the progress of molecular biology it has become possible to analyze the gene CYP21B which encodes the enzyme 21-hydroxylase [13, 23]. As a result we know today that the various forms of the disease are due to determinate genetic changes (mutations, deletions, changes to the clusters of bases, etc.) of the CYP21B gene which encodes the enzyme 21-hydroxylase. In this way, the results of hormonal evaluations, the stimulation of the adrenals with ACTH, and the results of the HLA phenotypes can be controlled and confirmed at the gene level. The accurate diagnosis of CAH can be confirmed by molecular gene analysis [24-28], but even today in clinical practice, molecular biology is not routinely available. The ACTH stimulation test remains the principal diagnostic tool. The reason for a quick diagnosis of this enzymatic disorder in clinical practice using the adrenal stimulation test is the simplicity of this test. Thus, we have presented an index, that based on the adrenal stimulation test, affords a quick approach and diagnosis of the severity of 21-OH CAH, especially in members of families highly suspicious for CAH and belonging to closed societies, which is easy to detect and with low-cost effectiveness that can be followed-up later with molecular gene analysis, if deemed necessary.

Finally, in our study population, a high incidence of a clinically asymptomatic form of 21-OH CAH was found, revealed only after the ACTH stimulation test. The proposed index Marina seems to be of high diagnostic value in classifying the disease severity.

Acknowledgements

This study arises from two research programs that have been approved and funded by the Ministry of Science and Technology of the Hellenic Democracy and the Prefecture of Piraeus, respectively, whom the authors gratefully thank.

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Lipid peroxidation and antioxidant status in hypertensive pregnancies

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Summary

Aims and Objective: The present study was designed to evaluate the lipid peroxidation and non-enzymatic antioxidant status in hypertensive complications during pregnancy (preeclamptic and eclamptic) compared with healthy pregnant and non-pregnant patients as controls. **Materials and Methods:** 25 healthy non-pregnant women as controls, 25 third trimester normal pregnant women and 25 preeclamptic and 25 eclamptic patients of the same trimester. Lipid peroxidation as a thiobarbituric acid reactive substance reduced glutathione, Vitamin E, Vitamin C and Vitamin A. **Results:** Lipid peroxidation was significantly elevated and significantly decreased levels of antioxidants were found in preeclamptic and eclamptic patients as compared with normal pregnant and control subjects. **Conclusion:** Our study gives support to those few studies considering lipid peroxidation as an important factor in the pathogenesis of preeclampsia and eclampsia. The decrease in antioxidants, probably of a compensatory nature responding to the increased peroxide load in preeclampsia and eclampsia, may reflect the severity of the disease.

Key words: Lipid peroxidation; Malondialdehyde, Preeclampsia; Eclampsia.

Introduction

Hypertensive disorders are the most common medical complications of pregnancy, and are the third leading cause of maternal mortality, after thromboembolism and non-obstetric injuries. Preeclampsia/eclampsia is a specific pregnancy syndrome, occurring in approximately 5% of all pregnancies, 10% of first pregnancies and 20-25% of women with chronic hypertension [1]. Lipid peroxidation has been suggested as a causative factor and is found to increase in pregnancy [2]. The metabolic effects of lipid peroxides may be linked to an imbalance between the production of prostacyclin and thromboxane A₂ [3] that is well documented in preeclampsia [4]. Lipid peroxides in preeclampsia has been suggested that poorly perfused placental tissue may evoke the free radical process and inception of generalized lipid peroxidation [5]. It is envisaged that increased free radical activity (oxidative stress) arises from increased production of free radicals or a deficiency in the protective antioxidant system. Preeclampsia is associated with endothelial dysfunction [6]. Such dysfunction could be caused by oxidative stress: the unsaturated lipids and thiol containing proteins in cell membranes are susceptible to free radical attack [7]. There is evidence of increased free radical activity in pregnancy-induced hypertension [8]. Specific antioxidants will play a role in diminishing the action of lipid peroxides.

In the present study, we tested the hypothesis that the normal balance between lipid peroxidation and antioxidant levels observed during uncomplicated pregnancies is impaired in preeclampsia and eclampsia. To test this

hypothesis we measured plasma antioxidant levels relative to lipid peroxide levels during normal pregnancy and compared this relationship with that of preeclamptic and eclamptic patients.

Materials and Methods

The present study was carried out jointly by the Departments of Biochemistry and Obstetrics and Gynecology from July 2000 to June 2004. The ethical committee of J.N. Medical College and District Civil Hospital of Belgaum approved the study protocol. Informed consent was given by all subjects. The study comprised 100 cases, of which 25 were normal healthy controls, 25 were normal healthy pregnant women in the third trimester, 25 were third trimester preeclamptic women and 25 women were eclamptic in the same trimester. The subjects (aged 20-40 years) selected for the present study were outpatients or patients admitted to the District Civil Hospital of Belgaum. The diagnosis of preeclampsia was based on the definition of ACOG [8]: 1) Systolic blood pressure greater than 140 mm Hg or a rise of at least 30 mm Hg or 2) Diastolic blood pressure greater than 90 mm Hg or a rise of at least 15 mm Hg (manifested on two occasions at least 6 hours apart), and 3) Proteinuria of 300 mg or greater in a 24-hour urine collection or protein concentration of 1 g/l (on two occasions at least 6 hours apart). Eclampsia was defined as the occurrence of seizures in women with preeclampsia. Subjects with normal pregnancies were normotensive throughout the gestation and had no proteinuria. None of the women had received antihypertensive medication until the study samples were taken. Blood pressure levels and proteinuria were determined at the time of sampling.

The subjects were of low socio-economic status. Obese women, women with diabetes mellitus under medication and untreated diabetes, women with severe anemia (< 6.0 g% of Hgb), alcoholics, and women suffering from any other systemic disorders were excluded from the present study.

Blood samples (5 ml) were drawn by venipuncture and collected in a heparinized tube. Malondialdehyde, a product of

Revised manuscript accepted for publication October 29, 2008

lipid peroxide detectable in blood, was used as an indicator of lipid peroxidation. Malondialdehyde concentrations were determined by using thiobarbituric acid [9]. Plasma was separated to determine the antioxidant levels like reduced glutathione (Beutler E *et al.* [10]), Vitamin E (Quaife and Baker [11]), Vitamin C (Evelyn and Malloy [12]) and Vitamin A (Bessey *et al.* [13]). Statistical data were expressed as mean \pm SD. Statistical significance was determined by ANOVA and the Bonferroni multiple comparison test.

Results

Table 1 summarizes the characteristics of the four groups. A statistically significant increase in the levels of circulating malondialdehyde, a marker of lipid peroxidation, was observed in the third trimester in normal pregnant women and in preeclamptic and eclamptic patients as compared to non-pregnant controls. Further increase was observed in preeclamptic and eclamptic patients when compared to normal pregnant women.

Non-enzymatic antioxidants like reduced glutathione, Vitamin E, Vitamin C and Vitamin A differed significantly in each group by analysis of variance.

Table 1. — Malondialdehyde (MDA) enzymatic antioxidants reduced glutathione, Vitamin E, Vitamin C, and Vitamin A levels in the non-pregnant, 3rd trimester normal pregnant and 3rd trimester toxemic (preeclamptic and eclamptic) women.

Groups	MDA n mol/ml	Reduced glutathione mg %	Vitamin E mg %	Vitamin C mg %	Vitamin A μ g %
Non-pregnant women (n = 25)	1.19 \pm 0.09	59.97 \pm 1.3	1.42 \pm 0.19	1.11 \pm 0.24	28.62 \pm 3.45
3 rd trimester normal pregnancy (n = 25)	1.79 \pm 0.14	50.77 \pm 6.91	0.87 \pm 0.15	0.96 \pm 0.33	22.84 \pm 5.07
3 rd trimester pre- eclamptic (n = 25)	2.93 \pm 0.54	41.35 \pm 4.23	0.57 \pm 0.16	0.66 \pm 0.24	18.93 \pm 5.28
3 rd trimester eclamptic (n = 25)	4.80 \pm 0.61	36.92 \pm 4.71	0.48 \pm 0.14	0.48 \pm 0.22	16.11 \pm 3.41
F value	347.849	113.075	167.781	30.891	36.729
p value	0.0005	0.0005	0.0005	0.0005	0.0005

Discussion

Oxidative stress during pregnancy and preeclampsia and eclampsia was evaluated in the present study by analyzing pro-oxidant and non-enzymatic antioxidants. Lipid peroxidation was considered as a marker for pro-oxidant, whereas reduced glutathione, Vitamin E, Vitamin C and Vitamin A were considered as antioxidants.

Free radicals by their unstable and transient nature are difficult to measure directly. Their tendency to cause lipid peroxidation has been used as an indirect measure. Markers of lipid peroxidation (such as MDA) have been increased during the progression of a normal pregnancy [14]. Non-enzymatic antioxidants like reduced glutathione, Vitamin E, Vitamin C and Vitamin A oppose the toxic actions of lipid peroxides and oxygen radicals by limiting the amount of lipid peroxide formation. In the lipophilic environment tocopherol acts as a protective

chain-breaking antioxidant. Vitamin C acts as an antioxidant by scavenging superoxides, hydroxyl radicals and various lipid hydroperoxides. In addition it can also restore the antioxidant properties of oxidized Vitamin E.

Hubel *et al.* [2] and Kharb [15] have shown that serum lipid peroxides increase during pregnancy and this increase was exaggerated in preeclampsia. This increased lipid peroxide level can increase the susceptibility of polyunsaturated fatty acid to peroxidative damage, presumably by free radicals that may lead to the formation of malondialdehyde.

We found a significant increase in lipid peroxide (MDA) levels in the third trimester of normal pregnant women compared to non pregnant women, and a further increase was observed in preeclamptic and eclamptic patients when compared to normal pregnant women as well as non-pregnant women. Our findings are in accordance with some researchers that show lipid peroxidation may be an important factor in the pathogenesis of pregnancy-induced hypertension [16].

Glutathione, an effective reductant, plays an important role in a variety of detoxication processes. It readily neutralizes the hydroxyl radicals, which are considered to be a major source of free radical damage. Decreased levels of glutathione in maternal blood might indicate decreased detoxification of free radical scavenging in preeclampsia [17]. Our study showed a significant decreased level of reduced glutathione and increased levels of MDA in preeclamptic and eclamptic patients as compared to normotensive pregnant patients as well as non-pregnant controls, which was supported by Pyska *et al.* [18].

Kharb [19] reported that vascular endothelial damage has been implicated in the pathophysiology of preeclampsia. Significantly lower levels of Vitamin E and Vitamin C were observed in preeclamptic women as compared to controls. Our study indicates that normal pregnant women had markedly reduced plasma alpha tocopherol, ascorbic acid, and retinol as compared to non-pregnant controls. A further decrease was observed in preeclamptic and eclamptic patients when compared to normal pregnant women.

Hypertensive complications of pregnancy are not the only disease group where enhanced lipid peroxidation and decreased levels of antioxidants are reported. Early attention, intensive management and better treatment with antioxidant vitamins are essential to improve the maternal and fetal outcome in pregnancy-induced hypertensive patients.

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Incidence of cervical cancer in Montenegro

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Summary

Objective and Method: To analyze the development of the number of cervical cancer cases and those who died of the disease in Montenegro. We used data on the number of cases and deaths over a eight-year period (2000-2007). **Results:** In Montenegro 550 women developed cervical cancer in that period, which is 45% of all cancers affecting the female genital organs. In the first five years, the average disease rate was 19.52/100,000, but in the last three years it amounted to 25,61/100,000 (around 24% higher). The trend suggests significant growth. The average mortality rate was 4.2/100,000. The trend suggests very slight growth. The majority of cases were aged between 40 and 49 years (196 cases out of 550 or 35.64%). **Conclusion:** Almost 2/3 of the cases (64.19%) were aged between 40 and 59 years. There is a need to organize screening of the entire female population aged between 20 and 65-70 years. There is also a need to establish a central registry for malignant diseases in Montenegro.

Key words: Cervical cancer; Incidence; Screening.

Introduction

Cervical cancer is the most frequent malignant disease of the female genital organs and accounts for 12% of all malignant diseases [1]. Cervical cancer strikes nearly half a million women around the globe and claims 270,000 lives each year. Around 75% of all diseased cases and 80% of those who die are from less developed regions of the world [2].

Average standardized incidence rate in less developed regions is 19.1/100,000 and is twice as high as the rate in developed regions (10.3/100,000), whereas mortality rate is 2.8 times higher in undeveloped regions (11.2/100,000) if compared to developed ones (4.0/100,000) [3]. Finland is the country with the lowest incidence rate (4.3/100,000) and in France the incidence rate is 9.8/100,000, whereas in Romania it is 23.9/100,000 [3]. Incidence rate in Serbia amounts to 24.3/100,000, whereas mortality rate is 6.8/100,000 [4].

Reduction in the number of diseased cases and women who die of cervical cancer in developed countries has resulted from years of screening aimed at early detection of premalignant and malignant changes in cervixes. The aim of screening is to cover as many women as possible, to examine them and conduct a Pap test. It has been shown that an increase in the number of the target population examined produces better screening effects than more frequent repetition of screening of the same women [5]. Predominantly the accepted opinion is that women aged between 20 and 65 years should be included in screening, even though some suggest that the threshold should be 70 years. Following three consecutive normal cytological smears with each taken after a one-year period, further screening of women in low-risk groups should be conducted every two or three years [6, 7].

In Montenegro well organized screening for the early detection of cervical cancer still does not exist. Some activities have been undertaken in that respect which is why annual work plans of health institutions (healthcare centers) contain the item – systematic examination of women – which mainly includes small numbers of women examined and repeated examinations of the same women.

There is no reliable registry of malignant diseases in Montenegro which is why valid data concerning diseases of the female genital organs can only be found in the records of the Council for Malignant Diseases of Female Genital Organs (CMDFGO) in the Clinical Center of Montenegro, Podgorica, which includes all women with an established diagnosis of malignant disease of the female genital organs.

Materials and Method

Data obtained from the CMDFGO in the Clinical Center of Montenegro, protocols of women who underwent surgery, and pathohistological examinations of women treated in the Department of Gynecology and Obstetrics at the Clinical Center were reviewed. All women with a diagnosis of cervical cancer are addressed to the CMDFGO where a final decision as to further treatment is made.

Data on number of the female population and mortality data were extracted from the Bureau of Statistics of the Republic of Montenegro (MONSTAT).

The observation period was eight years (2000-2007). Data for the period between 2000 and 2004 had been gathered earlier, and then the data between 2005 and 2007 were processed, and we have sought to consider the potential differences.

Results

Tables 1a and 1b show the distribution of female genital cancer cases in Montenegro.

Over a five-year period (2000-2004) 308 women in Montenegro developed cervical cancer, whereas over the last three years (2005-2007), 242 women developed cer-

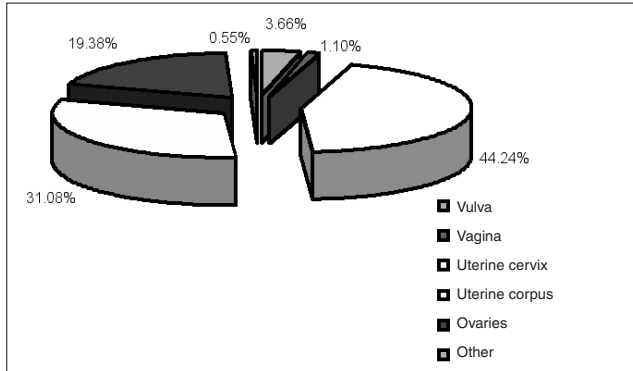
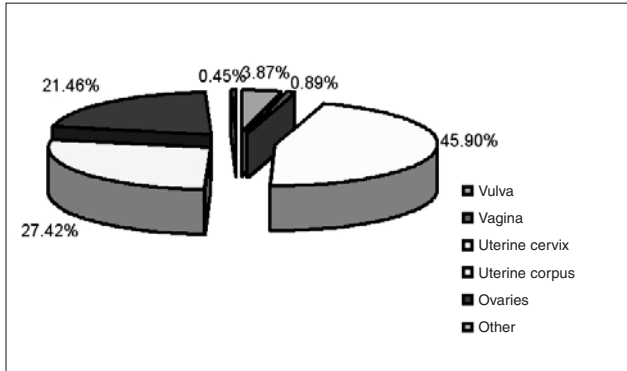


Figure 1a/1b. — Graphic distribution of Tables 1a/b.

Table 1a.

	2000	2001	2002	2003	2004	Total	
Vulva	7	4	8	3	4	26	3.87%
Vagina	3			2	1	6	0.89%
Uterine cervix	70	64	56	48	70	308	45.90%
Uterine corpus	47	36	25	40	36	184	27.42%
Ovaries	32	20	34	23	35	144	21.46%
Other				2	1	3	0.45%
Total	159	124	123	118	147	671	100.00%

*Other: choriocarcinoma 1, fallopian tube cancer 1, and malignant schwannoma 1.

Table 1b.

	2005	2006	2007	Total	
Vulva	11	3	6	20	3.66%
Vagina	1	2	3	6	1.10%
Uterine cervix	81	76	85	242	44.24%
Uterine corpus	60	47	63	170	31.08%
Ovaries	45	29	32	106	19.38%
Other	3			3	0.55%
Total	201	157	189	547	100.00%

**Other: choriocarcinoma 2, fallopian tube cancer 1.

Table 2.

Uterine cervical cancer	2000	2001	2002	2003	2004	2005	2006	2007
Cases (no.)	70	64	56	48	70	81	76	85
Rate	22.2	20.3	17.7	15.2	22.2	25.72	24.13	26.99
Decreased (no.)	8	19	11	10	15	17	14	
Rate	2.5	6.0	3.4	3.1	4.7	5.4	4.4	

*** Mortality data for 2007 have still not been processed by MONSTAT.

vical cancer which is an approximate 24% increase with respect to the annual average. Cervical cancer is the most frequent malignant disease of the female genital organs and accounts for 45% of all malignant diseases. It is followed by endometrial cancer accounting for around 27-31%, ovarian cancer with around 19-21%, and vulvar cancer with somewhat more than 3.5%.

Table 2 contains data on the number of diseased cases and women who died of cervical cancer, and disease and mortality rate per 100,000/year.

Disease rate (by year) moves within a range from 15.2/100,000 to 26.99/100,000. Average disease rate in the observed eight-year period in Montenegro is 21.83/100,000 which is at the level of non-developed regions.

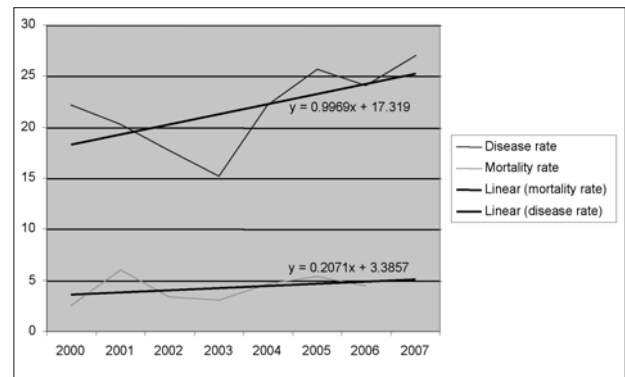


Figure 2.

Mortality rate over the seven-year period (2000-2006) is within a range of 3.1-6.0/100,000. Average mortality rate is 4.2/100,000 which is nearly at the level of developed regions.

The trend graphically shows the incidence of disease rate and mortality caused by cervical cancer.

Both of the observed rates indicate growth. Disease rate suggests more substantial growth over the last eight years, whereas the mortality rate suggests minor growth.

Tables 3a and 3b provide data regarding the number of cervical cancer cases relative to age (age groups, data about the number of females according to age group and calculation of specific rates over the last eight years).

Average disease rate in the first five years is 19.52/100,000, whereas over the last three years it amounts to 25.61/100,000 which is higher by around 24%.

Around 320,000 women live in Montenegro (according to the 2003 census there were 314,920 women). Tables indicate the number of people according to the five-year period for females aged 20 and more years.

