Journal of Ankara Medical School

ISSN 1300-5464

Vol 23, No 3, 2001

Journal of Ankara Medical School

Vol 23, No: 3, 2001

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Journal of Ankara Medical School

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Design & Preparation: ANTIP A.Ş. 0 (312) 310 63 05 - 362 30 30/7005 Hakan Ellidokuzoğlu

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Journal of Ankara Medical School

Published Quarterly by
ANKARA UNIVERSITY MEDICAL SCHOOL

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IMMUNOHISTOCHEMICAL ANALYSIS OF BASEMENT MEMBRANE FORMATION IN EMBRYOID BODIES DERIVED FROM EMBRYONIC STEM CELLS

H. Seda Vatansever* ❖ M. Kemal Özbilgin* ❖ V. Sevinç İnan*

SUMMARY

The central role of basement membranes in organ and cell biology has made them an important subject for researchers who are investigating their structure, organisation and regulatory functions. Our study employed indirect immunohistochemistry to investigate the formation of basement membranes in embryonic bodies (EB) derived from embryonic stem (ES) cells. Basement membranes initially appeared in embryonic bodies at the end of the first day. Depositions of basement membrane components - laminin, collagen IV, nidogen and perlecan - appeared in cultures as linear staining. Thick basement membrane depositions were also observed in embryonic bodies at the end of the first week using anti-laminin, anti-collagen IV, anti-nidogen and anti-perlecan antibodies. Using TEM, the ultrastructure and thickness of the basement membrane was observed to be similar to Reichert's membrane, a membrane consisting of parietal endoderm cells derived from primitive endoderm cells, secrete basement membrane components and the trophectoderm basement membrane. However, ES cells, which are derived from inner cell mass cells, cannot be differentiated from trophoectoderm cells; therefore, basement membranes in EBs may form mechanisms other than Reichert's membrane. It is likely that different basement membrane types are present in EBs but cannot be distinguished by immunohistochemistry.

Key Words: *Basement Membrane, Embryonic Stem Cells, Immunohistochemistry*

ÖZET

EMBRİYONİK KÖK HÜCRELERİNDEN FARKLILANAN EMBRİYOBLASTLARDA GELİŞEN BASAL MEMBRANIN İMMUNOHİSTOKİMYASAL OLARAK İNCELENMESİ

Organ ve dokulardaki hücrenin temel biyolojik fonksiyonlarında önemli rol oynayan basal membranın, yapısı, organizasyonu ve regülasyonu günümüzdeki araştırma konularındandır. Bu nedenle, embriyonik kök hücrelerinden gelişen embriyoblastlardan oluşan basal membranın yapısı elektron mikroskobik ve indirekt immunohistokimya teknikleri ile incelenmiştir. Basal membranın formasyonu ilk olarak 1 günlük kültür yapılmış embriyoblastlarda gözlendi. Basal membran proteinleri olan laminin, kollagen IV, nidogen ve perlekanın immunoreaktivitelerinin pozitifliği bir günlük kültürde bir çizgi şeklinde saptandı. Embriyoblastların 1 hafta sonraki kültür dönemlerinin analizlerin de ise, basal membranın kalın bir tabaka olarak laminin, kollagen IV, nidogen ve perlekan antikorları ile immunohistokimya tekniği ile boyandığı gözlendi. Yapısal ve kalınlık açısından embriyoblastlarda farklılanan basal membranın TEM yöntemi ile Reichert'ın membranına benzediği gözlendi. Reichert'ın membranı primitif endoderm hücrelerinden farklılanan ve basal membran proteinlerini salgılayan parietal hücreler (Smith and Strickland, 1981; Hogan et al., 1981; Dziadek et al., 1985; Paulsson et al., 1985) ve trofoektoderm hücrelerinin basal membranı (Hogan et al., 1984; Salamat et al., 1995) tarafından oluşturulur. Bununla beraber, embriyonun iç hücre kitlesinden faklılanan embriyonik kök hücreleri trofoektoderm hücrelerine faklılanamaz. Bu nedenle, embriyoblastlarda gelişen basal membranın Reichert' ın membranının gelişiminden farklı bir mekanizma ile geliştiği düşünülmektedir. Tabiki, immunohistokimyasal olarak embriyoblastlardan gelişen basal membranın tipi ayırt edilemez.

Anahtar Kelimeler: *Basal Membran, Embriyoblast, İmmunohistokimya.*

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Received: May 17,2001 Accepted: Aug 22,2001

Basement membranes, which are secreted during early embryonic development, are found in all vertebrates and invertebrates except for sponges (1). For this reason, the characterisation of the biological properties of basement membranes has become an important theme in cell and developmental biology. Basement membranes are abundant in proteinaceous sheets underlying epithelial and endothelial cells and surrounding muscle fibres, fat cells and peripheral nerves. Only a few tissues (bone and cartilage) completely lack basement membranes (1). When analysed using standard electron microscope fixation methods, basement membranes appear to be divided into three layers. These are identified with respect to their staining behaviour and localisation: the lamina rara, the lamina densa and the lamina fibroreticularis.

Basement membranes exhibit structural and functional differences at different stages of development and in different tissues. For example, in the renal glomerulus, the basement membrane is sandwiched between a fenestrated endothelium and epithelial cells, providing a barrier between blood and urine. In the mouse embryo, Reichert's membrane, a multi-layered basement membrane, acts as a filter allowing free access of nutrients between the embryo and mother (2). In addition, basement membranes may play a central role both in the initial stages of development and in the maintenance of polarity of epithelial and endothelial cells (3).

The molecular organisation of the basement membrane depends on the inter-molecular interaction between its different components (1). The major mass of the basement membrane is built up of only a few of these components, including laminin, nidogen (entactin), collagen IV, perlecan, fibulin (BM-90) and SPARC (BM-40) (1). It is generally assumed that the formation of basement membranes requires specific interactions between the various constituents. Interaction of components may contribute to the structural and functional heterogeneity of basement membranes. Furthermore, the interaction of one basement membrane component with cells may modulate the deposition of other components within the basement membrane. The specific roles played by the individual components during basement membrane assembly remains a major question. They may include self-assembly processes involving identical components as well as the formation of the basement membrane containing different components.

In this study, embryonic stem (ES) cells were derived from the inner cell mass of 3.5 day blastocysts, which retained their pluripotency when allowed to grow under undifferentiated conditions (4,5). ES cells can be maintained in culture for many passages in an undifferentiated state by culturing on STO cells or primary cultures of mouse embryo fibroblast-feeder layers and/or with a differentiation inhibitor of ES cells, namely the leukemia inhibitor factor (LIF). In their absence, ES cells spontaneously differentiate in vitro into embryonic bodies (EBs) (6,7). EBs were found to be divided into two distinct parts - the outer endodermal cell and inner ectodermal cell layers, bordered by a basement membrane, which is similar in appearance to the egg-cylinder stage of the five-day embryo (Figure 1).

In using EBs derived from ES cells, it is significant that the analysis of basement membrane formation provides a very convenient model for the study of the biosynthesis and assembly of the basement membrane components. Thus, in order to understand the ultrastructure of the basement membrane in EBs, we used an electron microscope and indirect immunoflourescence staining to examine basement membrane formation and component deposition (laminin, nidogen, collagen IV and perlecan) in EBs derived from differentiating ES cells.

MATERIALS AND METHODS

Embryoid Bodies

Stock frozen cultures of undifferentiated mouse ES cells were allowed to differentiate in DMEM 10% FBS using the hanging drop method (8). Drops of 10µl (5x103 cells per drop) were placed on the lower surface of plastic petri dish lids containing PBS. Cell aggregates were plated

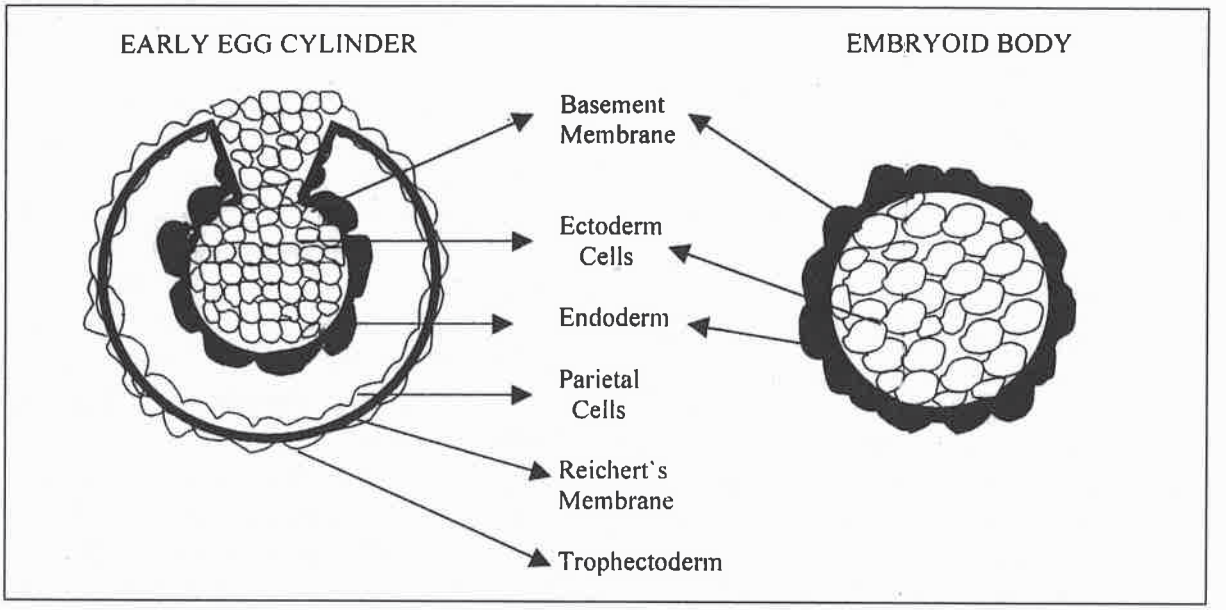

Figure 1: Analogy between structures in mouse egg cylinder and embryonic body. (Not to scale)

into plastic petri dishes after two hours in culture conditions, and the EBs were collected at the first day, second day and first week of culture. Specimens were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature prior to sectioning and immunostaining as above. EBs were washed in PBS prior to embedding in gelatin and freezing in Tissue-Tek (Sakura Finetek Europe).

Immunostaining of Frozen Sections

Rabbit polyclonal primary antibodies anti-EHS laminin (LA5 antiserum), which recognises all three subunits of laminin (9), anti-perlecan raised against recombinant domain III3 (10) and anti-collagen IV (Chemicon AB756) were used in this study. Rat monoclonal antibody against nidogen purified from the mouse EHS tumour was also used (15). Cryostat sections (7 µm) were washed in PBS for 30 minutes at 38°C, followed by a 10 minute wash in PBS at room temperature for the removal of gelatine. These sections were then incubated with a blocking solution consisting of 10% (v/v) normal goat serum (NGS Sigma G-9023). The primary antibodies (rabbit anti-laminin, rat anti-nidogen, rabbit anti-collagen IV and rabbit anti-perlecan) were then added. After washing with PBS, they were incubated with secondary antibodies [anti-rabbit IgG rhodamine-conjugated (Dako R-1569) for anti-laminin, anti-collagen IV and anti-perlecan antibodies; biotinylated anti-rat IgG (Dako E-0468) and streptavidin conjugated fluorescein isothiocyanate (FITC) (Amersham RPN 1323) for anti-nidogen antibody]. All immunostaining slides were mounted in a fluorescent mounting medium (DAKO). Slides were examined using a Leitz DMR photomicroscope with epifluorescence and phase-contrast microscopy and then photographed with HP5 film (Ilford, ISO 400).