Out of the total number of cases (8 years), 82 or 14.91% were younger than 40 years of age, while 196 or 35.64% were aged between 40 and 49 years. In the sixth life decade (50-59 years) 157 or 28.55% of women developed cancer, while in the seventh life decade there were 85 or 15.45% cases. Thirty women (5.45%), aged over 60 years developed the disease.

Table 3a (2000-2004).

	20-24	25-29	30-34	35-39	40-44	45-49	50-54	55-59	60-64	65-69	70-74	75-79	80+
No. of females	24,171	22,632	20,963	20,908	22,111	22,002	20,523	14,900	15,840	15,442	11,889	8,471	6,374
Uterine cervix	1	4	13	30	49	63	51	28	29	22	11	7	
Rate	0.8	3.5	12.4	28.7	44.3	57.3	49.7	37.6	36.6	28.5	18.5	16.5	

Table 3b (2005-2007).

	20-24	25-29	30-34	35-39	40-44	45-49	50-54	55-59	60-64	65-69	70-74	75-79	80+
No. of females	24,171	22,632	20,963	20,908	22,111	22,002	20,523	14,900	15,840	15,442	11,889	8,471	6,374
Uterine cervix		2	11	21	39	45	44	34	12	22	9	1	2
Rate		2.9	17.5	33.5	58.8	68.2	71.5	76.0	25.2	47.5	25.2	3.9	10.4

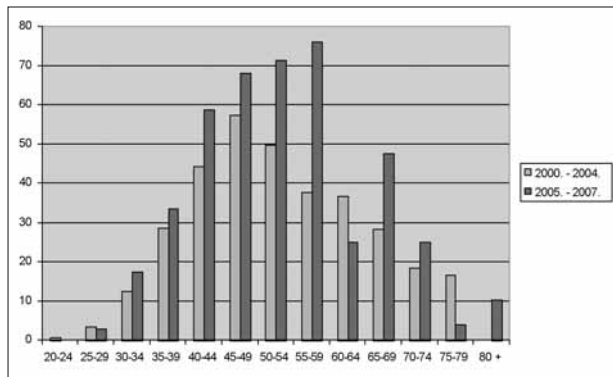


Figure 3. — Graphic of the data from Table 3.

Over the last three years (2005-2007) the number of women with cervical cancer has increased by around 24%. The increase differs in certain age groups. For women under 40 years, the increase is 15%, for those aged between 40 and 50 the increase is 29% (range from 16% in the group aged between 45 and 49 years of age, range from 50% for the group aged between 55 and 59 years of age). The rise in incidence over the last three years among women aged between 40 and 44 years is 25%, while it amounts to 30% among women aged between 50 and 54 years.

In the period between 2000 and 2004, the highest specific incidence rate was among women aged between 45-49 years, whereas over the last three years the highest specific incidence rate was among women groups aged between 50 and 59 years.

Table 4 provides data for all eight years on the number of cervical cancer cases by municipalities, number of the female population and calculation of disease rate.

Average cervical cancer rate in Montenegro over the period 2000-2007 is 21.8/100,000. As indicated in the table, disease occurrence in municipalities is quite uneven.

It was observed that in eight municipalities (out of a total of 21 in Montenegro) the cervical cancer rate is higher than the average. In those municipalities there were 354 cervical cancer cases, while the number of females was 155,543 (49.4% in Montenegro); therefore the average incidence rate is those eight municipalities is 28.4/100,000 and is higher by 23% than the average rate in Montenegro.

Table 4.

2000-2007	No. of women	Cervical cancer	Incidence rate
Andrijeвица	2844	4	17.58
Bar	20508	59	35.96
Berane	17523	33	23.54
Bijelo Polje	25057	22	10.97
Budva	8275	16	24.17
Cetinje	9603	21	27.34
Danilovgrad	8283	10	15.09
Herceg Novi	17027	26	19.09
Kolašin	4966	10	25.17
Kotor	12074	20	20.71
Mojkovac	5022	8	19.91
Nikšić	38113	53	17.38
Plav	6984	3	5.37
Pljevlja	18246	22	15.07
Plužine	2135		
Podgorica	86264	193	27.97
Rozaje	11336	16	17.64
Šavnik	1485	3	25.25
Tivat	6919	19	34.33
Ulcinj	10118	11	13.59
Žabljak	2138	1	5.85
Total	314920	550	21.83

Discussion

There is no a malignant disease registry in Montenegro. Registration and records of malignant diseases are not arranged or complete. The data we have processed were retrieved from various sources. The obligation of all women with malignant disease of the female genital organs to attend the CMDFGO at the Clinical Center in Podgorica has enabled us to analyze the data we have presented (the number of cervical cancer cases and mortalities, distribution by age of disease occurrence, age groups and place of residence-municipalities). It was impossible to analyze the complete data on stage of disease at the time of establishing the diagnosis, manner of treatment, and results of treatment. The majority of cases were treated in other localities in the region, while only a small number were treated in Montenegro. This imposes the need for the Council to keep more complete and quality records.

With good recordkeeping and gathering of all data (starting from initial diagnosis to the therapy prescribed and further monitoring) in one place – Center or Malignant

nant Disease Registry - regular (annual) evaluation of these data would create conditions for undertaking all appropriate measures for resolution of the problem arising from this disease, as is the practice in developed regions of the world.

The aim of well organized screening is to stop the rise in the number of cervical cancer cases, and reduce the number in later stages. It is well known that in the first years of screening the number of disease cases rises (higher number of newly detected cases). Maybe this rise in the number of the cases over the last three years has resulted from incomplete screening that has been conducted in our country. A more aggressive media campaign has been undertaken in the last four years to popularize systematic examinations of women aimed at early detection of cervical cancer.

Our data suggest that the growth in disease rate is more substantial, similar to the average rate in undeveloped countries, than the mortality rate which is like the average rate in developed countries. This might substantiate the assumption presented previously (the result of incomplete screening and media popularization of systematic examinations), while recognized knowledge and protocols are used in treatment which is why the mortality rate has slightly increased.

This paper is aimed at emphasizing the need to organize adequate screening and establish a central cancer registry. Screening should first be conducted with 20-year-old women, i.e., three years after first sexual intercourse. The threshold for screening is mainly 65 years of age, but some still advocate for 70 years of age.

In Montenegro there are 185,000 women aged between 20 and 64 years, i.e., 200,000 women aged between 20 and 69 years. It is quite possible to conduct complete screening of the entire female population once in three years for women aged between 20 and 65 or 70 years. That means that annually 62,000-67,000 women should undergo screening.

Conclusions

Cervical cancer is the most frequent disease of the female genital organs in Montenegro (around 45%).

Cervical cancer incidence in Montenegro shows constant growth.

Over the last three years it has grown from 19-20 to 25-26/100,000.

Almost two-thirds of the disease cases (64.19%) are aged between 40 and 59 years.

Incidence rate is uneven in various municipalities.

It is necessary to organize screening of all women aged between 20 and 65 years (or between 20 and 70 years) and it should be a state-run project.

There is a need to establish a central registry for malignant diseases which would contain all the data starting from initial diagnosis to the results achieved by therapy.

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Abortion rates and the role of family planning: A presentation of the Greek reality

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Summary

Objective: Due to lack of consistent evidence, it is widely believed that Greece holds one of the highest abortion rates in Europe. The aim of this study is to clarify these rates. **Study design:** The Greek Society of Obstetrics and Gynecology collected data demonstrating that abortion rates seem to be declining which coincides with the rise of the use of more effective methods of contraception. Data were collected both from public and private hospitals, and an attempt was made to correlate the data with the current trends of family planning and birth control. **Results:** A decline in abortion rates in Greece was found, which may be due to the better organization of counseling programmes in the field. **Conclusion:** The programmes organized by the Greek Family Planning Association under the guidance of the University of Athens has led to a decline in abortion rates in Greece.

Key words: Abortion; Greece; Law; Family planning.

Introduction

Abortion is defined as a procedure to terminate an unintended pregnancy. Women's access to safe abortion was negotiated in the Programme of Action of the International Conference of Population and Development (ICPD) in 1994. However, in 2000 an estimated 19 million women had unsafe abortions and some 68,000 died (14% of them was under the age of 20, and 955 lived in developing countries) [1-3]. It should be mentioned that there is lack of evidence in the field, even in developed countries, because induced abortions are often severely under-reported in national surveys. For instance, a comparison of the National Survey of Family Growth with information from other sources has shown that fewer than 50% of the abortions performed in the USA among women aged 15-44 were finally reported [4]. The same data also showed that abortion reporting is lower in Catholic women, black women, women aged 25-29 years old, women never married at the time of abortion or women with incomes below 200% the poverty level [1]. On the other hand, in an Australian study it was shown that the estimated abortion rate ranged from 17.9/1,000 in 1985, to 21.9/1,000 in 1995 and to 19.7/1,000 in 2003, with the total estimated number of abortions reaching 84,460 [5]. Another study showed that the abortion rate in Greece was estimated as 100-120 per 1,000 women in 2000 compared to 20-30/1,000 in Austria and 25/1,000 in the Netherlands (6). However, the Greek reality lacks evidence in the field.

Abortions in Greece were legalized in 1986 (law no. 1609). The law states (presidential act no. 304) an abortion is legal if carried out with the consent of the pregnant woman by an obstetrician-gynecologist, with an anesthesiologist present in an equipped hospital. If the

pregnant woman is under age 18, the consent of one of the parents or the legal guardian is required. Furthermore, one of the following conditions must be met:

- 1) The fetus must be below 12 gestational weeks;
- 2) When there is hard evidence of congenital anomalies, the fetus could also be up to 24 gestational weeks;
- 3) An abortion is also legal if a doctor confirms that the continuance of the pregnancy involves unavoidable risk for the life or health (physical or mental) of the pregnant woman up to 24 weeks;
- 4) Finally, an abortion is legal if the pregnancy resulted from a rape or other forms of coercion and the fetus is under 19 gestational weeks.

Greek organizations estimated that the number of illegal abortions in the 1970s was 300,000 as there was no information regarding contraceptive methods and thus abortion was thought to be the only solution for an unwanted pregnancy [7]. The Family Planning Association (FPA) was officially oriented in Greece in 1976 [6]. The members of the Greek FPA in 1995 pointed out the lack of guidance and care for women which manifested as an above average abortion number. The number was estimated at about 150,000 abortions per year. The lack of consistent evidence was unexpected and led to much consideration over the following decade. It should be mentioned that abortions were carried out mainly in the private clinics at that time. However, the data held by the Greek National Statistical Service referred only to abortions taking place in public hospitals. This total number of abortions was between 100,000 and 120,000 per year in 2000 [8], a number equal to that of births per year [6]. Furthermore, the representatives of the Greek FPA at the 2nd National Conference on Reproductive and Social Health in 2000 reported that unregistered abortions were estimated to reach 300,000 [6].

The main goal in the FPA policy in Greece is to minimize the number of unintended pregnancies by providing

Revised manuscript accepted for publication April 21, 2008

Table 1. — Public hospitals and rates of abortions in Greece.

No.	Institution	Number of abortions	Gestational age	Abortions/month	Women's mean age
<i>Athenian Hospitals</i>					
1	Ob/Gyn Hospital, "Helena Venizelou" (NHS)	1752	8-10 weeks	73.0	29.3
2	Ob/Gyn Clinic "Agia Olga" Hospital (NHS)	337	8 weeks	14.0	30.2
3	1 st Dept. of Ob/Gyn, Univ. of Athens	350	7-9 weeks	29.0	30.1
4	2 nd Dept. of Ob/Gyn, Univ. of Athens	40	7-9 weeks	3.3	22.2
<i>Hospitals of Thessaloniki</i>					
5	1 st Dept. of Ob/Gyn, Univ. of Thessaloniki	110	7-9 weeks	4.58	28.8
6	2 nd Dept. of Ob/Gyn, Univ. of Thessaloniki	21	7-8 weeks	1.0	22.2
7	3 rd Dept. of Ob/Gyn, Univ. of Thessaloniki	192	7-12 weeks	8.0	31.4
<i>Rest of Greece</i>					
8	Ob/Gyn Clinic, Grevena Hospital (NHS)	56	6-11 weeks	2.3	31.3
9	G. Clinic Aigion Hospital (NHS)	16	8 weeks	1.3	30.0
10	Ob/Gyn Clinic, Hios Hospital (NHS)	144	7-8 weeks	12	23.5
11	Ob/Gyn Dept., Univ. of Thessalia	68	10 weeks	5.7	28.4
12	Ob/Gyn Clinic, Kos Hospital (NHS)	159	8 weeks	13.0	26.3
13	Ob/Gyn Dept., Univ. of Patra	17	10 weeks	1.5	26.5
14	1 st Dept. of Ob/Gyn, Serres Hospital (NHS)	100	7.7 weeks	4.0	29.0
15	2 nd Dept. of Ob/Gyn, Serres Hospital (NHS)	99	7-8 weeks	4.12	28.5
16	Ob/Gyn Dept., "Hatzikosta" Hospital of Ioannina	213	6-12 weeks	8.6	35.4
17	Ob/Gyn Dept., "Mamatseio" Hospital of Kozani (NHS)	20	9 weeks	1.21	28.0
18	Ob/Gyn Dept., Hospital of Florina (NHS)	33	7-11 weeks	1.37	29.5
19	Ob/Gyn Dept., Hospital of Volos (NHS)	224	7-7 weeks	10.86	28.4
Total		3,960			

easy and ready access to contraception counseling. In an evaluation on the impact of that policy in the population as a whole, it is important to look both at pregnancy and abortion rates. In order to understand abortion rates in Greece, fertility rates should also be mentioned.

Methods

Collecting complete data on the number of abortions has always been problematic, mainly due to the facts that abortion, as an act, is a social taboo and that the majority of abortions are mainly carried out in the private sector, where they may remain unregistered.

In the year 2004-2005 the Greek Society of Obstetrics and Gynecology commenced a thorough survey and managed to collect the evidence demonstrated in the present study. The programmes were organized by the FPA under the guidance of the University of Athens.

Collecting data from the major public hospitals of Greece was relatively uncomplicated. The obstetrics department of each hospital was contacted and if the department performed abortions the following data were requested from the responsible doctor: the exact number of abortions, the mean age of the women subjected to an abortion and the mean gestation age of the fetus (Table 1). The same method was used in the private sector but the collected data remain questionable regarding the total or partial registration.

Results

The total number of abortions in Greece was 69,960. The mean age of women was 28.4 years, while the mean gestational age was 7.7 weeks (Table 1). It was revealed that the vast majority of abortions were carried out in private clinics (6% vs 94% in public and private hospitals, respectively) (Figure 1). Furthermore, the rates between urban and rural Greece, respectively, were 61% vs 39%.

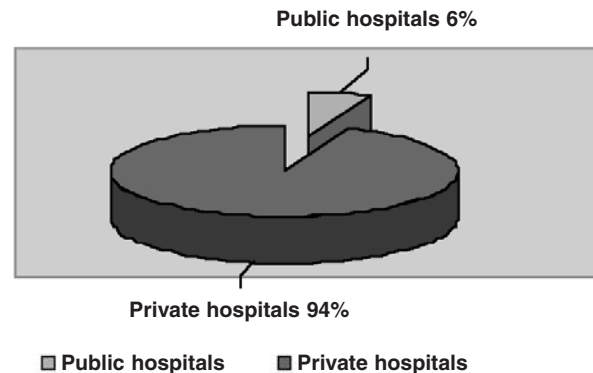


Figure 1. — Division of abortions in Greece.

Discussion

From our data, it is shown that the majority of women in a need of an abortion are still using private hospitals. The explanation for such a phenomenon is easy. The Non-aligned Women's Movement in 1992 argued that despite the fact that abortions had been legalized in Greece since 1986 and covered by insurance funds, some women were being treated in a hostile way by doctors and social workers in public hospitals [9]. On the other hand, abortions in the private sector were usually performed immediately without bureaucratic procedures and mainly in a more hospitable manner.

It is widely accepted that the vast majority of abortions, especially if performed early on, are without physical complications. Serious non fatal complications occur in about 3% of abortions [10]. The main pathophysiologic mechanisms involved are: infection which can result in sepsis, retention of some of the products of conception,

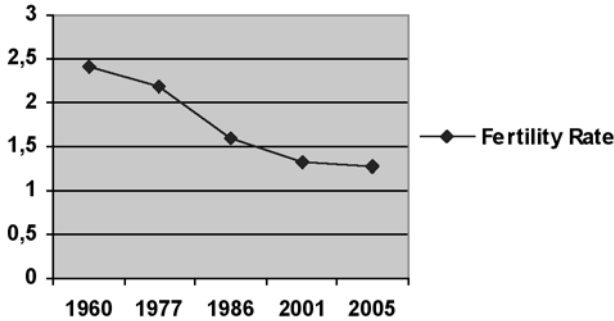


Figure 2. — Declining trend in fertility rates in Greece.

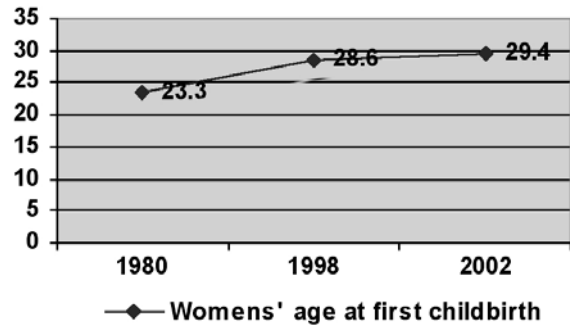


Figure 3. — Average age at first childbirth.

and injury with subsequent hemorrhage or even perforation of the uterus. Also, there is a standard risk involved in anesthesia. Finally, it should be mentioned that the operation could prove fatal and the estimated mortality risk is estimated at one and half greater than the maternal mortality rate. Risks are related to the duration of pregnancy [10].

Moreover, the risk of post abortion sterility ranges. It should be noted that in 2002 professors of Gynecology and Obstetrics, Bontis and Tarlatzis argued that from 1980 to 1999 there had been a decline of 41% in fertility rates in Greece. They added that 150,000 Greek women facing infertility problems had a history of at least one unsafe abortion [11]. Sterility may be due to blockage of the fallopian tubes following infection. The risk of ectopic pregnancy may also increase as well as the number of premature births. This may be attributed to damage done to the cervix intraoperatively. Sterility can also be attributed to psychogenic factors. Finally, a further complication affecting future childbearing is that of Rh-sensitization.

More specifically, in Greece the fertility rates (number of children for each woman of reproductive age) shows a steadily declining trend from 2.4 in the 1960s, to 2.25 in the 1980s, down to 1.28 in 2005 (Figure 2).

This decline is a European trend and is the main reason for the shrinking of the continent's population. However, the population of Greece still remains approximately constant, despite a less than 2.11% fertility rate which is the level needed to have a rising population. The main reason for this fact is the great number of immigrants, especially from non-European countries that enter Greece. The decline in the fertility rate could also be attributed to the changes in age in which most women give birth to their first child. This rates from 23.3 years in the 1980s, to 28.6 years in 1998, up to 29.4 in 2002 (Figure 3).

Another parameter should also be noted as Greece had a 5.1% of out-of-marriage births in 2005 while the European average (25 countries) was 33% [12]. Finally, it should be mentioned that Greek women start their sexual life comparatively later than men, at about 18 years of age, which is relatively lower than the European mean rate [13].

The current contraceptive choices that are used by Greek couples are provided by the Greek FPA as shown in Table 2.

Table 2. — Current (2003) Contraceptive choices in Greece (Greek Society of Family Planning).

Contraceptive method	Percentage of use
Oral contraception	4%
Emergency contraception	35%
IUD	5-10%
Condom	70-80%
Coitus interruptus (withdrawal)	15-20%
Female sterilization	0.2%
Male sterilization	0.07%

Significant differences from previous Greek data are the increasing use of condoms (70-80% in comparison to 41% in 1997) and emergency contraception use (35% in comparison to no previous data) [6, 15, 16]. The high percentage of use for the male condom and coitus interruptus (withdrawal) and the very low percentage of use of oral contraception, especially in relation to the world data (Figure 4), are mainly attributed to the lack of formalized sex education at school, the late beginning of visits to the gynecologist, fear of the "pill" use, and the low nationwide coverage from family planning centers.

The lack of responsible, fact-based sex education and consultation is translated to below average percentages for modern, safer contraceptive methods.

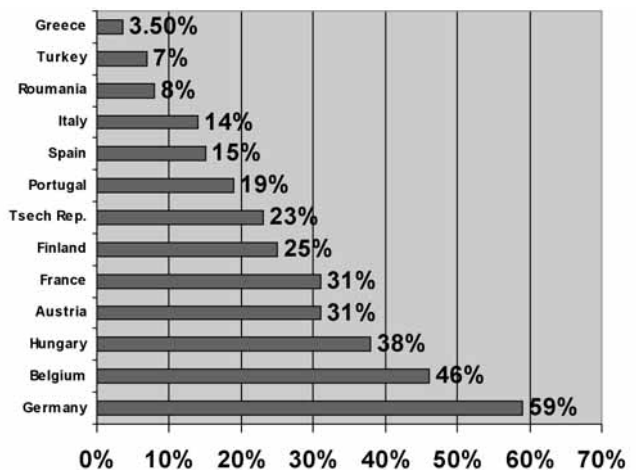


Figure 4. — IPPF Data (2003) on the use of oral contraception [17].

Although, many parents still believe that there is no need for sexual information at school due to personal taboos, Greek adolescents state that they prefer to discuss such matters with classmates or friends, or even to look for such data in television programmes or internet sites. Sexual education in Greece is not included as a part of the main school programme. However, the Ministry of Education has organized an extra 25 hours of teaching programmes on Health Education for adolescents (ages 14 to 18 years) which include topics on sexual education. These programmes are hosted in schools and other organizations, such as local unions, the military service, and athletic clubs. It should be noted that similar programmes have been organized by non governmental organizations, such as the Greek FPA, after requests from the local authorities or the school itself. All the above programmes are approved by the Ministry of Education. The FPA organizes information programmes in each of the 52 Greek prefectures trying to inform women and men regarding abortion, choices of contraception and sexually transmitted diseases. In the last two years, 150,000 students have been informed with such programmes.

The number of abortions in Greece according to our study shows a declining trend. The reasons for such decline are still questionable. Many explanations could be given such as easier contact to self-information by television or internet, a decline in personal parental taboos due to changes in life trends, the increasing use of condoms or emergency contraception, and last but not least, the efforts of the FPA for better and wider information in the field.

In contrast, much more should be achieved in the field. Still the question of contraception, especially of emergency contraception, is the fundamental issue in any discussion of pregnancy and abortion. Answering questions of the public regarding safety, effectiveness, and acceptability of contraception are the core considerations of the family planning policy. However the family planning policy also needs to entail providences for the safe termination of a pregnancy and the follow-up care needed. In many countries economic and manpower constraints may prevent the provision of additional facilities designed to meet the needs of a woman in terminating an unwanted pregnancy. Health personnel should be encouraged to be sensitive to the complexity of the problems that these women face, especially in social environments where family background, moral climate and legal institutions impose undue stress. Basic also in family planning policy should be sexual education integrated in the main school curriculum, taught by approved, specializing in the subject, well-informed teachers. It should be noted that the currently implemented extra-curriculum programmes should be expanded to include ages prior to adolescence. There is also a need for counseling procedures that are concordant with the values of society, social and ethnological groups which the pregnant woman represents, and the needs that are expected to arise during pregnancy. Clinical personnel should be made aware of the counseling services that exist and should be encouraged to work

closely with them, even though in some cases these services may not be provided through formal channels. All possible alternative ways of providing counseling should be considered, e.g., peer group counseling provided by religious leaders, paramedical personnel and various educational staff.

The abortion rates in Greece seem to have dropped and there are many factors involved. Changing social conditions and changing demographic patterns are producing new social and health trends. Moreover, the advent of 'emergency contraception' has had a significant role on diminishing the rate of abortions. Thus the need for thorough epidemiological studies is apparent, particularly in countries where existing record systems do not afford a comprehensive picture of the situation. We would like to state that the next goal of the Greek FPA is to achieve the organization of sexual educational programmes in every school and the provision of facilities in all the prefectures of Greece.

Conclusion

The decline in the number of abortions in Greece, evident in the last decade can mainly be attributed to the positive outcomes in the spread of extra-curriculum teaching programmes implemented by the Ministry of Education. It is characteristic that through the programmes organized by the Greek FPA under the guidance of the University of Athens and the approval of the Ministry of Education, 150,000 adolescents aged 14 to 18 years and their parents, received full, concise, up-to-date education and counseling on contraception, sexually transmitted diseases, emergency contraception and abortions.

Keypoints

1. A decline in abortion rates in Greece may be due to the better organization of counseling programmes in the field;
2. Fertility rates (number of children for each woman of reproductive age) in Greece shows a steadily declining trend from 2.4 in the 1960s, to 2.25 in the 1980s, down to 1.28 in 2005;
3. Significant differences from previous Greek data in current contraceptive choices are the increasing use of condoms (70-80% in comparison to 41% in 1997) and emergency contraception use (35% in comparison to no previous data).

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Serous cystadenoma with omental caking and ovarian torsion: an unusual case presentation

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Summary

Background: This case evaluates a patient with abdominal pain who presented with a pelvic mass and imaging studies suspicious for malignancy. **Case:** A 21-year-old, gravida 0, para 0, was admitted after presenting to the outpatient with abdominal pain. Ultrasound revealed a large 17 x 20 cm pelvic mass with solid and cystic components. CT scan revealed worrisome findings including the finding of “omental caking” and nodal enlargement. CA 125 was elevated at 85 U/ml. Final pathology after surgical removal of the mass showed evidence of serous cystadenoma with ovarian torsion without signs of malignancy. The patient had an unremarkable postoperative course and was discharged in stable condition. **Conclusion:** Although uncommon, pelvic masses that are benign may mimic malignant masses with extradnexal inflammation.

Key words: Serous cystadenoma; Omental caking; Torsion”.

Introduction

Evaluation of pelvic masses is a constant challenge. A broad differential is key to the development of an accurate clinical impression and design of an appropriate clinical plan. The case below provides an example of a large torsed ovary which presented in a manner suspicious for malignancy or other pathology.

Case Report

A 21-year-old, gravida 0, para 0, Hispanic female presented at the outpatient clinic with the complaint of mild but increasing abdominal pain, a feeling of “fullness” that had been persistent for one to two weeks, and increasing difficulty moving her bowels. Her past medical history included childhood asthma and remote removal of a tibial lesion. She was taking over-the-counter ibuprofen and laxatives for her symptoms and denied use of tobacco, alcohol, or illicit drugs. Her menses were reported to be regular without metrorrhagia and her last cycle had ended one week before.

Physical exam revealed a slightly distended abdomen with pain on deep palpation, greater in the lower quadrants bilaterally, but no guarding or rebound. Bimanual exam was difficult due to lack of abdominal relaxation. Rectal exam was negative for occult blood. Bowel sounds were normal. Other aspects of the physical exam were unremarkable. Vital signs were normal. CA 125 was elevated at 85 U/ml. AFP and LDH levels were normal at 1 ng/ml and 184, respectively. The patient was anemic with a hematocrit of 27.8 with other laboratory values unremarkable.

Ultrasound (US) revealed the presence of large pelvic masses containing solid and cystic components noting an area of several masses abutting each other measuring 17 x 19 x 6 cm (Figure 1). A separate cystic mass measuring 7 x 7 x 6 cm was

identified on the left ovary, which demonstrated no abnormal vascular flow. The right ovary could not be identified in the scan. Computed tomography (CT) revealed prominent intraperitoneal lymph nodes and minimal omental caking “suggesting intraperitoneal metastatic disease” (Figure 2).

At surgery, an approximately 20 x 10 cm apparently torsed right ovary and cyst were appreciated and right salpingo-oophorectomy was performed (Figure 3). Laparoscopy was not undertaken as the mass felt, under anesthesia, to be incorporated into the anterior abdominal wall. “Untwisting” the ovary was not considered as the visible ovarian tissue was dusky and appeared necrotic. Frozen pathologic analysis confirmed serous cystadenoma. A 7 cm left ovarian mass was also noted and cystectomy with ovarian preservation was performed.

Final pathology confirmed serous cystadenoma of both ovaries with evidence of ovarian torsion on the right. No malignancy was seen. Omental biopsy showed evidence of fibrosis only and peritoneal washings were negative for malignancy. The patient was discharged on post operative day 2 without complications.

Discussion

A medline search with Ovid and Pubmed from 1950 to March, 2008 using the keywords “serous cystadenoma” and “torsion” did not produce any literature documenting extra-adnexal inflammation with such radiological markers as omental caking or nodal adenopathy associated with ovarian serous cystadenoma. While rare, large ovarian serous cystadenomas with massive ovarian edema and with cystic and solid components have been previously reported [1].

Omental caking occurs when the native omentum is invaded by surrounding tumor [2]. These thick and solid omental masses indicate advanced disease is present [2]. Omental caking is seen in many tumors including the ovary, colon, uterus, breast, testes, muscle and pancreas [2].

Revised manuscript accepted for publication June 2, 2008

Fig. 1

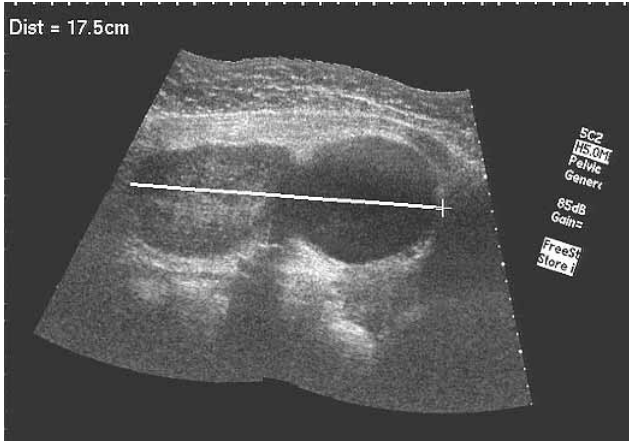


Fig. 3

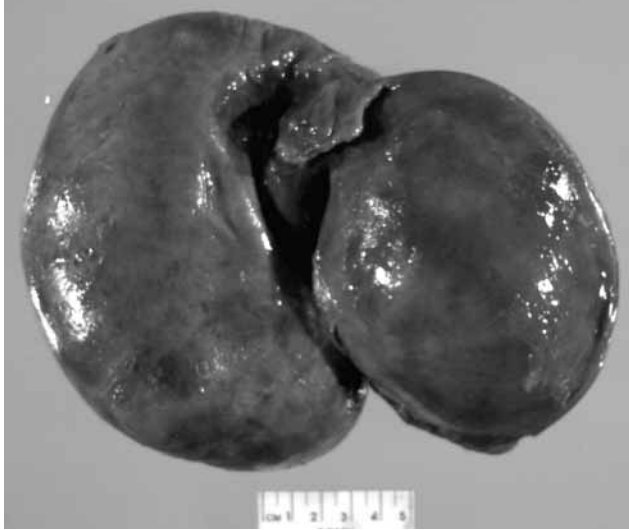


Fig. 2



Figure 1. — Ultrasound of mass.

Figure 2. — CT scan (arrow = area of omental caking)

Figure 3. — Gross pathology of right mass and ovary.

Serous cystadenomas are relatively common benign tumors [3]. Bennington *et al.* evaluated 225 serous cystadenomas and found that 141 of these tumors occurred in patients between the ages of 20 and 44. The same study showed that approximately 14% of these tumors were bilateral at the time of surgery [3].

Adnexal torsion is most commonly caused by an ovarian mass between 8 and 12 cm in size, however a torsed ovary can occur with an ovary of any size [4]. Adnexal torsion is most commonly associated with a benign process and typically occurs in individuals under 50 years of age [5]. The condition classically presents with acute and severe unilateral abdominal and back pain [4].

Progressive torsion is associated with venous and lymphatic obstruction and occurs prior to arterial compromise, causing cyanosis and edema within adnexal tissue often with fever and leukocytosis [4]. While conservative management is appropriate in many situations, when severe vascular compromise is evident, salpingo-oophorectomy is appropriate [4]. Laparoscopy has been shown to be a safe in removing large gynecologic tumors and has been associated with a reduced hospital stay [6].

The most frequent US finding associated with ovarian torsion is enlargement of the ovary with consistent phys-

ical exam findings including abdominal pain [7]. While the absence of ovarian Doppler flow is suggestive of torsion, the presence of ovarian Doppler flow can not rule out torsed ovary when an ovarian mass is present [7]. In this case, the right ovary could not be discerned due to the significant size of the associated mass. Of note, the 7 cm cystadenoma on the left would have placed that ovary at elevated risk for torsion if the cystectomy had not been performed.

The initial presentation of pelvic masses can often differ from the final pathology. This final diagnosis of bilateral serous cystadenoma with unilateral torsion of the right ovary differed significantly from imaging interpretations suggesting a more ominous process prior to surgery. The importance of maintaining a wide differential is vital to providing the highest level of care.

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Spontaneous uterine rupture during preterm labor in the second trimester of a twin IVF pregnancy without any apparent risk factor

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Summary

Uterine rupture is a rare complication of pregnancy. It can cause severe problems for the mother and child if not immediately diagnosed and treated. Some certain risk factors play a major role for occurrence. Multiple pregnancies, grand multiparity, previous cesarean sections or myomectomy operations, hysteroscopic manipulations and corrective uterine surgery, etc. are the main predisposing factors. In this case we report the successful management of a spontaneous uterine rupture in a primigravid female at the 29th week of a twin pregnancy after IVF treatment.

Key words: Uterine rupture; Spontaneous rupture.

Introduction

Spontaneous rupture of a non-laboring, unscarred uterus, especially in the second trimester, is extremely rare [1]. Uterine rupture during labor is a severe and fatal complication of pregnancy that can lead to mortality and morbidity of both mother and baby.

It may occur in a nontraumatic-unscarred uterus about one in 15,000 pregnancies. The incidence of uterine rupture has decreased significantly with the knowledge of leading causes and increased surgical interventions [2]. The maternal or fetal mortality rate is also high enough to make sure that emergent treatment is needed.

Most spontaneous uterine ruptures occur in patients with a scarred uterus during labor. However, they rarely occur without any apparent risk factor. We present a case of spontaneous rupture in a primigravid female at 29 weeks of a twin pregnancy after IVF (in vitro fertilization) treatment who was admitted to our clinic for mild subtle inguinal cramping pain and onset of pinkish bloody discharge with no history of any primary uterine operation.

Case Report

A 29-year-old primigravid female in the 29th week of a twin pregnancy after IVF treatment came to our clinic after 12 hours of subtle inguinal cramping pain and onset of pinkish bloody discharge. She had had no previous primary uterine surgery except for laparoscopic endometrioma extirpation in 2005. She had become pregnant on the first IVF trial. She was fine except for mild blunt pain on the left side of the abdomen. Her vital signs were within normal limits with no tachycardia and no fever. Two live fetuses with vertex and breech presentation appropriate for gestational age on transabdominal ultrasound (US) were seen. Placentas were located anteriorly and posteri-

orly, and amniotic fluid volumes were also within normal limits. On examination, the cervix was 8 cm dilated and effaced with minimal bleeding. Emergent cesarean section was planned. Preoperative laboratory values were hemoglobin: 11.1 g/dl, hematocrit: 33.5%, white blood cells: $14 \times 10^3/\text{ml}$, platelets: $264 \times 10^3/\text{ml}$, and CRP: 11.8 mg/l. Coagulation tests were also within normal limits.

During cesarean section, a minimally hemorrhagic collection was seen in the abdominal cavity and two separate amniotic sacs were seen bulging into the abdominal cavity through a 3 cm length of ruptured uterine segment. The rupture was vertical and extended up to the fundal area on the anterior aspect of the uterine wall. The babies were delivered through the ruptured area and then the uterine wall defect was repaired. The 1,185 and 1,350 gram neonates were transferred to the intensive care unit. The infants were discharged from the neonatal intensive care unit 28 and 26 days later without any sequels weighing 1,800 and 1,700 grams, respectively. The mother was discharged on the 3rd day after the operation without any complications.

Discussion

Traditionally, the primigravid uterus has been considered almost resistant to spontaneous rupture. Rupture of the gravid uterus continues to be one of the serious life-threatening complications of pregnancy [3]. Multiparity, multiple pregnancy, uterine corrective surgery like in an arcuate or septate uterus, and previous intrauterine intervention like myoma enucleation or uterine sharp curettage may predispose to uterine rupture. Induction of labor with oxytocin or ripening of the cervix may also easily cause rupture to both a previously sectioned or scarred uterus and unscarred primigravid uterus.

As is known the most common cause of uterine rupture is separation due to previous cesarean section. Classical cesarean scars constitute a high rupture tendency in comparison to a low segment transverse incision. The fundal and cervical areas of the uterus are affected from laceration,

Revised manuscript accepted for publication May 2, 2008

tion of a previous vertical scar. Although our case did not have uterine scarring, the cervix and fundal areas were included in the lacerated area.

The most common risk factor is previous uterine surgery, although this was not the case in our patient. Grosetti *et al.* studied 2,128 births with a low transversal scar after previous cesarean section retrospectively. They found a uterine rupture rate of 0.3/100 among women with repeated cesarean delivery without labor, 1/100 among women with spontaneous onset of labor, 1.4/100 among women with oxytocin-induced labor, and 2.2/100 among women with prostaglandin cervical ripening. Compared to women with a planned cesarean section, women with spontaneous onset of labor were more likely to have uterine rupture (OR: 4.0; 95% CI: 0.8-42.0). A greater relative risk was observed among women with oxytocin-induced labor (OR: 4.3, 95% CI: 0.3-60.0), and particularly those with prostaglandin-induced labor (OR: 8.7; 95% CI: 1.5-97.3, $p = 0.01$). They concluded that in women with uterine scarring, prostaglandin E2 induction of labor is a risk factor for uterine rupture [4].

In a multicenter study that studied the complication rates of laparoscopic myomectomy, it was found that the rupture rate was one in 2,050 patients who had undergone laparoscopic myomectomy [5]. This is one of the largest series reported on laparoscopic myomectomy and the first one to focus on complications. The complication rate appears to be better than acceptable in comparison with complication rates reported after laparotomic myomectomies. Laparoscopic myomectomy, when performed by an inexperienced surgeon, can be dangerous with a slightly high uterine rupture during pregnancy.

Clinical stigmata of uterine rupture commonly includes fetal bradycardia. In our case as soon as we saw 8 cm of cervical dilatation and active fetuses on the US we performed surgery immediately. Thus, we did not have enough time for cardiotocographic evaluation. Blood and bulging membranes were seen in the abdominal cavity. Although our patient had no severe pain or vaginal bleeding, she had a uterine rupture. It should be remembered that even complex uterine ruptures may happen during a pregnancy without any significant symptoms. We have to keep in mind that nonsignificant complaints of a pregnant

patient, whatever her gestational age, may alert us to the possibility of uterine rupture to prevent complications.

Treatment is immediate care and surgery as soon as possible. The best procedure for a ruptured uterus is the one which is shortest in duration, will not exacerbate the patient's state of shock and will get the patient off the operating table in the best condition [6]. Hypovolemia, hemorrhagic shock may occur suddenly due to high blood flow to the pregnant uterus. Correction of vascular deficits with crystalloid or blood is mandatory for early intervention for stabilization of the patient's condition. We incidentally detected a severe rupture and we repaired the torn uterus. It should be kept in mind that hysterectomy may be required in such cases. However once laceration has occurred and is successfully treated, there is always an increased risk for subsequent rupture.

In conclusion, we have reported the rare occurrence of a spontaneous uterine rupture in a preterm primigravid in labor with no known risk factors. It should be remembered that even an unscarred uterus can be ruptured and delaying diagnosis can cost the life of the mother and/or baby.

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Successful methotrexate treatment of an abdominal pregnancy in the pouch of Douglas

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Summary

Abdominal pregnancy is a rare localization of ectopic pregnancy. Early diagnosis and treatment are advised and the choice of treatment is crucial. A successful case of conservative treatment with combined systemic and intra-amniotic methotrexate is presented. This treatment option should be considered in the management of this potentially life-threatening condition.

Key words: Abdominal pregnancy; Ectopic pregnancy; Local methotrexate; Systemic methotrexate; Douglas pouch.