Transmission Electron Microscopy (TEM)

EBs were taken from culture after a week and fixed in 2.5% (w/v) glutaraldehyde, 2% (w/v) paraformaldyde in 1 M sodium cacodylate buffer (pH 7.2) for two hours and postfixed with 2% (w/v) osmium tetraoxide in the same buffer. EBs were rinsed twice for five minutes with 0.15 M sodium acetate buffer, stained with 2% (w/v) uranyl acetate in 0.15 M sodium acetate buffer (pH 6.0) for 45 minutes, dehydrated in graded concentrations of alcohol and transferred into an embedding component (L. R. White). Sections

(70 nm) were taken with a Huxley microtome and mounted on 3000 mesh grids for examination by electron microscope (Philips 300 operating at 80 Kv). Sections were stained with 2% (w/v) uranyl acetate solution in 0.15 M sodium acetate buffer and lead citrate to increase contrast.

RESULTS

Using the hanging drop method, ES cell clumps appeared within 24-36 hours as simple EBs. Each clump had an outer layer of endodermal cells and inner ectodermal cells. There was an apparent increase in the size of the clump after 24 hours, probably due to both clump aggregation and cell multiplication. A basement membrane between the outer endodermal cell layer and the inner ectodermal cells was clearly visible under the dissecting microscope after a week in culture (Figure 2).

EB ultrastructure was examined by TEM. The thickness of the basement membrane measured $5x10^{-6}m$ between the outer layer and inner cells (Figure 3).

Immunohistochemical Analysis of Laminin in Ebs

EBs were stained with an anti-laminin antibody after a week in culture. Immunoreactivity of laminin was located underneath the outer layer of EB cells (Figure 4a). In addition, some punctuate staining was found in the inner layer cells. Figure 4-b shows the same EBs under phase microscopy. No staining was observed in the EB control sections for immunohistochemistry (Figure 4-c).

Time Course of Deposition of Basement Membrane in EBs

In order to analyse the induction of the basement membrane deposition, EBs were collected from culture at different times. After two hours in culture, single ES cells began to aggregate and form EBs (Figure 5-a). Under phase-contrast microscopy, some crescents of endodermal-like cells were seen on EB surfaces; however, no complete layers of endodermal cells encircling the EBs were seen (Figure 5-a). Immunoreactivity of laminin was seen both extracellularly (cell surfaces) and intracellularly (Figure 5-b). After one

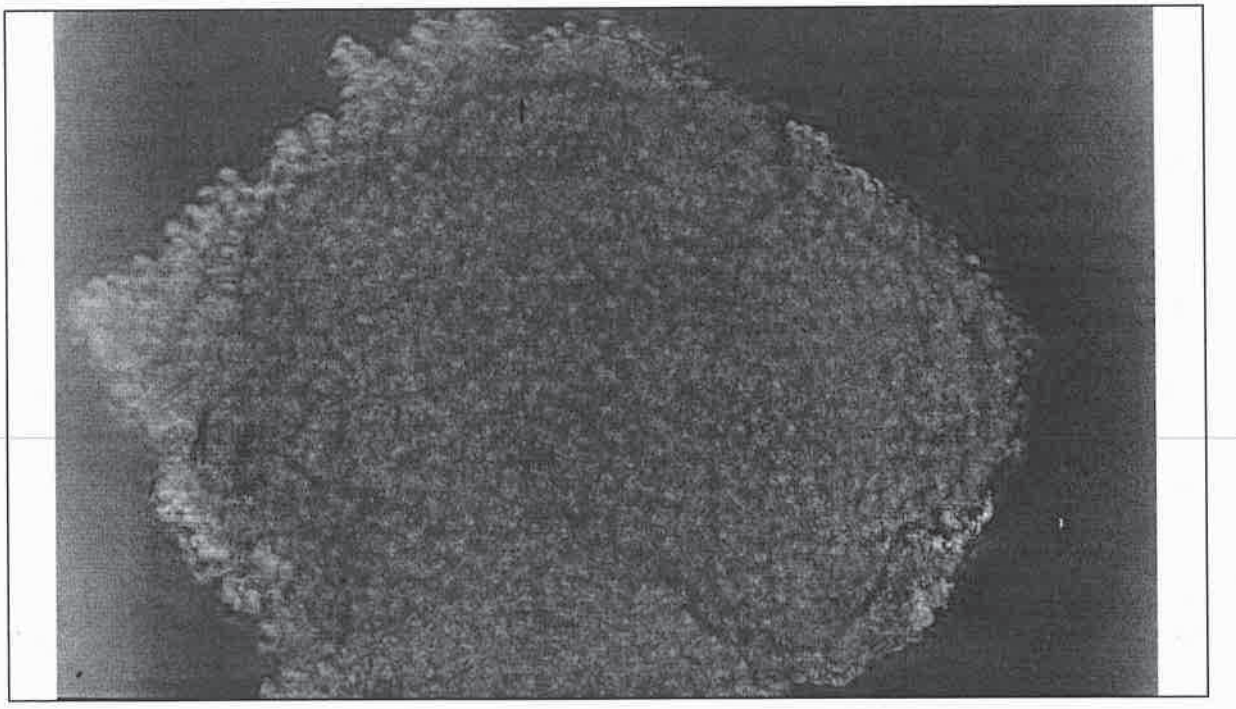

Figure 2: Phase micrographs of EBs produced using the hanging drop technique. After a week in culture, the basement membrane can be identified between the outer layer and inner cells (arrow). (Scale bar= 40 μm)