Introduction

Abdominal pregnancy occurs in approximately one in 8,000 births and 1.4% of ectopic pregnancies [1]. Primary abdominal pregnancy is rarely suspected due to the infrequency of this condition and the lack of specific symptoms. Delay in diagnosis is common and often an emergent laparotomy is required.

Case Report

A 26-year-old pregnant woman, gravida 2, para 1, was admitted to the emergency department of our hospital because of pelvic pain. Her last menstrual period was unknown. Bimanual pelvic examination revealed a tender normal-sized anteverted uterus with a close external cervical os and no adnexal masses. The rectal examination was painful. Ultrasound (US) examination revealed an empty uterus with normal adnexa and a gestational sac in the pouch of Douglas containing a yolk sac and a 12 mm embryo with positive cardiac activity corresponding to seven weeks menstrual age; no free intraperitoneal fluid was noted in either the pelvis or upper abdomen.

The patient was treated with transvaginal intra-amniotic instillation of methotrexate (MTX) 50 mg under sonographic guidance followed by systemic MTX in a single dose intramuscularly (50 mg/m²). The electrolytes, glucose, leukocyte count, hematocrit, renal and liver function tests were all within normal limits. She required minimal analgesia post-procedure and was discharged the next day after confirming the absence of embryonic cardiac activity.

Her beta human chorionic gonadotropin (β -hCG) level was 28; 110 mUI/ml at diagnosis. Quantitative β -hCG was followed each week and became undetectable seven weeks after the procedure.

Discussion

Pregnancies have been reported in every part of the peritoneal cavity, including the liver, spleen, omentum and Douglas pouch. Most abdominal pregnancies probably occur after tubal abortion or rupture, with subsequent reimplantation of the conceptus on a nearby peritoneal surface. Although the possibility to carry the pregnancy to term has been described, the prognosis is poor with an estimated maternal mortality rate of 5.1 per 1,000 cases, and a 7.7-fold higher risk than that of other ectopic pregnancies [2]. Because of the high maternal morbidity and mortality, early diagnosis is essential to avoid massive hemorrhage.

The presenting symptoms of abdominal ectopic pregnancy are similar to tubal ectopic pregnancy, with lower abdominal pain, amenorrhea, and vaginal bleeding the most common presenting symptoms in most series. In addition, early satiety, nausea, vomiting, diarrhea, rectal bleeding, or constipation are also common due to gastrointestinal irritation or obstruction [3].

The advanced modalities of vaginal US and sensitive β -hCG assays have greatly facilitated the diagnosis of early abdominal pregnancy. The optimal treatment of patients with abdominal pregnancies is dependent on numerous factors and will need to remain highly individualized. While the traditional treatment of advanced abdominal pregnancy was laparotomy, an early diagnosis has enabled new management by laparoscopy and recently, medical treatment by MTX. Few cases of abdominal pregnancies successfully treated only with MTX have been reported [4-6]. If an abdominal pregnancy is diagnosed early, combined systemic and transvaginal sonographically guided MTX treatment is effective as the definitive therapy. We would suggest consideration of conservative treatment with MTX as the first-line treatment of early abdominal pregnancy.

Revised manuscript accepted for publication February 20, 2008

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Xanthogranulomatous salpingitis: report of three cases and comparison with a case of pseudoxanthomatous salpingitis

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Summary

Xanthogranulomatous inflammation, an uncommon form of chronic inflammation, has been described in several organs including those of the female genital tract. A rare condition described as pseudoxanthomatous salpingitis or pseudoxanthomatous salpingiosis, which is often associated with endometriosis, has been distinguished from xanthogranulomatous inflammation of the fallopian tube based on its histological features. In the present report three cases of xanthogranulomatous salpingitis and one case of pseudoxanthomatous salpingitis are presented and their clinical, pathological and histochemical features are compared.

Key words: Fallopian tube; Ovary; Pseudoxanthomatous; Salpingitis; Xanthogranulomatous

Introduction

Xanthogranulomatous inflammation is characterized on microscopic examination by aggregates of lipid-laden foamy macrophages admixed with other inflammatory cells. On gross examination it presents as a golden yellow or bright yellow mass-like lesion that may mimic infiltrative cancer [1-5]. It is an uncommon form of chronic inflammation, mainly observed in the kidney and gall-bladder [2, 6-8]. A few cases of xanthogranulomatous salpingitis have been described in the literature, commonly related to pelvic inflammatory disease (PID) [9-12]. Xanthogranulomatous masses of the adnexae may be highly suspicious for malignancy, both clinically and radiologically, due to the vague symptoms, and the computed tomography (CT) and magnetic resonance (MR) imaging features of the mass, which may appear invasive with irregularly enhancing portions [13]. Frozen section evaluation is important in these cases.

A rare condition variously described as pseudoxanthomatous salpingitis [10, 14, 15], pseudoxanthomatous salpingiosis [16] or pigmentosis tubae [17] has been distinguished from xanthogranulomatous salpingitis based on the presence of pigmented histiocytes in the distended plicae and the relative absence of other inflammatory cells. The lesion is most often associated with endometriosis [10, 15], while a similar lesion has been described after pelvic irradiation [17]. In some reports the two conditions are not clearly separated [18], and the possibility that they belong to a spectrum of changes has been suggested.

The purpose of the present report is to present four cases of these uncommon conditions, including three cases with a diagnosis of xanthogranulomatous salpingitis and one case of pseudoxanthomatous salpingitis, and compare their clinical, pathological and histochemical features.

Case Reports

Case 1

A 50-year-old woman, gravida 3, para 3, presented with lower abdominal pain and fever of four days duration. One month before the patient had been hospitalized for pyelonephritis of the right kidney. She had had an intrauterine contraceptive device (IUD) for seven years. Adnexal masses were detected by gynecologic and ultrasonographic examination. The WBC was 12,900 and the reaction for C-reactive protein was positive. Serum CA125 was 54.7 U/ml and CA 15.3 was 29.6 U/ml. The preoperative diagnosis was pelvic inflammatory disease and the patient was treated for seven days with antibiotics. Subsequently, bilateral salpingo-oophorectomy and appendectomy were performed. Per-operative cultures of peritoneal fluid and purulent material from the right adnexa were negative.

A bisected 7 × 5 × 3 cm mass was received for frozen section evaluation. It involved the right adnexa, ovary and a distended fallopian tube, which showed reddish brown mucosa with bright yellow lobulated areas in the wall. The left adnexa consisted of a distended fallopian tube measuring 6 × 2.5 cm, and the ovary with a maximum dimension of 3 cm. The frozen section was negative for malignancy.

Histopathologic examination showed chronic inflammation in both fallopian tubes, extending to the right ovary. A large number of histiocytes with foamy vacuolated cytoplasm were present in the right fallopian tube. The histiocytes formed aggregates and were mixed with plasma cells, lymphocytes and neutrophils (Figure 1a). The findings in the right adnexa were consistent with a diagnosis of xanthogranulomatous inflammation. The appendix showed acute inflammation with serosal involvement.

Case 2

A 38-year-old woman, gravida 1, para 1, presented with lower abdominal pain and a low fever. An adnexal mass was detected. The patient's medical history was unremarkable. Her WBC was 9,000 and there was a positive reaction for C-reactive protein. Tumor markers were within normal limits. The clinical impression was pelvic inflammatory disease with an adnexal mass. After five days of antibiotic therapy, a right salpingo-oophorectomy was performed.

Revised manuscript accepted for publication April 21, 2008

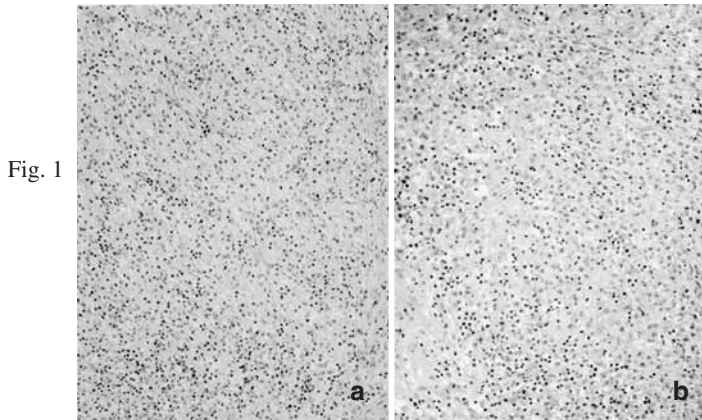


Fig. 1



Fig. 2

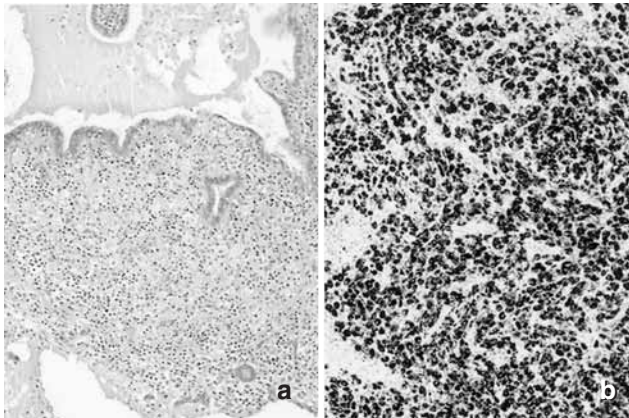


Fig. 3

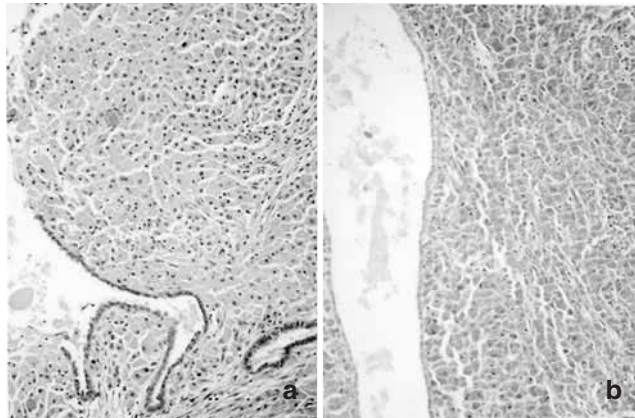


Fig. 4

Figure 1 (a-b). Case 1. a) Foamy histiocytes admixed with variable numbers of plasma cells, lymphocytes and neutrophils in the tubal wall (H&E, original magnification $\times 100$); b) PAS after diastase did not show strong positivity (original magnification $\times 100$).

Figure 2. Case 2. Enlarged fallopian tube with thickened yellow mucosa.

Figure 3 (a-b). Case 3. a) Histiocytes admixed with plasma cells, lymphocytes and neutrophils, in the distended tubal plicae (H&E, original magnification $\times 100$); b) Immunohistochemical stain for CD68 (original magnification $\times 100$).

Figure 4 (a-b). Case 4. Numerous histiocytes causing distention of the plicae (H&E, original magnification $\times 100$); b) PAS after diastase showed strong positive staining (original magnification $\times 100$).

A $9 \times 4 \times 4$ cm mass was received for histopathologic examination, including an enlarged fallopian tube, with thickened yellow mucosa showing polypoid projections and a yellow cloudy fluid (Figure 2), adherent to the ovary.

Histopathologic examination showed numerous histiocytes with foamy vacuolated cytoplasm, admixed with a large number of plasma cells, lymphocytes and neutrophils, in the distended tubal plicae. The findings were consistent with a diagnosis of xanthogranulomatous inflammation.

Case 3

A 47-year-old woman presented with lower abdominal pain. Adnexal masses were detected on gynecologic examination. Serum CA125 was 56.5 U/ml. A large solid and cystic mass was detected on CT scan. The patient underwent total abdominal hysterectomy and bilateral salpingo-oophorectomy with frozen section evaluation for suspected malignancy. A frozen section from the right adnexa performed at the time of surgery was negative for carcinoma.

On macroscopic examination, the uterus, measuring $7.5 \times 5 \times 4$ cm, did not show any remarkable lesion. Both fallopian tubes were enlarged and edematous, measuring 8×3 cm and 11

$\times 2.5$ cm, respectively, with soft consistency, yellow and brown areas and polypoid mucosal projections on cut section. The maximum dimension of the ovaries was 3 cm and 2.8 cm, respectively.

Histopathologic examination showed chronic inflammation with nodular collections of numerous histiocytes, often showing a foamy cytoplasm, admixed with a large number of other inflammatory cells in the tubal wall (Figure 3a). The findings were consistent with a diagnosis of xanthogranulomatous inflammation of the fallopian tubes, extending to the right ovary. Michaelis-Gutmann bodies were not found. The endometrium showed only focal collections of lymphocytes. An ill-defined lesion from the surface of the colon was also removed and showed xanthogranulomatous inflammation.

Case 4

A 48-year-old woman presented with abdominal pain. Transvaginal US revealed uterine leiomyomas, a dilated left fallopian tube and a left adnexal mass consisting of two cystic spaces separated by a septum. Tumor markers were within normal limits. The patient underwent explorative laparotomy. Intraoperative frozen section of the ovarian lesion showed a benign cyst con-

sistent with endometriosis, and total abdominal hysterectomy with left salpingo-oophorectomy was performed.

On macroscopic examination, the uterus measured 11 × 8 × 6 cm and showed four leiomyomas, one 4 cm in the maximum dimension, and an endocervical polyp. The ovary was enlarged, measuring 6 × 5 × 5 cm, and exhibited two cystic spaces with dark red contents. The fallopian tube was also enlarged, measuring 7 × 1.7 cm, and on cut section showed a brown-red mucosa with small polypoid projections. In the lumen there was a small amount of dark red fluid.

Histopathologic examination showed endometriosis of the ovary. The fallopian tube exhibited distention of the plicae, with numerous histiocytes within the lamina propria, that contained light brown pigment (Figure 4a). Lymphocytes and plasma cells were present in small numbers focally. The findings were consistent with a diagnosis of pseudoxanthomatous salpingitis.

Histochemical and immunohistochemical stains

Immunohistochemical stain for CD68 (PGM-1, 1:50, Dako-Cytomation, Glostrup, Denmark) (Figure 3b) was positive in the histiocytes of all cases. PAS after diastase showed weak positivity of histiocytes in cases 1 to 3 and strong positive staining in case 4 (Figure 1b and 4b, respectively). Masson-Fontana stain showed negative or focally positive staining in cases 1 to 3 and positive staining in extensive areas of case 4.

Discussion

In this study three cases with histopathologic features of xanthogranulomatous salpingitis and one case with features of pseudoxanthomatous salpingitis are presented. The microscopic and histochemical findings of the lesions and the associated disorders support the distinction of the two entities.

Xanthogranulomatous salpingitis is characterized by histiocytes with foamy vacuolated cytoplasm admixed with multiple types of inflammatory cells [10, 11], as observed in our first three cases. In contrast, pigmented histiocytes are a common finding in the stroma of well-developed endometriotic lesions and similar cells have been observed at a distance from endometriotic foci, including the mucosa of the fallopian tube [19], a condition described as pseudoxanthomatous salpingitis. These cells contain degradation products of blood, especially ceroid or lipofuscin, while hemosiderin is often less conspicuous [15]. The histiocytes in our case 4 were mostly pigmented and showed staining reactions consistent with the presence of lipofuscin or ceroid. Furthermore, the mucosal plicae in cases 1-3 often showed bridging, resulting in an altered architecture, as commonly observed in chronic salpingitis, in contrast to case 4, in which there was expansion of plicae with less prominent architectural distortion.

There was a recent history of pyelonephritis in one of our cases of xanthogranulomatous salpingitis. Only a few cases of xanthogranulomatous inflammation of the fallopian tube have been described in the literature [3, 9-12], and an association with pelvic inflammatory disease has been reported [10, 11]. In one of these [11] the presence of an IUD was reported, as in our case 1. The precise pathogenesis of xanthogranulomatous inflammation,

characterized by collections of macrophages containing large amounts of lipid with tissue destruction, is not well understood. Suggested mechanisms include defective lipid transport, immunological disorders, infection by low-virulence organisms, reaction to specific infectious agents, and obstruction [6, 20]. In some cases of xanthogranulomatous inflammation microbiologic investigations have shown negative results, although in several cases bacterial cultures were positive for *E. coli*, *Proteus vulgaris*, *M. gonorrhoea*, and *Bacterioides* [9-11]. Interestingly, in a study of interval (delayed) appendectomy specimens, 36.4% of the latter were reported to show features of xanthogranulomatous inflammation compared to none in the acute appendicitis group [21].

The causes of development of pseudoxanthomatous salpingitis or salpingiosis are not clear, but the process might be due to hemorrhage directly into the fallopian tube lumen with a macrophage response [22]. Pseudoxanthomatous salpingitis was associated with endometriosis in eight of 11 cases reviewed by Furuya *et al.* [10]. In the present small series only case 4 showed evidence of endometriosis in the left ovary and this was associated with features of pseudoxanthomatous salpingitis in the ipsilateral fallopian tube.

In summary, the clinicopathologic features in this small group of patients support the distinction between xanthogranulomatous and pseudoxanthomatous salpingitis, although the presence of cases with mixed features is possible. Knowledge of these entities is important for clinicians, radiologists and pathologists, since both lesions may show features reminiscent of a malignant lesion. In these cases a suggestive preoperative diagnosis or a correct intraoperative evaluation may lead to less aggressive surgical treatment.

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Bowel obstruction due to endometriosis in the rectovaginal septum

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Summary

It is very rare that endometriotic lesions in the rectovaginal septum cause ileus. We report a case of bowel obstruction due to endometriotic lesions in the rectovaginal septum in a 22-year-old woman whose barium enema presented with apple-core-like findings. Diagnostic and treatment modalities were discussed. Preoperative and postoperative gonadotropin-releasing hormone analog and aromatase inhibitor therapy promote relief of clinical symptoms, a reduction of tumor volume and a better approach to radical surgery.

Key words: Endometriosis; Rectovaginal septum; Bowel obstruction.

Introduction

Endometriosis is classified into three types: peritoneal, ovarian, and rectovaginal [1]. Rectovaginal endometriosis (RVE) is the deepest and most serious type, causing obstinate, severe dysmenorrhea, dyspareunia, and chronic pelvic pain. Histologically, RVE is considered a specific entity of deep endometriosis. Deeply infiltrating endometriosis is a particular form of endometriosis that penetrates > 5 mm under the peritoneal surface and is typically associated with marked proliferation of smooth muscle cells and fibrosis, and strongly associated with pelvic pain [2]. The etiology of endometriosis is still a matter of debate. The notion that rectovaginal endometriosis and peritoneal endometriosis are two distinct entities was first proposed by Donnez *et al.* [3]. Recent evidence indicates that endometriotic nodules, being anchored to the cervix, may originate from the rectovaginal space, as reported by Donnez and Squifflet [4], whereas Koninckx and Martin [5] have suggested that endometriotic nodules localized in the rectovaginal space are a severe form of deep endometriosis, resulting from the natural evolution of peritoneal endometriosis.

Endometriosis infiltrating the posterior vaginal and anterior rectal walls usually causes severe symptoms, and studies of the treatment of this particular form have focused primarily on pain relief [6], whereas information regarding the ileus is scattered and scanty. We present a rare case of endometriosis of the rectovaginal septum that caused rectal obstruction.

Case Report

A 22-year-old Japanese woman (gravida 0) presented in the emergency outpatient department with increasing abdominal discomfort and nausea that she had begun to notice five months earlier. Her past medical and surgical history was uneventful. An abdominal X-ray showed an ileus due to obstruction of the

upper part of the rectum. The apple-core-like finding was confirmed by a barium enema (Figure 1). At rectoscopy, the rectal mucosa appeared regular and normal, but indirect signs of external rectal compression with negative biopsies were shown. Magnetic resonance imaging (MRI) was performed and revealed a T2WI high signal and a T1WI iso-signal in the rectovaginal space. Because her ileus occurred at the beginning of her menstruation and MRI findings, she was admitted to our gynecologic department. A bimanual and rectovaginal examination was performed on the patient. She had a 70 x 40 mm, painful nodule involving the upper portion of the rectovaginal septum. The uterus was fairly mobile, and no adnexal mass was palpated. A red papillary lesion at the posterior fornix histologically consistent with vaginal endometriosis was present. The serum concentration of the tumor marker CA125 was 280 U/ml whereas the serum concentrations of CA19-9 and CA 72-4 were within the normal range. Diagnostic laparoscopy was carried out and showed that the uterus, ovaries, pelvic organs, and peritoneum were macroscopically normal. After three courses of leuprolide treatment (at a dose of 3.75 mg/ four weeks), the tumor volume had decreased in size by approximately 70%. The CA125 level dropped to 10 U/ml after treatment. Low-anterior rectal resection was performed with immediate end-to-end anastomosis. The pathological diagnosis was endometriosis in the rectovaginal space. The patient's postoperative course was uneventful. She received an additional three courses of leuprolide therapy postoperatively, followed by adjuvant anastrozole therapy (at a dose of 1 mg daily) for 17 months. Subsequent examination revealed no evidence of disease recurrence and minor anal stenosis. No additional bowel or urinary complications were observed. At the time of her last follow-up, eight months after stopping anastrozole therapy, the patient was without evidence of disease recurrence.

Discussion

When a bowel obstruction is diagnosed, endometriosis can be suspected when gynecological symptoms such as dyspareunia, infertility, or dysmenorrhea are present. Similarly, bowel symptoms should be looked for when genital endometriosis is diagnosed [7]. When an obstructive syndrome is present, or in cases of symptomatic

Revised manuscript accepted for publication May 2, 2008



Figure 1. — Image of the barium enema. The apple-core-like finding was confirmed.

advanced endometriosis (defined as reactional fibrosis invading the muscularis of the intestine), the only treatment is surgical resection. Surgery is the only viable option because endometriotic tissue in the bowel muscularis undergoes muscle cell hyperplasia and fibrosis, which are resistant to medical treatment. Approximately 10% of women with intestinal endometriosis initially develop symptoms after menopause or after bilateral oophorectomy [7], which is why surgical castration (ovariectomy or bilateral salpingo-oophorectomy) is not part of the treatment for intestinal endometriosis.

Excising rectovaginal endometriosis is a technically challenging process. Almost all the methods involve separating the adherent rectum from the back of the vagina and cervix and dissecting down into the rectovaginal septum. As this is done, the disease can be left on the side of the rectum to be stripped off after the dissection [8] or can be left on the vaginal side of the septum and excised vaginally [9]. Where deeper involvement of the rectal muscularis or mucosa occurs, it is necessary to excise a full-thickness part of the rectum. This can be done by excising a disc of rectal wall [10] or by performing an anterior segmental rectal resection.

Heilier *et al.* [11] have shown a significantly different expression of aromatase in the peritoneal and ovarian endometriotic tissues and deep endometriotic nodules of the rectovaginal septum, which strengthens the theory of three distinct clinical entities. In addition, it was demonstrated that deep endometriotic lesions do not respond to progestin treatment as much as endometrium and superficial endometriotic lesions, suggesting a difference in the susceptibility of deep lesions [12]. Recently, aromatase

inhibitors have been proposed for the treatment of endometriosis; pilot studies have combined type II aromatase inhibitors (anastrozole and letrozole) with progesterone, progestins, or oral contraceptive pills [13, 14]. These studies have suggested that aromatase inhibitors not only reduce pain symptoms but also eradicate the disease either as an alternative to surgery [13] or as a postoperative prevention of recurrence [15].

In conclusion, although rare, endometriosis in the rectovaginal septum should be considered in a patient with rectal obstruction who presents with ileus.

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Conservative management of cervical ectopic pregnancy: case report and review of literature

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Summary

Purpose of investigation: The aim of the study is to describe the management of a case of cervical ectopic pregnancy at six weeks. **Case:** A 34-year-old patient presented with six weeks of amenorrhea and a cervical pregnancy diagnosed by transvaginal ultrasound. Obstetrical anamnesis showed previous cesarean section and celiac disease as medical complications. At six weeks and one day 50 mg intramuscular methotrexate (MTX) was started and repeated three days later. At six weeks + six days the patient had vaginal bleeding so she was submitted to an emergency surgical procedure consisting of dilatation and curettage followed by a Foley balloon tamponade, which was gradually deflated and removed after two days. **Conclusion:** Early diagnosis and an appropriate MTX regimen in combination with adjuvant conservative procedures allow successful treatment of a cervical pregnancy, preserving the uterus and future reproductive outcome. However further studies are needed to define the best approach for management of cervical pregnancy.

Key words: Cervical pregnancy; Methotrexate.

Introduction

Ectopic pregnancy is defined as a pregnancy in which the implantation of the embryo occurs outside the uterine cavity. Of all reported pregnancies, 0.4-2% are extrauterine [1, 2].

The improved accuracy of transvaginal ultrasonography (TVS) early in pregnancy and the quantitative measurement of the β -unit of human chorionic gonadotropin (β hCG) allow early detection of ectopic pregnancies [3, 4]. Early detection has led to a steady decline in maternal deaths associated with this condition since the early 1970s [5].

Indications and criteria for the different management options are described in the literature and in clear guidelines from institutions such as the Royal College of Obstetricians and Gynaecologists.

Methotrexate (MTX), in a single dose protocol, is widely used in the medical management of ectopic pregnancy, associated or not with surgery.

We report a case of a cervical ectopic pregnancy successfully treated with MTX, subsequent cervical curettage and intracervical Foley catheter placement with gradual deflation of the balloon.

Case Report

A case of a 34-year-old patient with a cervical pregnancy diagnosed by TVS at six weeks was referred to our division. Obstetrical anamnesis showed a previous cesarean section and celiac disease as medical complications. TVS showed a cavity 19.3 mm x 6.81 mm in diameter located on the right lateral wall of the cervix, containing a yolk sac (Figure 1). Serum β hCG

levels, before and after MTX administrations, are shown in Table 1. Hematologic exams were substantially normal while clinical symptoms revealed pelvic pain. At six weeks and one day 50 mg intramuscular MTX was started and repeated three days after. At six weeks + six days the patient had vaginal bleeding so she was submitted to an emergency surgical procedure consisting of dilatation and curettage followed by a Foley balloon tamponade, which was gradually deflated and removed after two days.

Severe hemorrhage during suction curettage or other adverse effects were avoided. Histological examinations of the specimen confirmed the cervical pregnancy.

Table 1. — *Seriate β hCG levels before and after MTX administrations.*

Weeks of pregnancy	β hCG levels
5 weeks + 2 days	3,245 mIU/ml
5 weeks + 4 days	8,941 mIU/ml
5 weeks + 5 days	12,396 mIU/ml
6 weeks + 1 days, MTX 50 mg IM	
6 weeks + 2 days	20,038 mIU/ml
6 weeks + 4 days, MTX 50 mg IM	20,231 mIU/ml

Discussion

Extrauterine pregnancy contributes substantially to maternal mortality in all parts of the world and, despite fluctuations in the incidence in different countries, it will remain a challenge to clinicians in the future.

Early diagnosis allows the clinician to consider a more conservative approach such as expectant management or medical therapy, also because recognized risk factors are known [6-8]. The etiology is still unknown but there is evidence of its association with cervico-uterine instrumentations, such as in the case reported.

Revised manuscript accepted for publication March 20, 2008



Figure 1. — TVS showed a cavity of 19.3 mm x 6.81 mm in diameter located on the right lateral wall of the cervix containing a yolk sac.

In our country there are no clear guidelines for cervical pregnancy but analysis of the literature showed that if a cervical pregnancy is present and diagnosed early, treatment with MTX is effective as definitive therapy [9-11] and appears to be a convenient method for the treatment of the majority of cervical pregnancies before 12 weeks of gestation.

In contrast Hung *et al.* [12] reported prognostic factors for successful MTX treatment of cervical pregnancy, concluding that cervical pregnancy presenting with a serum β hCG concentration of $\geq 10,000$ mIU/ml or gestational age > 9 weeks or crown rump length ≥ 10 mm are factors associated with a higher unsatisfactory rate of primary MTX treatment. These observations suggest that an appropriate selection of candidates for MTX treatment for cervical pregnancies is needed because currently there are no specific recommendations for the best treatment of this entity [13], and also in consideration of the low incidence of the problem, occurring in one out of 8,628 deliveries [14, 15].

Particularly the main problem of conservative treatment is life-threatening hemorrhage after pregnancy evacuation.

Ushakov *et al.* reported in 1997 that the use of a cervical canal tamponade with a Foley catheter balloon led to reliable hemostasis in 92.3% of cases in which this method was used [15].

Cosin *et al.* reported combined use of MTX and arterial embolization to avoid surgical intervention in cases in which hemorrhage occurs after chemotherapy treatment [16] while some authors have suggested vaginal ligation of the cervical branches of the uterine arteries as an emergency surgical procedure [17, 18].

Conclusion

The case reported confirms the observations reported in the literature but further studies are needed to define the best approach for the management of cervical pregnancy.

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Infertility in a new 46, XX male with positive SRY confirmed by fluorescence in situ hybridization: a case report

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Summary

The 46, XX male syndrome (de la Chapelle syndrome or 46, XX testicular disorder of sex development) is a rare form of sex reversal with complex mechanisms leading to a large spectrum of clinical manifestations ranging from ambiguous genitalia in the newborn to normal male phenotype. Therefore, diagnosis is established either pre- or early postnatal, or in adult life due to male infertility. In some cases, subtle clinical signs during childhood and puberty may be overlooked. A 28-year-old married man presented with azoospermia without erectile dysfunction. Between 9-14 years he was examined for the small testes and under-masculinized external genitalia but the diagnosis was not further clarified. At presentation, hormonal laboratory evaluation revealed hypergonadotropic hypogonadism. Chromosome analysis showed a 46, XX karyotype and translocation of SRY (testis-determining factor) from chromosome Y to chromosome X was identified by fluorescence in situ hybridization (FISH). Despite early subtle clinical signs of abnormal sexual development in this new 46, XX male syndrome, medical investigations were triggered by infertility.

Key words: 46, XX male; Infertility; Hypogonadism; Fluorescence in situ hybridization; Karyotype.

Introduction

Chromosomal anomalies are seen in about 10% of infertile men leading to testicular dysgenesis and oligo- or azoospermia. The 46, XX male syndrome is associated with infertility but also testosterone insufficiency. Due to major consequences on health and social life of the affected individuals, efforts have to be made towards an early diagnosis of the disease.

Case Report

In April 2005, a 28-year-old married man was referred to the outpatient service of the Department of Endocrinology of the University of Medicine and Pharmacy Cluj-Napoca with primary infertility and azoospermia. Between three and six years the patient was treated for stuttering and a minor form of anxiety. At the age of nine he was medically examined for underdeveloped external genitalia and small bilateral testes were noted. However, the diagnosis was not further clarified and therapy with human chorionic gonadotropin in a total dose of 6000 IU was administered.

At the age of 28, the patient claimed no erectile dysfunction and stated he had a normal sex life, with sexual intercourse two-three times a week. He and his wife were childless after three years of unprotected sexual activity. Physical examination revealed a height below the 5th percentile of normal height (1.67 m, body mass index = 23.0 kg/m²) and a female distribution of fat mass. The testes were small and firm, the pubic hair had a female distribution and the patient had bilateral minor diffuse gynecomastia. An intelligence score of 85 was established. Testicular ultrasound revealed bilateral small testes (right and

left testes: 2.8 and 1.6 ml, respectively). Osteopenia (T score L₁-L₄ = -2.1 SD, T score femoral neck = -1.3 SD, T score total hip = -1.1 SD) was detected by dual X-ray absorptiometry (DPX-NT, GE, USA). The serum total testosterone was 11.57 nmol/l (normal range 9.9-27.8 nmol/l), free testosterone 0.031 nmol/l (normal range 0.019-0.145 nmol/l), FSH 43.96 mU/ml (normal range 1.5-12.4 mU/ml) and LH 25.31 mU/ml (normal range 1.7-8.6 mU/ml) suggesting hypergonadotropic hypogonadism. On conventional chromosome analysis a 46, XX karyotype was found (Figure 1). Analysis of metaphases and interphases of 46, XX cells using fluorescence in situ hybridization (FISH) showed a translocation of the SRY gene from the distal short arm of chromosome Y to chromosome X (Figure 2). The diagnosis was therefore of infertility and azoospermia secondary to testicular dysgenesis and SRY positive 46, XX male syndrome. Hormone replacement therapy with 40 mg testosterone undecanoate daily was initiated. An ongoing pregnancy was achieved by the patient's wife through sperm donation.

Discussion

In humans, the 46, XX male syndrome is a rare form of gonadal dysgenesis caused by an aberrant balanced or unbalanced Y-to-X interchange during paternal spermatogenesis leading to the translocation of Y-specific loci to the paternal X chromosome. Also, a chromosome Y-to-autosome translocation is possible [1]. In our patient, FISH evidenced a Y-to-X translocation.

The Y-chromosome gene, SRY, is the first gene involved in testis development [2]. Proof that this is the most important or even only Y chromosomal gene required for sex determination came from the production of mice transgenic for the mouse SRY gene who developed testes and external male genitalia but the testes were smaller than in

Revised manuscript accepted for publication March 19, 2008

Fig. 1

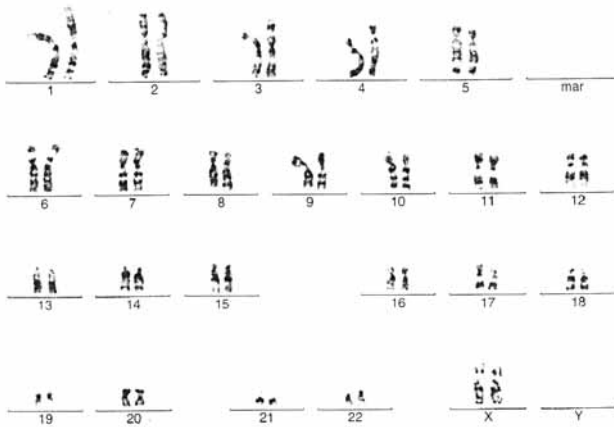


Figure 1. — 46, XX karyotype of a 28-year-old male with infertility.

Figure 2. — Fluorescence in situ hybridization (FISH) of metaphases and interphases of 46, XX cells with specific probe for the SRY gene revealed the presence of a translocation of the SRY gene from the distal short arm of chromosome Y to chromosome X. Detection and amplification of hybridization signals were done using an immunocytochemical reaction. Hybridization signals were analyzed using a fluorescence microscope.

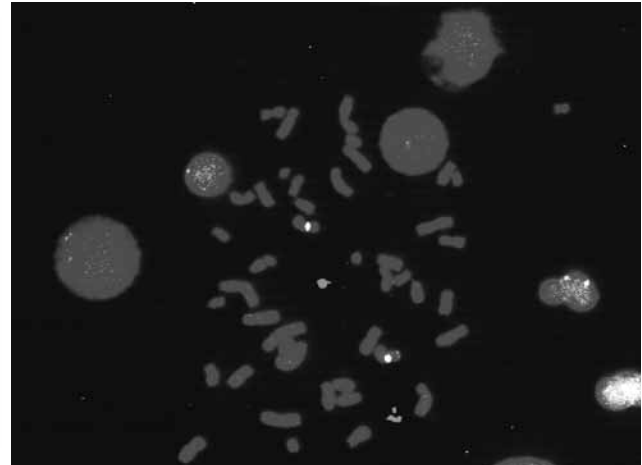


Fig. 2

normal mice and did not produce sperm [3]. In about 80% of cases, the SRY gene is part of the translocated fragment which is of variable length.

A feature of the 46, XX male syndrome is the large spectrum of clinical signs. A genotype-phenotype correlation has been described, with intersex and gynecomastia seen in 46, XX males that lack evidence of the SRY gene [4, 5]. On the other hand, few cases of SRY-negative 46, XX males with normal genitalia, complete masculinization and infertility were reported [6, 7]. In SRY-positive individuals, in general, the greater amount of Y material present, the more virilized the phenotype.

In our case, Y-to-X translocation was associated with small, firm testes and under-masculinized external genitalia in childhood. Testes of reduced size represent a major feature of testicular dysgenesis, especially when associated with cryptorchidism. However, in some XX (SRY+) cases, normal sized testes or atrophic, soft testes [8] may be encountered. In adult life, low-normal testosterone levels were measured in our patient, despite clinical signs of testicular insufficiency such as bilateral gynecomastia, pubic hair with a female pattern and female distribution of fat mass. However, serum LH levels were increased above 10 mU/l and the patient had low bone mass, thus, testosterone replacement therapy was initiated. In conclusion, phenotypic variability of 46, XX males cannot be totally explained only by the presence or absence of SRY. Other mechanisms have been proposed such as disruption of normal SRY expression by position effect [9], X inactivation [10], or mutations of other genes that may play a role in the definition of the phenotype.

In summary, infertility was the presenting symptom in this new first Romanian case of 46, XX male syndrome. A careful investigation of underdeveloped genitalia during childhood would have led to earlier diagnosis of this anomaly.

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A rare case of uneventful pregnancy in a woman with Cogan's syndrome

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Summary

Cogan's syndrome is a rare multisystem disorder of unknown etiology which is characterized by nonsyphilitic interstitial keratitis, vestibuloauditory dysfunction and systemic vasculitis. We present the clinical manifestations and the follow-up of a 39-year-old pregnant woman with Cogan's syndrome. The treatment and especially the histologic findings of the placenta are discussed.

Key words: Cogan's syndrome; Placental choriangiomas.

Introduction

Cogan's syndrome was first described by Morgan and Baumgartner in 1934 [1]. Later on, the clinical entity was also described in five cases by Cogan [2]. In 1963, the atypical form of Cogan's syndrome was first described by Bennett [3].

The disease is characterized by nonsyphilitic interstitial keratitis associated with vestibuloauditory dysfunction which resembles Meniere's disease and may lead to complete bilateral hearing loss within two years [4, 5]. The interval between ocular and ear involvement could range from three months in the typical syndrome up to 11 years in the atypical syndrome. However, Cogan's syndrome is considered to be a multisystem disease with a wide clinical spectrum [4-6]. Approximately 12% to 15% of patients develop vasculitis involving vessels of all sizes in various organ systems [7]. Ten percent of cases are complicated with aortic insufficiency which can be life threatening [8]. Polyarthralgias or arthritis can also accompany the disease. Neurologic findings, such as epilepsy or encephalitis may also be found.

The syndrome is classified in two forms:

i) Typical form (according to Cogan's criteria) [2]: 1. Ocular symptoms, typically an isolated non-syphilitic interstitial keratitis that could be associated with conjunctivitis, conjunctival or subconjunctival bleeding, or iritis; 2. Audiovestibular symptoms similar to those of Meniere's syndrome usually progressing to deafness in one to three months; 3. An interval between the onset of ocular and audiovestibular manifestations of less than two years.

ii) Atypical form (according to Haynes criteria) [5]: 1. Inflammatory ocular manifestations, including episcleritis, scleritis, retinal artery occlusion, choroiditis, retinal hemorrhage, papilloedema, exophthalmos or tendonitis, with or without interstitial keratitis. Patients with isolated conjunctivitis, subconjunctival hemorrhage or iritis are also classified as having atypical Cogan's syndrome if

these inflammatory ocular manifestations are associated with Meniere-like episodes within an interval of two years; 2. Typical ocular manifestations associated, within two years, with audiovestibular symptoms different from Meniere-like episodes; 3. A delay of more than two years between the onset of typical ocular and audiovestibular manifestations.

It is a rare disease which primarily affects young adults with an age range of 2.5-60 years [9]. It should be noted that it is not a hereditary condition. Although the disease was thought to be caused by infection, today it is believed to be an autoimmune disorder [4, 5]. However, an upper respiratory infection is present in onset of 20% of cases. Anterior uveitis, detected in some cases, may suggest a possible immune mechanism. Cogan's syndrome has been detected in patients with antiphospholipid antibodies or Crohn's disease [10, 11].

There is no report about the histology of the placenta in women with Cogan's syndrome in the literature and so the presentation of Cogan's syndrome during pregnancy, its manifestations, follow-up and the placental findings are discussed in our case report.

Case Report

This is the case of a 39-year-old woman (para 1, gravida 1) who presented at eight weeks of gestation to our department. The personal history revealed Cogan's syndrome (with pain in the right ear and the ipsilateral eye) known for five years which was treated with the use of systematic administration of prednisone (1 mg/kg) per day and prednisolone acetate ophthalmic solution (1%). Symptoms progressively improved, and one year after the onset of the disease, only hearing loss and tinnitus remained. No recurrence was mentioned the following four years.