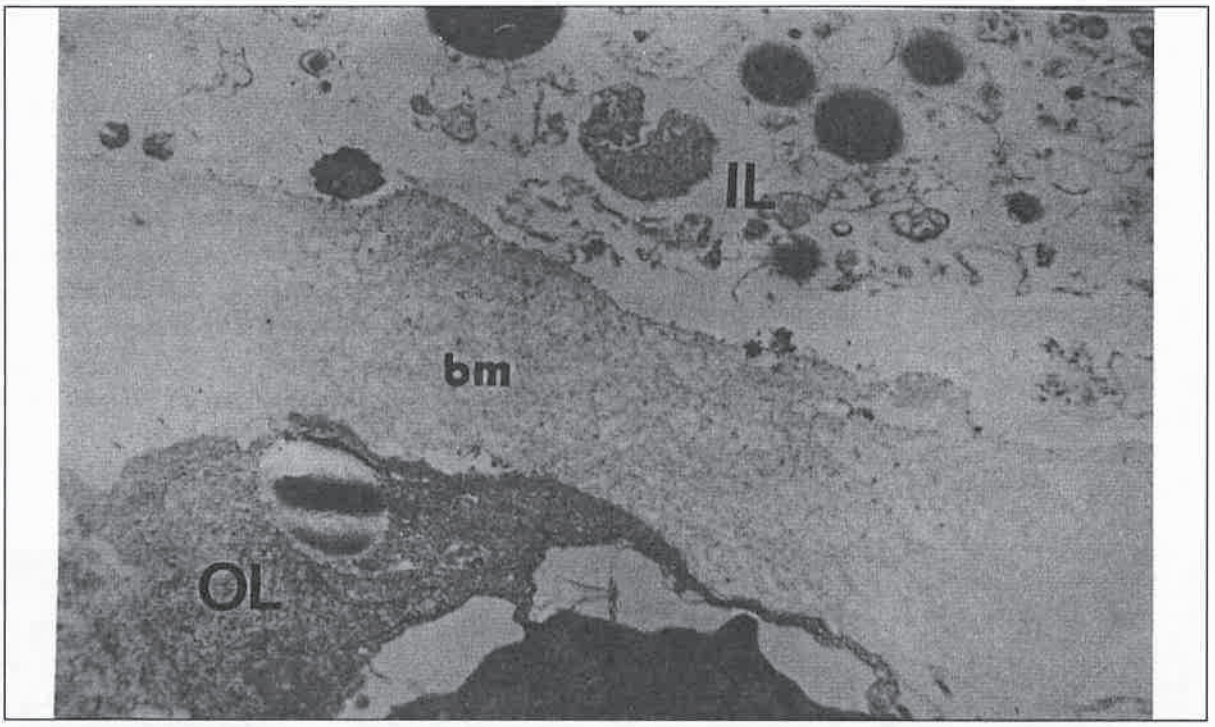

Figure 3: Electron microscopy of EBs after one week in culture. A sheet-like structure (basement membrane) seen beneath the outer layer. (BM:basement membrane; OL:Outer endodermal cell layer; IL:Inner ectodermal cell layer. Scale bar= 2 μm)