At the 12th week of gestation, the patient complained of pain, photophobia and bilateral lacrimation in her eyes. The ophthalmic examination revealed extensive peripheral interstitial keratitis. Audiometry was stable. Topically used steroids were recommended for the ocular symptoms. Her pregnancy was uneventful and she had a vaginal delivery at 38 weeks of gestation. One year post-labor no sign of the disease was discovered. The pathological examination of the placenta showed a mature placenta, measuring 18 x 14.5 cm, 1.5 cm thick, and weighing

Revised manuscript accepted for publication April 7, 2008

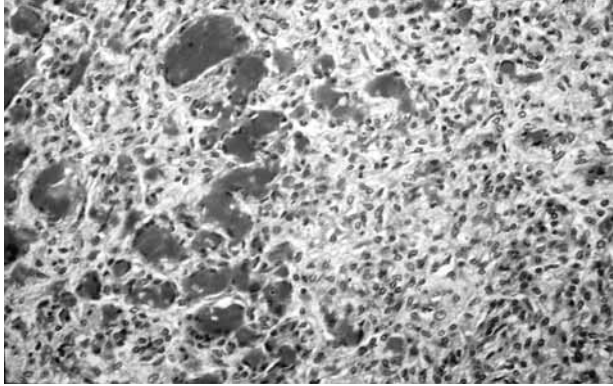


Figure 1. — Histological section of placenta with chorioangioma (hematoxylin-eosin x 200).

650 g. The multiple sections of the placenta showed whitish areas (more than 10), 0.2-1.5 cm in diameter. The histological examination revealed a mature placenta with multiple infarcts, old and new, and extensive calcification. Many foci of chorioangiomas were observed as well as a chorioangioma measuring 2 cm in the greatest diameter (Figure 1). No inflammatory changes of the placenta were observed.

Discussion

Ischii *et al.* described that the ligamentum spirale cochlea is infiltrated with plasma cells and lymphocytes in Cogan's syndrome; endolymphatic hydrops in the cochlea, collapse of the saccule and fibrosis in the posterior semicircular canal are also some of the characteristic findings [12].

As indicated in the literature, the symptoms could be correlated with a generalized vascular illness [13] such as aortic insufficiency and vasculitis (12-15%) [4, 7]. The median interval between the syndrome's diagnosis and vasculitis onset is seven months (range 3 weeks to 8 years) [7]. Vasculitis could affect the skin, kidneys, subcutaneous nodules, distal coronary arteries, and muscles. Pathologic reports reveal vasculitis in the dura, brain, gastrointestinal system, kidneys, spleen, aorta and the coronary arteries [7, 14, 15]. Characteristic findings are acute vasculitis and fibrosis in any artery or vein. Generalized dilatation and narrowing of the coronary ostia in the region of the aortic valve may be found [16]. Inflammation, necrosis and fibrosis were noted in the vessels during microscopic examination with the characteristic neutrophils, mononuclear cells, plasma cells, eosinophils and giant cells [14-16].

In our case, a remarkable feature was the presence of chorioangiomas of the placenta. Chorioangiomas of the placenta is a rare change of unknown etiology [17] believed to result from long-standing placental hypoperfusion or low-grade tissue hypoxemia. It is reported in preeclampsia, diabetes mellitus, drug ingestion and various placental and umbilical cord anomalies. In our case, the exact cause of chorioangiomas could not be determined but the chronic illness of the mother and the drug use may be responsible.

The treatment of the syndrome includes administration of systematic and local corticosteroids and/or immuno-

suppressive treatment in order to prevent progression of inner ear deafness. An initial dose of one to two mg/kg per day of oral prednisone is usually recommended. If corticosteroids are not effective, immunosuppressive drugs such as azathioprine, cyclophosphamide and cyclosporine are commonly used [4, 10, 18, 19]. However, the best results seem to have been obtained with methotrexate use [19].

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Pelvic packing method (after two laparotomies): a salvage procedure to control intractable pelvic hemorrhage after vaginal hysterectomy: a case report

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Summary

Background: Hysterectomy is one of the most commonly performed operative procedures in the world and hemorrhage continues to be a serious complication of both obstetrical and gynecologic surgeries. The pelvic packing technique is a useful alternative to control pelvic bleeding when standard measures fail. **Case:** A 45-year-old premenopausal woman with a history of pelvic pain and obstructive voiding symptoms underwent vaginal hysterectomy. Intraabdominal bleeding persisted after surgery and relaparotomy was performed. After routine surgical techniques failed to achieve adequate hemostasis, a pelvic packing technique was successfully used to tamponade the pelvic bleeding. **Conclusion:** When traditional methods of controlling pelvic hemorrhage fail, pelvic packing can be used as an unusual method for intractable pelvic hemorrhage. We successfully used the pelvic packing technique in a premenopausal patient with intractable hemorrhage after vaginal hysterectomy and this technique saved the patient's life.

Key words: Pelvic packing; Pelvic hemorrhage.

Introduction

Hysterectomy is one of the most commonly performed operative procedures in the industrialized world and occurs most frequently during the childbearing years. Hemorrhage remains to be a serious complication of both obstetrical and gynecologic surgeries. Hemorrhage after hysterectomy might be the result of failure to ligate a significant blood vessel securely, bleeding from the vaginal cuff, slippage of a previously placed ligature, or avulsion of tissue before clamping. Most surgical bleeding can be avoided with adequate exposure and good surgical technique [1].

The management of serious and refractory hemorrhage of the pelvis include insertion of topical hemostatic agents and bilateral ligation of uterine or internal iliac arteries [2]. Abdominal and pelvic packing is a valuable method in patients with uncontrollable bleeding but few data exist regarding early and late outcome [3].

Here we provide a case with successful management of intractable hemorrhage after vaginal hysterectomy with abdomino-pelvic packing in a premenopausal patient.

Case Report

A 45-year-old premenopausal woman was admitted with a history of pelvic pain, obstructive voiding symptoms and defecatory problems (constipation, tenesmus). Her past medical history included no operation or systemic illness. On general examination, she was afebrile with a blood pressure (BP) of 129/60 mmHg and a pulse rate of 78 beats/min. Abdominal examination was normal. On gynecologic examination, protrusion of the cervix and uterus toward the introitus and complete eversion of the vagina were noted. Rectoceles and cystoceles were accompanied by uterine prolapse. The patient was diagnosed as having pelvic organ prolapse (Stage IV). Her hematologic workup demonstrated hemoglobin = 12.4 g/dl, hematocrit = 34.2%, WBC = 3.17 K/ul, and platelet count = 145,000 10³/ul. Surgical management was performed to relieve symptoms and to restore vaginal anatomy. The patient was taken to the operating room and vaginal hysterectomy, and anterior and posterior vaginal colporrhaphy were carried out under general anesthesia. She was managed with hourly observations of temperature, pulse and blood pressure as per gynecologic department protocol postoperatively. Six hours after the operation, she developed lower abdominal pain. On examination, her temperature was 37.5°C, pulse rate was 120 beats/min and BP was 95/54 mmHg. Abdominal examination revealed diffuse tenderness. Ultrasonographic examination revealed intraperitoneal hemorrhage. An urgent request was sent for cross-matching of 4 U of blood and for full blood count. The patient's hematologic workup demonstrated hemoglobin = 6.52 g/dl, hematocrit = 19.7%, WBC = 8.78 K/ul, and platelet count = 132,000 10³/ul. The patient seemed to enter a state of shock, leading to an immediate exploratory; when removing intraperitoneal blood, suspicious bleeding points were observed. No bleeding sites were found during abdominal observation; 2000 ml of hemoperitoneum was evacuated. The decision was made to discontinue the operation. A suction drain was applied and the abdominal incisions were closed. The patient was transfused 3U of blood intraoperatively. She was managed with 30-min observations of temperature, pulse and blood pressure postoperatively. Although no hematological abnormalities were found, the patient continued to bleed intraperitoneally despite an intravenous transfusion of 6 U of blood, 4 U of fresh frozen plasma and platelets. Fifteen hours after the second operation, her temperature was 36.2°C, pulse rate was 137 beats/min and BP was 77/64 mmHg. The patient's hematologic workup demonstrated hemoglobin = 3.03 g/dl, hematocrit = 7.79%, WBC = 5.35 K/ul, platelet count = 101,000 10³/ul. A total of 1000 ml hemoperitoneum was drained with a suction drain. The multidisciplinary team was activated

Revised manuscript accepted for publication February 14, 2008



Figure 1. — Pelvic packing method as a lifesaving procedure.

to care for the patient's emergency condition. Due to the deteriorating clinical condition of the patient and the continuous bleeding, it was decided to proceed to a third operation (second exploratory laparotomy) as a lifesaving measure. She was transferred to the operating room. An attempt was made to find any bleeding sites during abdominal observation, and multiple small bleeding points were found. Bilateral hypogastric artery ligation was performed. Small bleeding sites were carefully ligated and the vaginal cuff was resutured. Finally pelvic packing were used with laparotomy pads as a lifesaving method (Figures 1 and 2). The hematologist and blood transfusion services helped to determine the degree of depletion of blood and clotting factors, and provided appropriate quantities of these for replacement during surgery.

The patient was transferred to the intensive care unit after surgery. She received 4U of blood and 2 U of fresh frozen plasma (FFP) and platelets. If there had been no response to the clotting factors, recombinant activated factor VIIa was contemplated. She made a good recovery within 48 hours and was transferred to the gynecologic unit. Laparotomy pads were removed after 48 hours of surgery. She was discharged home in good health 18 days after the third operation.

Discussion

Despite adequate technical skills and careful dissection, serious hemorrhage can suddenly complicate almost any operative procedure. The diagnosis of intraperitoneal bleeding in a postoperative patient can be difficult initially. Peritoneal signs are subtle and can be masked at the initial examination of the abdomen. Sometimes it is difficult for the surgeon who performed the original operation to convince him- or herself that bleeding is persistent and intervention is urgently needed. Sometimes a consultation with a colleague is helpful [1, 4].

For our patient, the multidisciplinary team was activated to decide which course to take for her emergency condition. After six hours of vaginal hysterectomy surgery, our patient seemed to enter a state of shock, leading to an immediate exploratory. An experienced surgeon knows that the most common reason for intraperitoneal blood and postoperative shock is loss of surgical hemostasis (a vessel has become untied) [1]. We performed exploratory laparotomy but could not find any bleeding in the pelvic and abdominal areas. It is not unusual to open the abdomen and find no active bleeding sites, which is somewhat disconcerting since the problem might reoccur after the abdomen is again closed [4].

Due to the deteriorating clinical condition of the patient and the continuous bleeding, we decided to reoperate on the patient as a lifesaving measure; bilateral hypogastric artery ligation was performed. Small bleeding sites were carefully ligated and the vaginal cuff was resutured. As is known, one of the methods of controlling severe pelvic hemorrhage is ligation of both hypogastric arteries [2]. However we could not totally control the hemorrhage after ligation of the hypogastric arteries and small bleeding sites, so finally we used the pelvic packing method as a lifesaving method.

Packing is a lifesaving technique for the temporary control of severe injury and it is used as a damage control procedure [3]. Several modifications to pelvic packing have been described. Dildy *et al.* used an effective pressure pack (e.g., pillow cases, gauze sheets, plastic X-ray cassette drapes, or orthopedic stockings) to control severe pelvic hemorrhage [5]. Cirese *et al.* used emergency pelvic packing to control intraoperative bleeding after a Piver type-3 procedure [6]. Awonuga *et al.* utilized a modified packing technique (which consists of ribbon gauze, a penrose drain, and Kocher's forceps) [3].

The pelvic packing technique is simple, and requires little equipment and training. This approach attempts to compress the bleeding vessels against the pelvic floor. It can be used for compression of bleeding vessels that cannot be controlled by other means [1, 4]. When traditional methods of controlling pelvic hemorrhage fail, it can be used as an unusual method for intractable pelvic hemorrhage [1, 4]. We successfully used the pelvic packing technique in a premenopausal patient with intractable hemorrhage after vaginal hysterectomy and this technique saved the patient's life.

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Fetal ovarian cysts: report of two cases and literature review

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Summary

In the present study we report two cases of fetal ovarian cysts. In the first case the cyst was initially discovered during prenatal ultrasound examination, while in the second case the cyst was identified during autopsy examination of a stillborn fetus. Pathologic examination of both specimens revealed similar histology, which was mainly characterized by the presence of an ovarian cortex comprising several maturing and primordial follicles which were occasionally biovular, and a focal lining of luteinized theca cells. Another prominent feature was the presence of multiple deposits of dystrophic calcifications within the cyst wall. Both cysts were diagnosed as follicular in origin.

Key words: Fetal; Ovarian cysts; Follicular; Pathologic.

Introduction

Since the introduction of prenatal sonography in routine clinical practice, a significant increase in the detection and reporting of fetal ovarian cysts has been noted [1]. However, the overwhelming majority of all relative literature has focused mainly on the diagnosis, sonographic appearance and management of these lesions, with only sporadic studies commenting on their pathologic features [2]. Although hormonal parameters, of both fetal and maternal derivation are, reportedly, considered to be responsible for ovarian hyperstimulation in the fetus, the exact etiology of fetal ovarian cysts remains unknown [3]. Nevertheless, a correlation of the pathologic findings with the remaining clinical and laboratory data may significantly contribute to further elucidate the pathogenetic mechanism leading to the *in utero* formation of an ovarian cyst.

In the present study we report two cases of fetal ovarian cysts with emphasis on their pathologic features and compare our findings with those of previously published cases or series. A concise review of the existing literature has also been attempted, focusing on the particular difficulties and diagnostic pitfalls associated with the pathology of fetal and neonatal ovarian cysts.

Case Reports

Case 1

Prenatal ultrasound (US) examination of a 32-year-old pregnant woman at 37 weeks' gestation revealed the presence of a fetal ovarian cyst: the cyst measured 4 cm in the largest diameter and was further characterized by features of a retracting clot pattern, suggesting cyst torsion. No other pathology was found.

Two days after birth, the infant was submitted to cystectomy, after a repeat US confirmation of the torsion pattern of the cyst. On gross examination the specimen appeared as a cystic mass measuring 4 x 3 cm and was filled with hemorrhagic contents (Figure 1). Its surfaces were smooth and the thickness of the walls ranged from 0.3-0.5 cm. The entire specimen was subsequently serially sectioned and studied histopathologically. Microscopic examination showed a cyst containing liquid and organized hematoma. The cyst wall was composed of ovarian cortex with some maturing and several primordial follicles which were occasionally biovular, and was focally lined by luteinized theca cells (Figure 2). Elements of past and recent hemorrhage as well as multiple deposits of dystrophic calcifications were also observed covering the cyst wall to a large extent. On the basis of these findings the diagnosis of hemorrhagic follicular ovarian cyst was rendered.

Our little patient had a fine postoperative course and is now a healthy baby, two months after surgery.

Case 2

During autopsy examination of a female stillborn infant (gestation age: 36 wks) a cyst was found in the right ovary. The cyst measured 2.7 cm in the largest diameter and was filled with clear serous fluid. Its surfaces were smooth and the thickness of the walls ranged from 0.2-0.4 cm. Histological examination of the specimen showed that the cyst wall was composed of ovarian cortex with maturing and primordial follicles, and contained several deposits of microcalcifications (Figure 3). The diagnosis of a follicular ovarian cyst was rendered.

Discussion

The presence of small cystic structures is not a rare finding in fetal and neonatal ovaries. Routine prenatal US imaging as well as autopsy studies have demonstrated that an extremely high percentage (up to 34%) of neonates harbor these lesions [4-6]. However, in most cases these findings simply represent normal cystic ovarian follicles which tend to regress spontaneously within the first months of age and are therefore considered to be

Revised manuscript accepted for publication March 3, 2008

Fig. 1

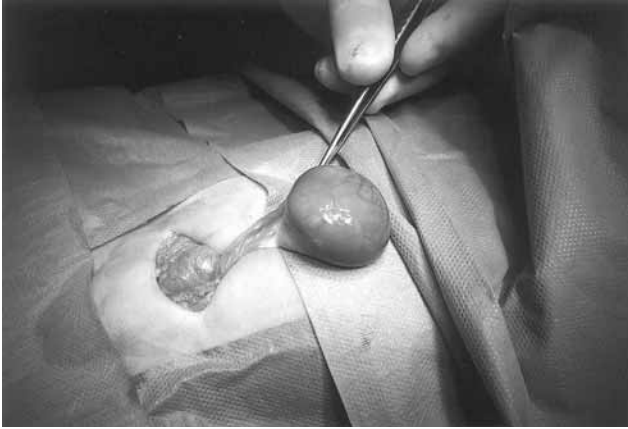


Fig. 3

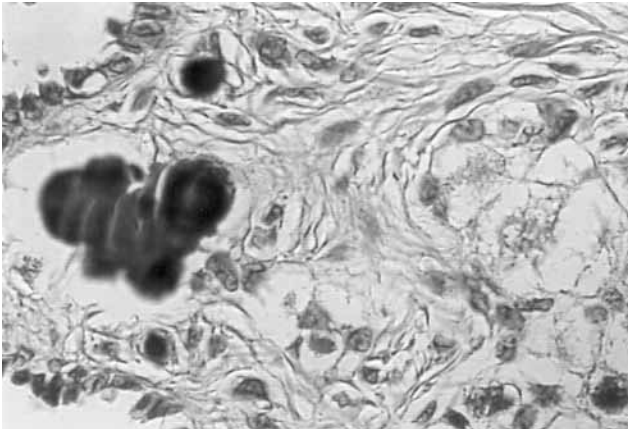
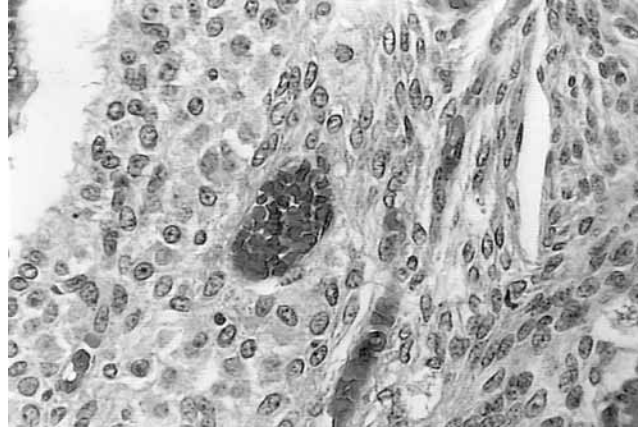


Fig. 2



of limited clinical significance [2, 4, 7, 8]. In contrast, pathological ovarian cysts are associated with several potential complications of variable severity, ranging from intracystic hemorrhage or cyst rupture to ovarian torsion which may further lead to intestinal obstruction or perforation, urinary obstruction and even sudden death of the infant [3, 9]. In rare cases of extremely large cysts, respiratory distress or pulmonary hypoplasia and polyhydramnios have also been described [10]. The distinction between a maturing cystic follicle and a true ovarian cyst is therefore extremely important from the clinical point of view and is mainly based on the size of the lesion, with cystic follicular structures designated as normal follicles when measuring less than 2-2.5 cm and as follicular cysts when they are larger than the above diameter [1, 11].

The pathologic features of fetal and neonatal ovarian cysts have been thus far described – to our knowledge – in very few studies [2]. An interesting histological finding in both of our cases was the presence of multiple deposits of dystrophic calcifications which were covering the cyst walls to a large extent. Extended calcification has been previously described in neonates and children in association with ovarian torsion and subsequent autoamputation and necrosis [12, 13]. Nussbaum *et al.* in their study of 17 neonatal ovarian cysts found dystrophic calcifications in the walls of nine out of ten twisted follicular ovarian cysts, and presumed that this finding was most likely the result of infarction [2]. The presence of calcifications

Figure 1. — Ovarian cyst of a female newborn (operative image, case 1). The smooth external surface and the hemorrhagic content of the cyst are evident.

Figure 2. — Histological section of the cyst wall (case 1) showing luteinized cells (hematoxylin-eosin x 250).

Figure 3. — Microcalcifications at the cortex of a fetal ovarian cyst (case 2) (hematoxylin-eosin x 250).

within the wall of a fetal ovarian cyst may give rise to a suspicion of a gonadoblastoma. However, the absence of the remaining histological features of this rare neoplasm (nests containing a mixture of germ cells and sex cord derivatives resembling immature Sertoli and granulosa cells) [14], and the history of cyst torsion and hemorrhage are the decisive diagnostic clues.

Although rarely reaching the preovulatory follicular stage, folliculogenesis and atresia also occur prenatally [15]. Thus, the wall of a follicular ovarian cyst in a neonate usually consists of ovarian cortex with several primordial and maturing follicles. Some of these follicles may contain multiple oocytes. Gougeon reported a frequent occurrence of multiovular follicles and multinuclear oocytes in the adult human ovary, which was independent of age, hormonal status or ovarian pathology [16]. In contrast to these findings, Manivel *et al.* in their autopsy study of 310 ovaries, reported that the occurrence of multiovular follicles is a much more frequent finding in children in comparison to older individuals, probably reflecting an abnormal folliculogenesis which disappears as the child matures, and should not be mistaken for a neoplastic process [17]. Interestingly, previous investigators observed an increased detection of structures mimicking neoplasms such as gonadoblastomas and sex-cord stromal tumors in association with follicular ovarian cysts in autopsy material obtained from stillbirths, infants and children, and further explained this phenomenon as the

result of the massive physiological reduction of oocytes which normally occurs during the initial developmental stages of the ovary [18].

With regard to the therapeutic approach of fetal and neonatal ovarian cysts, it should be emphasized that the decision of surgical intervention should be made on the basis of several parameters including not only the cyst size but also other parameters, such as the presence and type of symptoms and the US imaging findings [1, 3]. Although the optimal management of these lesions remains a controversial issue, it is generally agreed that asymptomatic cysts, with a diameter of less than 4 cm and a simple, non-complex US pattern may be conservatively observed with periodic ultrasound or aspirated in utero [1-3]. In the presence of larger, symptomatic or ultrasonographically complex cysts, surgery must be performed immediately after birth because of the significant risk of life-threatening complications, and all efforts should be made to preserve as much ovarian tissue as possible [1, 3, 7].

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Unusual presentation of a dermoid cyst that derived from the bladder dome presenting as subserosal leiomyoma uteri

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Summary

Background: Dermoid cysts are benign neoplasms deriving from ectoderm and mesoderm and can be found in a variety of locations. Uterine leiomyomas are benign tumors that grow within the muscle tissue of the uterus. Only a few cases have been reported and cited in the literature on dermoid cysts of the urinary bladder, and can be confused with urogenital masses. This paper reports the first case of a dermoid cyst deriving from the bladder dome presenting as subserosal uterine leiomyoma. **Case:** A 44-year-old woman was admitted with a history of chronic pelvic pain. After gynecological examination and ultrasonography a subserous uterine myoma was suspected. Tumor resection was performed and the histopathological diagnosis was a dermoid cyst. **Conclusion:** To our knowledge this is the first case report of a woman with a dermoid cyst presenting as a subserous uterine myoma.

Key words: Dermoid cysts; Bladder dome.

Introduction

Dermoid cysts are benign neoplasms that contain structures such as hair, fluid, teeth, or skin glands and are derived from ectoderm and mesoderm. Dermoid cysts can be found in a variety of locations such as the mouth, head, spine, and ovary, and sometimes can be difficult to distinguish from other more common benign masses [1].

Uterine leiomyomas are benign tumors that grow within the muscle tissue of the uterus. Between 20-50% of women of childbearing age have uterine fibroids. They may be subserosal, intramucosal, or submucosal in location within the uterus [1].

Dermoid cysts of the urinary bladder can be confused with urogenital masses and only a few cases have been reported and cited in the literature [2, 3].

This paper reports the first case in which a dermoid cyst deriving from the bladder dome presenting as a subserosal uterine leiomyoma was treated surgically.

Case Report

A 44-year-old woman was admitted with a history of chronic pain with severe dysmenorrhea and lower abdominal pressure. Her past medical history included no operation or systemic illness. On general examination, she was afebrile with a blood pressure of 100/60 mmHg and a pulse rate of 80 beats/min. Abdominal examination was normal. On gynecologic examination, the vulva, vagina and cervix were normal and a 10×10 cm pelvic mass originating from the uterus was noted. Ultrasonographic examination revealed a 10×10×10 cm subserosal uterine mass originating from the uterus. The patient was diagnosed as having a subserosal uterine myoma. Her hematologic

workup demonstrated hemoglobin = 12.3 g/dl, hematocrit = 37.7%, WBC = 8.41 K/ul, and platelet count = 323,000 10³/ul. Surgical management was planned to relieve the symptoms of pelvic pain due to the mass. The patient was taken to the operating room and after exploratory laparotomy, a 10×10×9 cm solid pelvic mass was identified originating from the bladder dome (Figure 1). Solid mass excision was performed. The patient was discharged home in good general condition three days after surgery and the histopathological diagnosis after the operation was a dermoid cyst.

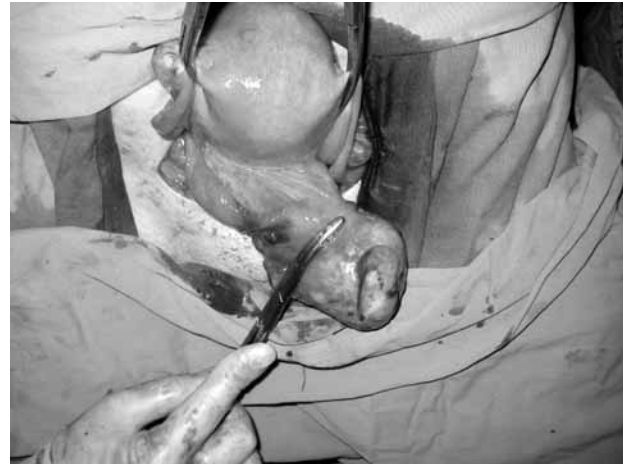


Figure 1. — Intraoperative view of the dermoid cyst deriving from the bladder dome.

Discussion

Bladder dermoid is a rare presentation and there are few cases in the literature [3-6]. Okeke *et al.* described a dermoid cyst of the urinary bladder as a differential diagnosis of bladder calculus [3], Agrawal *et al.* presented a

case of a dermoid cyst of the bladder in which the diagnosis was made cystoscopically and confirmed histopathologically [3]. Agbreta *et al.* described a dermoid cyst of the urachus [4]. Kamimura *et al.* presented a 46-year-old man complaining of transient dysuria who underwent retrovesical tumor resection and the histopathological diagnosis showed a dermoid cyst [5].

Subserosal uterine leiomyomas are located beneath the serosal surface. They grow out toward the peritoneal cavity, and can be broad-based or pedunculated. The pedunculated ones may attach themselves to adjacent structures like the bowel and omentum, and develop a secondary blood supply. Subserosal leiomyomas should be considered in the differential diagnosis of benign and malign pelvic masses [1].

We have described the case of a 44-year-old woman with a dermoid cyst that derived from the bladder dome presenting as a subserosal uterine myoma, which to our knowledge is the first report of a woman with a dermoid cyst presenting as a subserous uterine myoma.

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Anti-Lewis alloimmunization: report of seven cases

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Summary

Objective: The purpose of this study was to investigate the perinatal results of seven pregnant women with anti-Lewis antibodies and evaluate the need to screen for these antigens during routine prenatal care. **Setting:** São Paulo University Hospital, São Paulo, Brazil. **Population:** 200 Rh-negative pregnant women with a positive indirect Coombs test, managed during a 6-year period. **Methods:** The charts of all patients were reviewed to collect pertinent data and the variables were analyzed. **Main outcome measures:** Indirect Coombs test titer, intrauterine transfusion, mode of delivery, gestational age at birth, birthweight, neonatal transfusion, duration of neonatal hospitalization and perinatal mortality. **Results:** All newborn infants were classified as adequate for gestational age at birth and none needed intrauterine or neonatal transfusions. All infants, except one, were discharged in good health on the third day after birth. **Conclusions:** Alloimmunized pregnancies (Lewis antigens) have good perinatal results.

Key words: Anti-Le^a; Anti-Le^b; Alloimmunization in pregnancy; Antenatal screening; Hemolytic disease of the newborn.

Introduction

Red cell alloimmunization is an immune disorder caused by incompatibility between maternal and fetal red blood cell antigens [1]. Due to its high prevalence and immunogenicity, D-antigen incompatibility is the most frequent cause of significant perinatal hemolytic disease (PHD).

However, red blood cells have over 300 other surface antigens and at least 43 of these are capable of producing hemolytic disease [2]. The following blood group system (and antigens) are the most frequently associated with PHD: Rh (D, C, E, c, e, f, Cw), Kell (K, k-celano, Kp^a, Kp^b, Js^a, Js^b), MNS (M, N, s, S), Kidd (JK^a, JK^b), Duffy (Fy^a, Fy^b) and Lutheran [3]. The Lewis system is represented by two red cell antigens: Le^a and Le^b. Since most species of anti-Lewis antibodies are exclusively immunoglobulin M (IgM), which cannot cross the placenta, they rarely cause perinatal hemolytic disease (PHD) [4].

In Brazilian laboratories, when the indirect Coombs test is positive, the patient's serum is routinely tested using a panel of antigens which include the following systems: Rh: (D, C, E, c, e, f, Cw, variants), Lewis (Le^a, Le^b), Kell (K, k (celano), Kp^a, Kp^b, Js^a, Js^b), MNS (M, N, s, S), Kidd (JK^a, JK^b), Duffy (Fy^a, Fy^b), Diego (DI, Di^a, Di^b). While it is scientifically correct to try to identify exactly the specific antibodies present in the maternal blood, for clinical practice, only the detection of antibodies capable of producing hemolysis would be relevant. Motivated by the economic aspects of this question, we decided to study the prevalence of women sensitized to the Lewis system in our obstetric population and review the perinatal results of these pregnancies.

The purpose of this study was to investigate the perinatal results of seven pregnant women with anti-Lewis antibodies and evaluate the need for screening of these antigens during routine prenatal care.

Methods

This was a retrospective observational descriptive study of all (200) Rh-negative pregnancies with a positive indirect Coombs test managed at the Prenatal Unit of São Paulo Federal University (UNIFESP-EPM) during a 6-year period (2000-2005). These 200 pregnant women with a positive Indirect Coombs test were screened for Rh system antibodies and also for others red cell antibodies. In 15 of these women, other red cell antibodies were detected: seven Lewis, three MNS, three Kell and two Diego.

The charts of patients with anti-Lewis antibodies were reviewed and the following information was collected: indirect Coombs test titer, intrauterine transfusion, mode of delivery, gestational age at birth, birthweight, neonatal transfusion, duration of neonatal hospitalization and perinatal mortality. The study was approved by the Ethics Committee of the São Paulo Federal University (protocol no. 1407/05).

Results

Table 1 presents the results of the seven patients. All newborn infants were classified as adequate for gestational age at birth and none needed intrauterine or neonatal transfusions. All infants, except case 7, were discharged in good health on the third day after birth.

Discussion

The relative frequency of non-D alloimmunized pregnant patients has been rising in the last decades. A large study involving over 18,000 pregnant women reported 1.6% (299) had irregular antibodies. In this study, there were seven pregnant women sensitized to the Lewis antigens in a group of 200 Rh-negative pregnant patients managed at our tertiary center over a 6-year period [3].

Revised manuscript accepted for publication January 25, 2008

Table 1. — Perinatal results of seven pregnant women with anti-Lewis antibodies.

Case	Indirect Coombs titer	MCA Doppler	Delivery	GA (weeks)	Weight (g)	Apgar score (1 st and 5 th minute)
1	1/8	Normal	Vaginal	38	3440	9/10
2	1/2	Normal	C section	40	3660	7/9
3	1/8	Normal	Vaginal	37	3510	8/10
4	1/4	Normal	C section	38	2890	9/9
5	1/4	Normal	Vaginal	39	3600	9/10
6	1/8	Normal	C section	38	3350	9/9
7*	1/8	Normal	Vaginal	25	650	—

MCA: middle cerebral artery. GA: gestational age.

* Fetal death at 25 weeks.

Anti-E antibodies are the most common cause of non-D alloimmunization in pregnancy. In a retrospective study (1959 to 2004), Joy *et al.* [5] identified 283 pregnant patients with anti-E antibodies, 32 of them at risk for PHD. According to these authors, five of the 16 cases with indirect Coombs titers > 1:32 needed intrauterine or neonatal transfusions and there was one case of fetal hydrops and one perinatal death attributed to the anti-E antibodies. Additionally, two cases with titers > 1:32 had elevated middle cerebral artery peak systolic velocity.

There are no reports of PHD related to anti-Lewis alloimmunization. This may be attributed to two factors: most red blood cells express this antigen only after birth and most, if not all, antibodies against these antigens are IgM, which do not cross the placenta. In this study, as expected, the women with anti-Lewis antibodies had excellent perinatal results, with normal healthy infants who did not need transfusions.

The only fetal death was unrelated to anti-Lewis sensitization and occurred in a patient who had lost a previous child due to severe muscular dystrophy.

In summary, we believe this study contributed to reinforce the argument against ordering anti-Lewis antibody screening in routine prenatal care. This is especially true in developing countries, such as Brazil, where public health resources are limited and the money spent on this exam could be used in other more relevant tests to identify significant maternal and fetal disorders.

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CLINICAL AND EXPERIMENTAL OBSTETRICS & GYNECOLOGY

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General index - Volume XXIX, 2008

No. 1, January-February-March

EDITORIAL ARTICLE

- In vitro* fertilization is expensive: when should a couple be advised to stop trying with their own gametes and seek other options? Review of three cases - J.H. Check 5

ORIGINAL ARTICLES

Reproductive Biology Section

- Ovulation induction and pregnancy in a woman with premature menopause following gonadotropin suppression with the gonadotropin releasing hormone antagonist, cetrorelix - a case report* - J.H. Check, B. Katsoff 10
- Pregnancy outcome following in vitro fertilization-embryo transfer (IVF-ET) in women of more advanced reproductive age with elevated serum follicle stimulating hormone (FSH) levels* - J.H. Check, B. Katsoff, D. Brasile, J.K. Choe, J. Amui 13
- Difficulty of embryo-transfer (ET) and pregnancy rate based on the uterocervical angle* - A. Komaki, X. Qian, N. Shibata, N. Koyama 16

General Section

- Attitudes towards contraception in three different populations* - G. Galazios, P. Tsikouras, V. Liberis, N. Koutlaki, G. Vlachos, A.T. Teichmann, G. Maroulis 22
- Women's health measures in two North Carolina regions sampled from the Basic Automated Birth Yearbook (BABY) datasets: experimental findings, methodological limits and future directions* - E.S. Sills, T.M. Kling, S.S. Sills, M.J. Burns, L.P. Carroll, L.D. Parker, K.M. Wittkowski 27
- Misoprostol and first trimester pregnancy termination* - X. Grapsas, V. Liberis, G. Vassaras, P. Tsikouras, G. Vlachos, G. Galazios 32
- Association study between catechol-O-methyltransferase polymorphisms and uterine leiomyomas in a Japanese population* - J. Morikawa, S. Yoshida, S. Kennedy, N. Takemura, M. Sugimoto, K. Kitao, M. Deguchi, N. Ohara, T. Maruo 35
- Vaginal cysts: a common pathologic entity revisited* - A. Kondi-Pafiti, D. Grapsa, K. Papakonstantinou, E. Kairi-Vassilatou, D. Xasiakos 41
- Frequency of rate of body temperature chart at mid cycle in pregnant women and the subsequent effect on pregnancy* - M. Kawamura, M. Ezawa, T. Onodera, T. Nagashima, R. Toyooka, M. Yagishita 45
- Clinical symptoms and histopathological findings in subjects with adenomyosis uteri* - T. Cirpan, O. Yenieli, M. Ulukus, A. Ozbal, G. Gundem, S. Ozsener, I. Mete Itil, O. Zekioglu 48
- Laparoscopic management of adnexal masses in postmenopause* - E. Sturlese, G. Retto, A. Mancuso, A. Tripodi, M. Tripodi, V. Palmara, R. De Dominicis, V. Arancio 54
- Continuous oral or intramuscular medroxyprogesterone acetate versus the levonorgestrel releasing intrauterine system in the treatment of perimenopausal menorrhagia: a randomized, prospective, controlled clinical trial in female smokers* - T. Küçük, K. Ertan 57
- Cervicovaginal fetal fibronectin (FFN) for prediction of preterm delivery in symptomatic cases: a prospective study* - H.M. Tanir, T. Sener, Z. Yildiz 61
- A prospective randomized trial of labor induction with vaginal controlled-release dinoprostone inserts with or without oxytocin and misoprostol+oxytocin* - H.M. Tanir, T. Sener, C. Yildiz, M. Kaya, I. Kurt 65

CASE REPORTS

- Ovarian endometriotic cyst in a patient with Ellis-van Creveld's syndrome: case report* - G. Retto, E. Sturlese, V. Arancio, V. Palmara, A. Tripodi, M. Tripodi, R. De Dominicis 69

<i>Anaesthetic burns</i> - A. Govind, N. Lakhi	71
<i>Conservative treatment of an early ectopic pregnancy in a cesarean scar with systemic methotrexate - case report</i> - A.C. Iyibozkurt, S. Topuz, F. Gungor, I.H. Kalelioglu, E. Cigerli, S.E. Akhan	73
<i>Congenital cystic adenomatoid lung malformation: report of two cases and literature review</i> - J. Argeitis, D. Botsis, E. Kairi-Vassilatou, D. Hasiakos, K. Papakonstantinou, A. Kondi-Pafiti	76
<i>Spontaneous heterotopic pregnancy leading to intrauterine abortion and subsequent ruptured ectopic pregnancy with a βhCG of 125 mIU/ml: a case report</i> - P. Trompoukis, C. Chaidogiannos, A. Zourla, S. Grapsas, C. Salampassis, I. Papoulias, D. Kassanos	81
<i>Tubal ectopic pregnancy associated with an extraskelatal chondroma of the fallopian tube: case report</i> - M.N. Varras, Ch. Akrivis, G. Tsoukalos, Ch. Plis, St. Karadaglis, I. Ladopoulos	83
<i>Leiomyomatosis with multiple extrauterine pulmonary sites: an unusual case report</i> - K. Bakalianou, C. Iavazzo, N. Salakos, G. Paltoglou, K. Papadias, A. Kondi-Pafiti	86

No. 2, April-May-June

EDITORIAL ARTICLE

<i>A 59-year-old woman gives birth to twins - when should a fertility specialist refuse treatment?</i> - J.H. Check	93
---	----

REVIEW ARTICLES

<i>Placenta percreta presenting in the first trimester: Review of the literature</i> - J.C. Papadakis, N. Christodoulou	98
<i>Intrauterine devices and extrauterine pregnancy. A literature review</i> - C. Iavazzo, N. Salakos, N. Vitoratos, K. Bakalianou, E. Deligeoroglou, H. Dalainas, A. Kondi-Pafiti, A. Malamitsi-Puchner	103

ORIGINAL ARTICLES

<i>Analysis of perioperative morbidity according to whether the uterine cavity is opened or remains closed during abdominal myomectomy - results of 423 abdominal myomectomy cases</i> - M. Gavai, E. Berkes, T. Fekete, L. Lazar, Z.F. Takacs, Z. Papp	107
<i>Autofluorescence reveals menstrual phase in the endometrium</i> - A.C.Y. Chen, H.K. Chiang, W.Y. Liang, C.C. Yuan	113
<i>Study of pubertal development in Abruzzo (Italy) and analysis of factors implicated in puberty variability</i> - G. Mascaretti, C. Di Berardino	117
<i>An objective measurement to diagnose micrognathia on prenatal ultrasound</i> - G. Palit, Y. Jacquemyn, M. Kerremans	121
<i>Laparoscopic treatment of ovarian dermoid cysts</i> - P. Tsikouras, V. Liberis, G. Galazios, A. Savidis, A.T. Teichmann, T. Vogiatzaki, S. Zervoudis, G. Maroulis	124
<i>Misoprostol use as a method of medical abortion</i> - N. Salakos, C. Iavazzo, K. Bakalianou, O. Gregoriou, G. Paltoglou, K. Kalmantis, D. Botsis	130
<i>"Chromohysteroscopy" for evaluation of endometrium in recurrent miscarriage</i> - T. Küçük, S. Deveci	133
<i>CA 19-9 can be a useful tumor marker in ovarian dermoid cysts</i> - A. Coskun, G. Kiran, O. Ozdemir	137