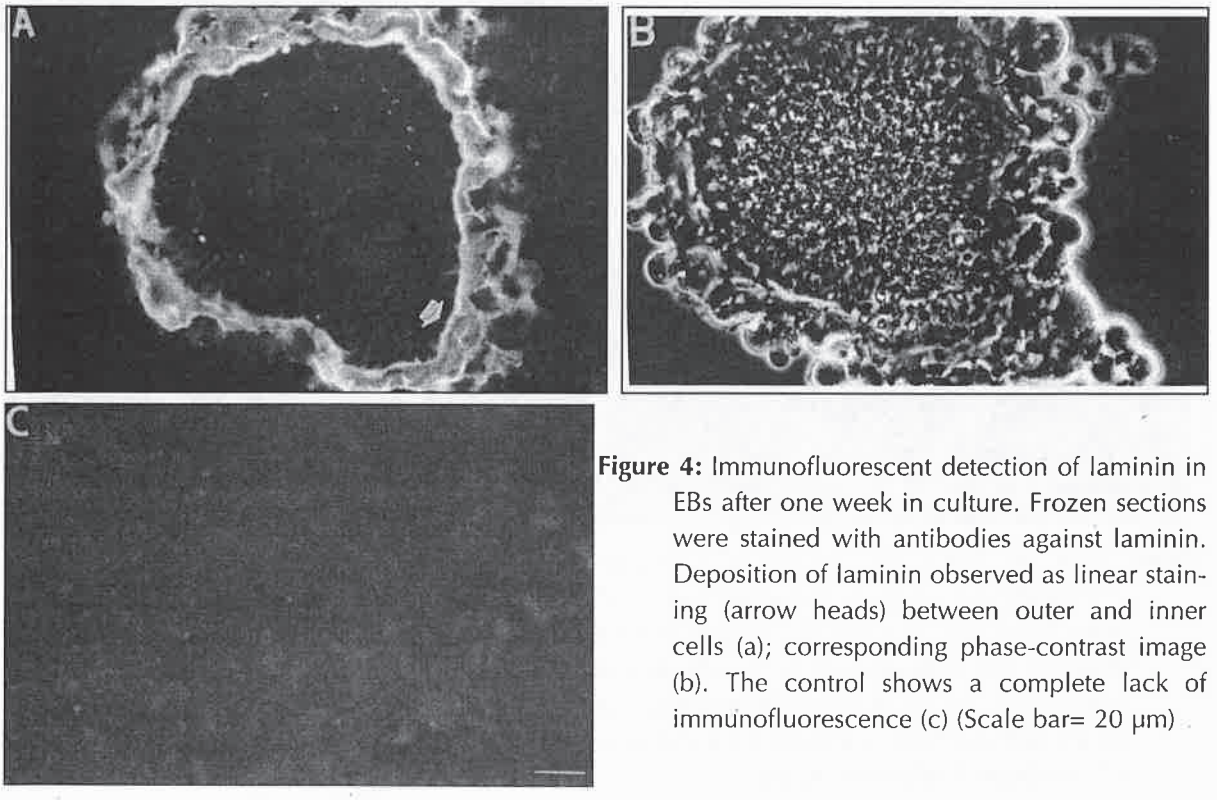

Figure 4: Immunofluorescent detection of laminin in EBs after one week in culture. Frozen sections were stained with antibodies against laminin. Deposition of laminin observed as linear staining (arrow heads) between outer and inner cells (a); corresponding phase-contrast image (b). The control shows a complete lack of immunofluorescence (c) (Scale bar= 20 μm)

day in culture, ES cell clumps became simple EBs (Figure 5-c). A comparison of Figures 5-a and 5-c shows the apparent increase in the size of EBs (four times greater than that of two-hour cultures) due to clump aggregation and cell multiplication. The outer cells of EBs appear as a layer that can be identified by phase contrast microscopy (Figure 5-c). Continuous linear staining with LA5 antiserum was seen between the outer layer and the inner cells in the periphery of EBs (Figure 5-d). Following two days in culture, the basement membrane was observed between outer and inner cells as a thick layer stained with laminin (Figure 5-e). Some immunoreactivity of laminin was seen to be associated with a few inner cells as well (Figure 5-e).

Deposition of Other Basement Membrane Components

EBs were stained with antibodies against collagen IV, nidogen and perlecan after two days and one week in culture (Figures 6-a, 6-c and 6-e, and Figures 6-b, 6-d and 6-f, respectively). Strong immunoreactivities of collagen IV (Figures 6-a, 6-b) nidogen (Figures 6-c, 6-d) and perlecan (Figures 6-e, 6-f) were seen as a continuous linear layer under the outer EB cells.

DISCUSSION

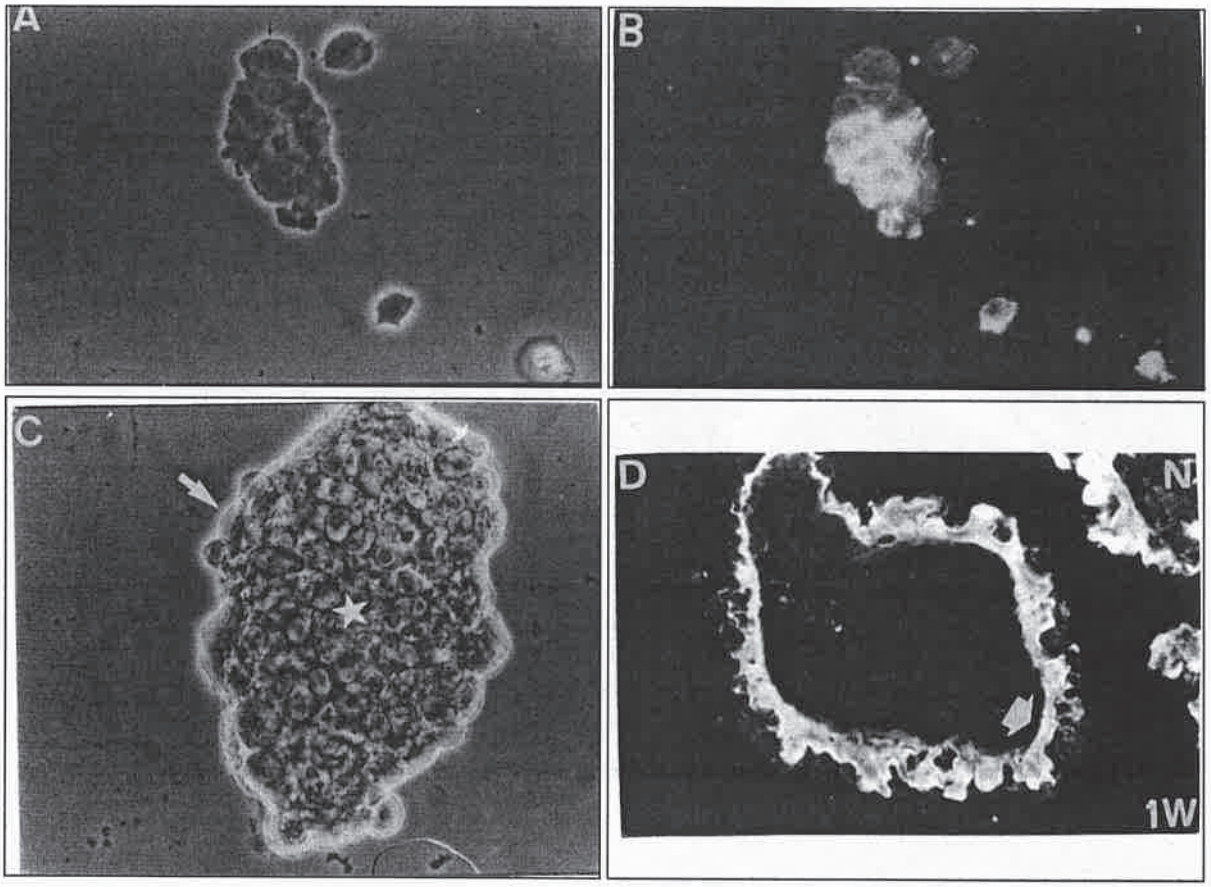

Figure 5: Immunofluorescent staining of EBs after two hours, one day and two days in culture. EBs were stained with LA5 antiserum against laminin. After two hours, primitive endoderm-like cells in outer layer (black arrow) were observed in phase-contrast image, and laminin immunoreactivity was shown as extracellular staining (b). After one day, EB growth and outer (white arrow) and inner cell layers (star) are clearly detectable in phase-contrast image (c). Immunoreactivity of laminin is also clearly detected between outer layer and inner cells (arrow heads) after one day (d) and two days (e) in culture. (Scale bar= 20 μm)

ES cells that are allowed to aggregate in the absence of LIF differentiate in vitro into EBs (6,7). Cell differentiation is promoted via cell-to-cell interactions similar to a compaction of the preimplantation mouse embryo (11). The use of the hanging drop technique is ideal for the rapid differentiation of defined EBs because it allows ES cells to aggregate spontaneously and quickly and permits EB size to be controlled by cell density (8). After cell differentiation, the EBs consisted of two parts, the outer layer and inner cells, which were separated by a basement membrane. The outer layer cells have the characteristics of both visceral and parietal endoderm cells (8). After a week in culture, a thick basement membrane was seen between the outer layer and inner cells by using TEM for EBs (Figure 3). The thickness of this basement membrane was estimated to be about $5x10^{-6}m$, giving it a resemblance to Reichert's membrane, a multilayer basement membrane seen at the blastocyst stage of mouse and rat development (12,13). Reichert's membrane is built up by both parietal endoderm cells, which are derived from primitive endoderm cells and produce basement membrane components (14-16), and the trophectoderm basement membrane (13,17). However, ES cells, which are derived from ICM cells, cannot differentiate into trophoectoderm cells. Therefore, EB basement membranes may form by a mechanism different from that of Reichert's membrane. Thus, it is likely that different basement membrane types are present in EBs but cannot be distinguished by immunohistochemistry. However, this basement membrane was deposited with major basement membrane formation.

Deposition of laminin was seen in our experiments as linear staining between the outer layer and inner cells, with some punctuate staining on or in the inner EB cells. Immunoreactivity of laminin was detected mainly in association with the outer layer cells (see below). Immunoreactivity of laminin was first demonstrated by immunohistochemistry using LA5 antiserum. The antibodies for laminin recognise all three subunits of type-1 laminin (9) and therefore do not distinguish individual subunits. When ES cells were allowed to differentiate after the hanging drop, they started to aggregate after two hours. However, basement membrane formation in EBs began on Day 1.

Indirect immunohistochemistry was used to detect basement membrane components including collagen IV, nidogen and perlecan to determine if any other basement membrane formation occurred. After 2 days in culture, immunoreactivities of collagen IV, nidogen and perlecan were extracellular, and their deposition was in the basement membrane between the EB outer and inner layers (Figure 6).

In recent years, the complexity of basement membrane deposition and components collagen IV and laminin has been revealed. The distribution of the two proteins vary in different basement membranes (18-21). In addition, recent studies have demonstrated that the deposition of the basement membrane is disrupted without laminin (22). The diversity of laminin and collagen IV may be important in the development and remodelling process and in providing tissue-specific variability of basement membrane structure and function. Also, interactions between laminin and other components, especially nidogen, play a role in basement membrane assembly (23). However, further research is needed to produce a clearer picture of basement membrane formation and assembly in the adult and during embryo development.