CASE REPORTS

<i>Neurofibroma of the vaginal wall</i> - S. Baulies, M.T. Cusidó, P.J. Grases, B. Úbeda, M.A. Pascual, R. Fábregas	140
<i>Mucinous cystadenoma in a female patient with 45,X/46,XY karyotype</i> - T. Bulakbasi, S. Erkanli, O. Ozer, F. Bolat, Z. Yilmaz, F.I. Sahin	144
<i>Secondary amenorrhea with normal ovulatory cycles in a young virgin with normal follicle stimulating hormone levels - a case report</i> - B. Katsoff, J.H. Check	147
<i>Tuberculosis of the cervix and infertility: report of a rare case</i> - P. Guié, P. Iovenitti, K. N'guessan, J. Tegnán, K. Koffi, G. Carta, S. Anongba	149
<i>Irregular dental structures in a benign ovarian cystic teratoma (dermoid cyst): case report</i> - V. Liberis, P. Tsikouras, E. Sivridis, M. Dadidou, N. Koutlaki, G. Galazios	151
<i>Prenatal diagnosis of type I sacrococcygeal teratoma and its management</i> - E.S. Saygili-Yilmaz, K.K. Incki, M. Turgut, S. Kelekci	153
<i>Uterus didelphys with blind hemivagina and ipsilateral renal agenesis complicated by pyocolpos and presenting as acute abdomen 11 years after menarche: presentation of a rare case with review of the literature</i> - M. Varras, Ch. Akrivis, St. Karadaglis, G. Tsoukalos, Ch. Plis, I. Ladopoulos	156

No. 3, July-August-September

EDITORIAL ARTICLE

- Action of progesterone receptor modulators on uterine leiomyomas* - N. Ohara 165

ORIGINAL ARTICLES***Reproductive Biology Section***

- Improved pregnancy outcome for women with decreased ovarian oocyte reserve and advanced reproductive age by performing in vitro fertilization-embryo transfer* - J.H. Check, J. Pinto, J.R. Liss, J.K. Choe 167

- Normal pregnancy resulting from a non-pronuclear oocyte at the time of examination for fertilization* - R.O. Burney, J. Gebhardt, Y. Shu, B. Behr, L.M. Westphal 170

General Section

- Inherited thrombophilia screening in Greek women with recurrent fetal loss* - A. Mougou, G. Androutopoulos, M. Karakantza, E. Theodori, G. Decavalas, N. Zoumbos 172

- HPV detection and genotyping as an earlier approach in cervical cancer screening of the female genital tract* - W.M. Krambeck, R.M. Cadid , E.M. Dalmarco, C.M.M. de Cordova 175

- Maternal serum mannose-binding lectin in severe preeclampsia* - N.  elik, H. Ozan 179

- Association of Fas-670 gene polymorphism with risk of cervical cancer in North Indian population* - D.M. Kordi Tamandani, R.C. Sobti, M. Shekari 183

- Silicone gel mammary prostheses: immune pathologies and breastfeeding* - G. Zoccali, N. Lomartire, G. Mascaretti, M. Giuliani 187

- Ultrasound assessment of endometrial thickness: correlation with ovarian stimulation and pregnancy rates in IVF cycles* - D. Giannaris, A. Zourla, C. Chrelias, C. Loghis, D. Kassanos 190

- Cranial imaging spectrum in hypertensive disease of pregnancy* - S. Topuz, I. Kaleliođlu, A.C. Iyibozkurt, S. Akhan, R. Has, M. Tunaci, L. Ibrahimoglu 194

- The effect of buserelin acetate on the uterus of adult rats: morphological aspects* - C.R. Trindade, A.F. Camargos, F.E.L. Pereira 198

- Use of GnRH antagonists in ovarian remnant syndrome experimentally induced in rats* - A. Risvanli, S. Kumru, N. Akpolat, S. Yildiz 202

- Usefulness of symphysis-fundal height in predicting fetal weight in healthy term pregnant women* - U. Indraccolo, L. Chiocci, P. Rosenberg, L. Nappi, P. Greco 205

- Mid-trimester maternal serum AFP levels in predicting adverse pregnancy outcome* - P. Gkogkos, G. Androutopoulos, P. Vassilakos, G. Panayiotakis, G. Kourounis, G. Decavalas 208

- Knowledge and general consideration about Pap test screening among women from Finland and Greece* - G. Iatrakis, S. Zervoudis, P. Peitsidis, M.M. Nikolaki, D. Biba, E. Sotiropoulou 211

CASE REPORTS

- Pregnancy and delivery following sonohysterographic lysis to treat recurrence after hysteroscopic lysis of severe intrauterine adhesions: a case report* - F. Taniguchi, H. Suginami 215

- A case of body stalk anomaly at 12 weeks of gestation* - G. Adonakis, N. Spinos, J. Tourikis, H. Hasiotou, K. Loutzi, G. Kourounis 218

- Partial placenta increta and methotrexate therapy: three case reports* - S. Pinho, S. Sarzedas, S. Pedroso, A. Santos, M. Rebord o, T. Avillez, E. Casal, M. Hermida 221

- Placenta percreta presenting in the first trimester and resulting in severe consumption coagulopathy and hysterectomy: a case report* - J.C. Papadakis, N. Christodoulou, A. Papageorgiou, M. Rasidaki 225

- A case with diffuse uterine leiomyomatosis and review of the literature* - A. Coskun, O. Ozdemir, M.A. Vardar, G. Kiran, D. Arıkan, C. Ersoz 227

- Successful treatment of advanced endometriosis with extremely high CA 125 and moderately elevated CA 15-3 levels* - M.T. Canda, N. Demir, O. Sezer, L. Doganay 231

No. 4, October, November, December

EDITORIAL ARTICLES

- Advances in oocyte cryopreservation - Part I: slow cool rapid thaw technique* - J.H. Check 237
Regulatory mechanism of Bcl-2 in uterine leiomyomas - N. Ohara 240

REVIEW ARTICLES

- High-grade cervical intraepithelial neoplasia, human papillomavirus and factors connected with recurrence following surgical treatment* - M.C. Ramos, B.H. Pizarro De Lorenzo, M.A. Michelin, E.F.C. Murta 242
Human papilloma virus in adolescence - P. Christopoulos, K. Papadias, K. Panoulis, E. Deligeoroglou 248

ORIGINAL ARTICLES**Reproductive Biology Section**

- Correction of failed fertilization despite intracytoplasmic sperm injection with oligoasthenoteratozoospermia but with acrosomes present by oocyte activation with calcium ionophore - case report* - J. Pinto, J.H. Check 252
Early endometrial changes following successful implantation: 2 and 3-dimensional ultrasound study - E. Zohav, I. Bar-Hava, S. Meltzer, J. Rabinson, E.Y. Anteby, R. Orvieto 255

General Section

- Increased expression of GRP94 protein is associated with decreased sensitivity to adriamycin in ovarian carcinoma cell lines* - L.Y. Zhang, X.C. Zhang, L.D. Wang, Z.F. Zhang, P.L. Li 257
Role of dietary habits on fetal anomaly development: Review of 315 consecutive fetal anomaly cases - F.B. Cebesoy, O. Balat, E. Dikensoy, M.G. Ugur, I. Kutlar, H. Kalayci 264
Non classical congenital adrenal hyperplasia due to 21-hydroxylase deficiency in families from a Greek island with a closed society - E. Trakakis, A. Chryssikopoulos, G. Basios, P. Trompoukis, A. Sarandakou, M. Spyropoulou, E. Salamalekis, P. Karanikolopoulos, G. Salamalekis, P. Skarpas, D. Kassanos 267
Lipid peroxidation and antioxidant status in hypertensive pregnancies - S.B. Patil, M.V. Kodliwadmath, S.M. Kodliwadmath 272
Incidence of cervical cancer in Montenegro - B. Čolaković, V. Čolaković-Popovic 275
Abortion rates and the role of family planning: A presentation of the Greek reality - N. Salakos, K. Bakalianou, O. Gregoriou, C. Iavazzo, G. Paltoglou, G. Creatsas 279

CASE REPORTS

- Serous cystadenoma with omental caking and ovarian torsion: an unusual case presentation* - P. Brezina, J. Woelk, D. Brezina, J. Devente 284
Spontaneous uterine rupture during preterm labor in the second trimester of a twin IVF pregnancy without any apparent risk factor - C. Ficicioglu, G. Yildirim, F. Arioglu, N. Cetinkaya 287
Successful methotrexate treatment of an abdominal pregnancy in the pouch of Douglas - R. Crespo, J.M. Campillos, A. Villacampa, B. Madani, R. Navarro, J.J. Tobajas 289
Xanthogranulomatous salpingitis: report of three cases and comparison with a case of pseudoxanthomatous salpingitis - E. Kostopoulou, A. Daponte, A. Kallitsaris, R. Papamichali, G. Kalodimos, I.E. Messinis, G. Koukoulis 291
Bowel obstruction due to endometriosis in the rectovaginal septum - N. Takai, T. Ueda, M. Nishida, K. Nasu, H. Narahara 295
Conservative management of cervical ectopic pregnancy: case report and review of literature - A. Corticelli, M. Grimaldi, E. Caporale 297
Infertility in a new 46, XX male with positive SRY confirmed by fluorescence in situ hybridization: a case report - C.E. Pepene, I. Coman, D. Mihu, M. Militaru, I. Duncea 299
A rare case of uneventful pregnancy in a woman with Cogan's syndrome - K. Bakalianou, N. Salakos, C. Iavazzo, K. Danilidou, K. Papadias, A. Kondi-Pafiti 301
Pelvic packing method (after two laparotomies): a salvage procedure to control intractable pelvic hemorrhage after vaginal hysterectomy: a case report - A. Kale, U. Kuyumcuoğlu 303
Fetal ovarian cysts: report of two cases and literature review - D. Grapsa, C. Salakos, V. Tsapanos, N. Salakos, K. Bakalianou, A. Kondi-Pafiti 306
Unusual presentation of a dermoid cyst that derived from the bladder dome presenting as subserosal leiomyoma uteri - U. Kuyumcuoğlu, A. Kale 309

BRIEF COMUNICATION

- Anti-Lewis alloimmunization: report of seven cases* - L.M.M. Nardoza, G.R. Lobo, A.F. Moron, L. Camano, E. Araujo Jr., H.A. Guimarães Filho 311

Index of Authors in alphabetical order

Vol. XXXV, 2008

- Adonakis G., 218
 Akhan S.E., 73, 194
 Akpolat N., 202
 Akrivis Ch., 83, 156
 Amui J., 13
 Androutsopoulos G., 172, 208
 Anongba S., 149
 Anteby E.Y., 255
 Arancio V., 54, 69
 Araujo Jr. E., 311
 Argeitis J., 76
 Arikan D., 227
 Arioglu F., 287
 Avillez T., 221
 Bakalianou K., 86, 103, 130, 279, 301, 306
 Balat O., 264
 Bar-Hava I., 255
 Basios G., 267
 Baulies S., 140
 Behr B., 170
 Berkes E., 107
 Biba D., 211
 Bolat F., 144
 Botsis D., 76, 130
 Brasile D., 13
 Brezina D., 284
 Brezina P., 284
 Bulakbasi T., 144
 Burney R.O., 170
 Burns M.J., 27
 Čolaković B., 275
 Čolaković-Popovic V., 275
 Cadidé R.M., 175
 Camano L., 311
 Camargos A.F., 198
 Campillos J.M., 289
 Canda M.T., 231
 Caporale E., 297
 Carroll L.P., 27
 Carta G., 149
 Casal E., 221
 Cebesoy F.B., 264
 Çelik N., 179
 Cetinkaya N., 287
 Chaidogiannos C., 81
 Check J.H., 5, 10, 13, 93, 147, 167, 237, 252
 Chen A.C.Y., 113
 Chiang H.K., 113
 Chiocci L., 205
 Choe J.K., 13, 167
 Chrelias C., 190
 Christodoulou N., 98, 225
 Christopoulos P., 248
 Chryssikopoulos A., 267
 Cigerli E., 73
 Cirpan T., 48
 Coman I., 299
 Corticelli A., 297
 Coskun A., 137, 227
 Creatsas G., 279
 Crespo R., 289
 Cusidó M.T., 140
 Dadidou M., 151
 Dalainas H., 103
 Dalmarco E.M., 175
 Danilidou K., 301
 Daponte A., 291
 de Cordova C.M.M., 175
 De Dominicis R., 54, 69
 Decavalas G., 172, 208
 Deguchi M., 35
 Deligeoroglou E., 103, 248
 Demir N., 231
 Deveci S., 133
 Devente J., 284
 Di Berardino C., 117
 Dikensoy E., 264
 Doganay L., 231
 Duncea I., 299
 Erkanli S., 144
 Ersoz C., 227
 Ertan K., 57
 Ezawa M., 45
 Fábregas R., 140
 Fekete T., 107
 Ficicioglu C., 287
 Galazios G., 22, 32, 124, 151
 Gavai M., 107
 Gebhardt J., 170
 Giannaris D., 190
 Giuliani M., 187
 Gkogkos P., 208
 Govind A., 71
 Grapsa D., 41, 306
 Grapsas S., 81
 Grapsas X., 32
 Grases P.J., 140
 Greco P., 205
 Gregoriou O., 130, 279
 Grimaldi M., 297
 Guié P., 149
 Guimarães Filho H.A., 311
 Gundem G., 48
 Gungor F., 73
 Has R., 194
 Hasiakos D., 76
 Hasiotou H., 218
 Hermida M., 221
 Iatrakis G., 211
 Iavazzo C., 86, 103, 130, 279, 301
 Ibrahimoglu L., 194
 Incki K.K., 153
 Indraccolo U., 205
 Iovenitti P., 149
 Iyibozkurt A.C., 73, 194
 Jacquemyn Y., 121
 Kairi-Vassilatou E., 41, 76
 Kalayci H., 264
 Kale A., 303, 309
 Kalelioğlu I., 73, 194
 Kallitsaris A., 291
 Kalmantis K., 130
 Kalodimos G., 291
 Karadaglis St., 83, 156
 Karakantza M., 172
 Karanikolopoulos P., 267
 Kassanos D., 81, 190, 267
 Katsoff B., 10, 13, 147
 Kawamura M., 45
 Kaya M., 65
 Kelekci S., 153
 Kennedy S., 35
 Keremans M., 121
 Kiran G., 137, 227
 Kitao K., 35
 Kling T.M., 27
 Kodliwadmth M.V., 272
 Kodliwadmth S.M., 272
 Koffi K., 149
 Komaki A., 16
 Kondi-Pafiti A., 41, 76, 86, 103, 301, 306
 Kordi Tamandani D.M., 183
 Kostopoulou E., 291
 Koukoulis G., 291
 Kourounis G., 208, 218
 Koutlaki N., 22, 151
 Koyama N., 16
 Krambeck W.M., 175
 Küçük T., 57, 133
 Kumru S., 202
 Kurt I., 65
 Kutlar I., 264
 Kuyumcuoğlu U., 303, 309
 Ladopoulos I., 83, 156
 Lakhi N., 71
 Lazar L., 107
 Li P.L., 257
 Liang W.Y., 113
 Liberis V., 22, 32, 124, 151
 Liss J.R., 167
 Lobo G.R., 311
 Loghis C., 190
 Lomartire N., 187
 Loutzi K., 218
 Madani B., 289
 Malamitsi-Puchner A., 103
 Mancuso A., 54
 Maroulis G., 22, 124
 Maruo T., 35
 Mascaretti G., 117, 187
 Meltcer S., 255
 Messinis I.E., 291
 Mete Itil I., 48
 Michelin M.A., 242
 Mihu D., 299
 Militaru M., 299
 Morikawa J., 35
 Moron A.F., 311
 Mougiou A., 172
 Murta E.F.C., 242
 N'guessan K., 149
 Nagashima T., 45
 Nappi L., 205
 Narahara H., 295
 Nardoza L.M.M., 311
 Nasu K., 295
 Navarro R., 289
 Nikolaki M.M., 211
 Nishida M., 295
 Ohara N., 35, 165, 240
 Onodera T., 45
 Orvieto R., 255
 Ozan H., 179
 Ozbal A., 48
 Ozdemir O., 137, 227
 Ozer O., 144
 Ozsener S., 48
 Palit G., 121
 Palmara V., 54, 69
 Paltoglou G., 86, 130, 279
 Panayiotakis G., 208
 Panoulis K., 248
 Papadakis J.C., 98, 225
 Papadias K., 86, 248, 301
 Papageorgiou A., 225
 Papakonstantinou K., 41, 76
 Papamichali R., 291
 Papoulias I., 81
 Papp Z., 107
 Parker L.D., 27
 Pascual M.A., 140
 Patil S.B., 272
 Pedroso S., 221
 Peitsidis P., 211
 Pepene C.E., 299
 Pereira F.E.L., 198
 Pinho S., 221
 Pinto J., 167, 252
 Pizarro De Lorenzo B.H., 242
 Plis Ch., 83, 156
 Qian X., 16
 Rabinson J., 255
 Ramos M.C., 242
 Rasidaki M., 225
 Rebordão M., 221
 Retto G., 54, 69
 Risvanli A., 202
 Rosenberg P., 205
 Sahin F.I., 144
 Salakos C., 306
 Salakos N., 86, 103, 130, 279, 301, 306
 Salamalekis E., 267
 Salamalekis G., 267
 Salamapasis C., 81
 Santos A., 221
 Sarandakou A., 267
 Sarzedas S., 221
 Savidis A., 124
 Saygili-Yilmaz E.S., 153
 Sener T., 61, 65
 Sezer O., 231

- Shekari M., 183
Shibata N., 16
Shu Y., 170
Sills E.S., 27
Sills S.S., 27
Sivridis E., 151
Skarpas P., 267
Sobti R.C., 183
Sotiropoulou E., 211
Spinou N., 218
Spyropoulou M., 267
Sturlese E., 54, 69
Sugimoto M., 35
Suginami H., 215
- Takacs Z.F., 107
Takai N., 295
Takemura N., 35
Taniguchi F., 215
- Tanir H.M., 61, 65
Tegnan J., 149
Teichmann A.T., 22, 124
Theodori E., 172
Tobajas J.J., 289
Topuz S., 73, 194
Tourikis J., 218
Toyooka R., 45
Trakakis E., 267
Trindade C.R., 198
Tripodi A., 54, 69
Tripodi M., 54, 69
Trompoukis P., 81, 267
Tsapanos V., 306
Tsikouras P., 22, 32, 124, 151
Tsoukalos G., 83, 156
Tunaci M., 194
Turgut M., 153
- Úbeda B., 140
Ueda T., 295
Ugur M.G., 264
Ulukus M., 48
- Vardar M.A., 227
Varras M.N., 83, 156
Vassaras G., 32
Vassilakos P., 208
Villacampa A., 289
Vitoratos N., 103
Vlachos G., 22, 32
Vogiatzaki T., 124
- Wang L.D., 257
Westphal L.M., 170
Wittkowski K.M., 27
Woelk J., 284
- Xasiakos D., 41
- Yagishita M., 45
Yenieli O., 48
Yildirim G., 287
Yildiz C., 65
Yildiz S., 202
Yildiz Z., 61
Yilmaz Z., 144
Yoshida S., 35
Yuan C.C., 113
- Zekioglu O., 48
Zervoudis S., 124, 211
Zhang L.Y., 257
Zhang X.C., 257
Zhang Z.F., 257
Zoccali G., 187
Zohav E., 255
Zoumbos N., 172
Zourla A., 81, 190

Clinical and Experimental Obstetrics & Gynecology

an International Journal



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Montréal, Qué. H3W 2H3 (Canada)
Tel. +514-4893242 - Fax +514-4854513 - E-mail: canlux@qc.aira.com - www.irog.net

ISSN: 0390-6663

Published three monthly

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