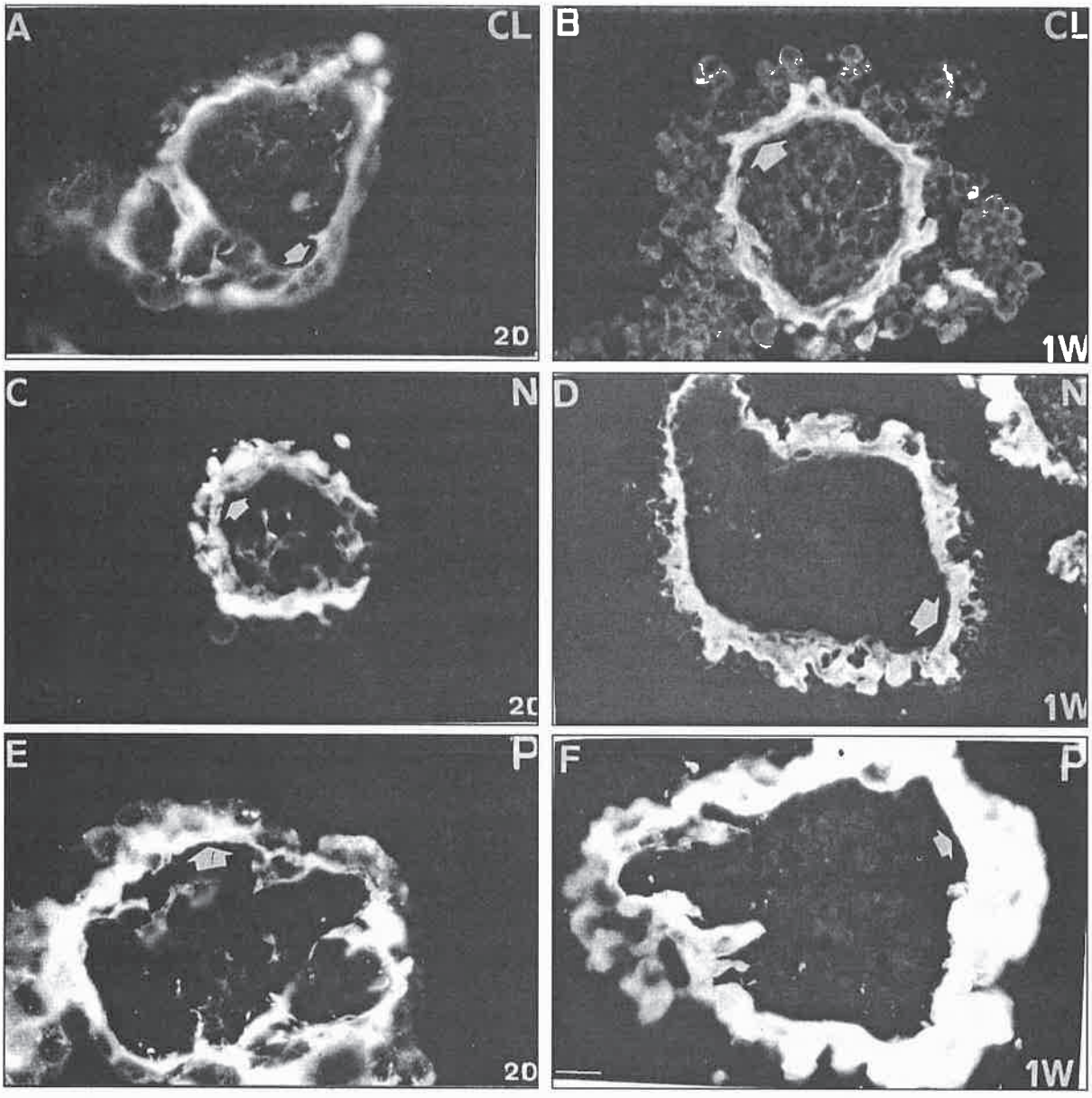

Figure 6: Immunofluorescent detection of collagen IV, nidogen and perlecan in EBs after two days and one week in culture. Immunoreactivity of collagen(CL) IV (a,b), nidogen (N) (c,d) and perlecan (P) (e,f) were seen between outer and inner layers of EBs (arrow heads) at two days (2D) and one week (1W) in culture. (Scale bar= 20 μm)

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MHC-CLASS II CELLS IN THE OVIDUCT PLAY A ROLE DURING THE MENSTRUAL CYCLE AND EARLY PREGNANCY?

V. Sevinç İnan* ❖ H. Seda Vatansever* ❖ M. Kemal Özbilgin* ❖ Muzaffer Sancı** ❖ Kenan Ertopçu**

SUMMARY

The epithelium of the oviduct may play significant roles in defending oviductal tissue and fertilized ovum from infection. The aim of this study was to determine the role of MHC-class II cells by analyzing them via HLA-DR immunoreactivity in the human oviduct epithelium during the menstrual cycle and early pregnancy. Oviducts from 21 healthy women undergoing tubal sterilization were collected and fixed in 10% formalin. After dehydration in ethanol, the oviducts were embedded in paraffin. Sections were immunostained with monoclonal mouse-anti human HLA-DR antibody, and immunoreactivity was determined using immunoperoxidase technique under light microscopy. While increased HLA-DR immunoreactivity was observed in columnar epithelial cells during the proliferatory phase of the menstrual cycle, immunoreactivity was withdrawn from the epithelium in the secretory phase and in early pregnancy. These results indicated that human oviduct epithelium has positive immunoreactivity for MHC-II+ cells and that increased immunoreactivity correlated with ovulation. Varied distribution of HLA-DR immunoreactivity in the oviduct epithelium may play a significant role in the regulation of local immunocompetence during the menstrual cycle. Increased HLA-DR immunoreactivity in the proliferatory phase may be needed against invading microorganisms. At the same time, a decrease in HLA-DR immunoreactivity in the secretory phase and early pregnancy may be required to endure preimplantation of the foreign embryo.

Key Words: *Oviduct, HLA-DR, İmmunohistochemistry,*

ÖZET

TUBA UTERİNADAKİ MHC-Class II HÜCRELERİNİN, MENSTRUAL SİKLUS VE ERKEN GEBELİK DÖNEMLERİNDEKİ ROLÜ

Tuba uterina lümenini döşeyen epitel dokusu, bu bölgeye gelebilecek patojenlere karşı ilk bariyeri oluşturmaktadır. Doku uygunluk kompleksi-MHC (Major Histocompatibility Kompleks) class II hücrelerinin, bu bölgede varlığının gösterilmesi lokal immün cevap sırasında, tuba uterinanın antijen sunucu olarak rol oynayabileceğini düşündürmektedir.

Bu çalışmada, menstrual siklus ve erken gebelik döneminde insan tuba uterina epitelindeki doku uygunluk kompleksi-II hücrelerinin rolünün, HLA-DR immunoreaktivitesi ile değerlendirilmesi amaçlanmıştır. Tuba uterina örnekleri, 21 sağlıklı kadından, mini laparotomi ile Pomeroy usulü yapılan tüp ligasyonunda alınarak % 10 formalinde tesbit edildi. Alkol serileri ile dehidrate edildikten sonra, ksilende şeffaflandırılan dokular parafin bloklara gömüldü. İmmünohistokimyasal boyama için, 6 mikron kalınlığındaki kesitler deparafinize edildi. Anti HLA-DR birincil antikoru uygulandıktan sonra, immunoperoksidaz tekniği kullanılarak immünoreaktivite ışık mikroskobu altında değerlendirildi.

Tuba uterinanın tek sıralı prizmatik epitel hücrelerinde, menstrual siklusun proliferasyon fazında HLA-DR immunoreaktivitesi belirgin pozitif olarak gözlenirken, sekresyon fazında ve erken gebelik döneminde ise immunoreaktivitenin azaldığı saptandı. Bu çalışma sonucunda, tuba uterinanın ovulasyon öncesi dönemde positif HLA-DR immünoreaktivitesinin mikroorganizmalara karşı gerekli immün cevabın sağlanmasında; ovulasyon sonrası dönemde azalmış immunoreaktivitenin ise implantasyon öncesi yabancı olan embriyonun optimal yaşaması ve korunmasına yönelik gerekli lokal immünokompetansın sağlanmasında önemli rol oynayabileceği düşünülmüştür.

Anahtar Kelimeler: *Tuba Uterina, HLA-DR, Menstrual Siklus*

The oviduct, which conveys secondary oocytes from the ovaries to the body of the uterus, is a dynamic and cyclically changing structure. Cyclical fluctuations in the circulating levels of estradiol and progesterone are responsible for cyclical and structural changes of the

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Received: May 17,2001 Accepted: Sep 06,2001

oviduct (1). The oviduct contains multiple cell types. This cellular diversity assists in the maturation and transportation of gametes and fertilized ovum and in the early development of the embryo (2).

The epithelium is the first protective barrier against foreign pathogens invading the human oviduct (2). These pathogens are blocked by initializing local immunity, which is greatly affected by sexual maturation and ovarian steroids (3). Local immunity may arise from Major Histocompatibility Complex (MHC) class II proteins. Human Leukocyte Antigens (HLA-DR, -DQ, -DP), which are members of the MHC-class II group, are transmembranous glycoproteins with a central role in cell-to-cell interactions in the initiation of immune response, especially in the early phase of T lymphocyte activation. Constitutive expression of MHC-class II antigens has been identified in a limited number of cells, mainly bone marrow-derived cells such as macrophages, B lymphocytes, monocytes, dendritic cells and Langerhans' cells (4).

The female reproductive tract represents an environment in which the physiological need for controlling MHC-class II antigens may be particularly delicate. Different needs for immunocompetence can be assumed to be at hand during different phases of the normal menstrual cycle (5).

The purpose of this study was to investigate the immune role of the oviduct by assessing HLA-DR immunoreactivity throughout the menstrual cycle and early pregnancy.

MATERIALS AND METHODS

Isthmic portions of oviducts were obtained from 21 healthy women undergoing tubal ligation by the Pomeroy technique in the Department of Family Planning of the Aegean Social Insurance Institute, Obstetric & Gynecology Hospital. Tubal ligation was performed, with patient consent, in conjunction with the termination of unwanted pregnancies.

Subjects were classified into three groups: (i) proliferatory phase (5th-14th day of cycle, n=7), (ii) secretory phase (15th-28th day of cycle, n=7) and (iii) early pregnancy (6th-7th week of gestation, n=7). At the same time, histology of endometrial biopsies was obtained and histologic dating of the endometrium performed, as described by Noyes et al (1950). The mean age of patients was 34±5 years (age range: 29-39). All patients had regular menstrual cycles, and there were no significant differences among the average ages of the women in the three groups. None were receiving any hormones or medication likely to interfere with ovulation.

Tissue samples were preserved in 10% formaline solution for evaluation under light microscopy. Samples were embedded in paraffin blocks after dehydration with graded ethanol. Paraffin sections (5(m) were deparaffinized with xylene and rehydrated through a graded ethanol series.

Indirect immunoperoxidase was used for immunohistochemistry. Sections were washed with phosphate-buffered saline (PBS) and treated with 0.1% trypsin solution. They were then washed with PBS and pre-treated with 0.3% hydrogen peroxide for 10 minutes at room temperature to inactivate endogenous peroxidase activity. They were washed again in PBS and incubated overnight with the primary antibody (1:100 dilution monoclonal mouse anti-human HLA-DR antibody - DAKO-M 0746) in a humidity chamber at 4(C. They were then incubated with anti-mouse immunoperoxidase antibody (Universal Dako LSAB2 Kit). Color reaction was developed using a Dako AEC Substrate System (Dako) containing 3-amino-9-ethylcarbazole. Sections were counterstained with Mayer's hematoxylin and covered with mounting medium. Normal mouse serum was used for immunoreactivity on negative control sections.

HLA-DR staining was graded semi-quantitatively according to the following scale: strong staining intensity (+++); moderate staining intensity (++); weak staining intensity (+); absence of staining (-).

RESULTS

HLA-DR immunoreactivity in the oviduct

TABLE 1: Summary of HLA-DR distribution in oviduct epithelium during menstrual cycle and early pregnancy

	Proliferatory Phase	Secretory Phase	Early Pregnancy Stage
Oviduct epithelium	+ ++	++	+

epithelium according to menstrual cycle and early pregnancy is summarized in Table 1. Positive HLA-DR immunoreactivity was detected in the epithelial layer and the lamina propria of the oviduct in all phases of the menstrual cycle and early pregnancy, but the intensity of immunoreactivity differed during these phases.

In the proliferatory phase, intense immunostaining was detected in columnar epithelial cells, especially in epithelial cell cytoplasm and under the epithelial layer (Figure 1-A). In this phase, HLA-DR+ cells were found in the epithelium and in the lamina propria, especially near the capillaries. Observation was significantly better under higher magnification (Figure 1-B). An increase in immunoreactivity was detected during the proliferatory phase. Immunoreactivity decreased after ovulation, but HLA-DR+ cells were observed in the intraepithelial region in the beginning of this phase (Figure 1-C). Weak immunoreactivity of the cytoplasm of epithelial cells was still detected during the secretory phase (Figure 1-D).

In the specimens taken during early pregnancy, immunoreactivity was detected in the oviduct epithelium, with HLA-DR distribution similar to that of the secretory phase

(Figure 1-E). Weak immunoreactivity was seen only in the cytoplasm of the epithelial cells.

There was no immune staining in the controls (Figure 1-F).

DISCUSSION

In this study, positive HLA-DR immunoreactivity in the human oviduct, which exhibited MHC-class II antigens, was observed during the menstrual cycle and early pregnancy. However, HLA-DR immunoreactivity in the oviduct epithelium showed some differentiation throughout the menstrual cycle. While strong HLA-DR immunoreactivity was detected in the columnar

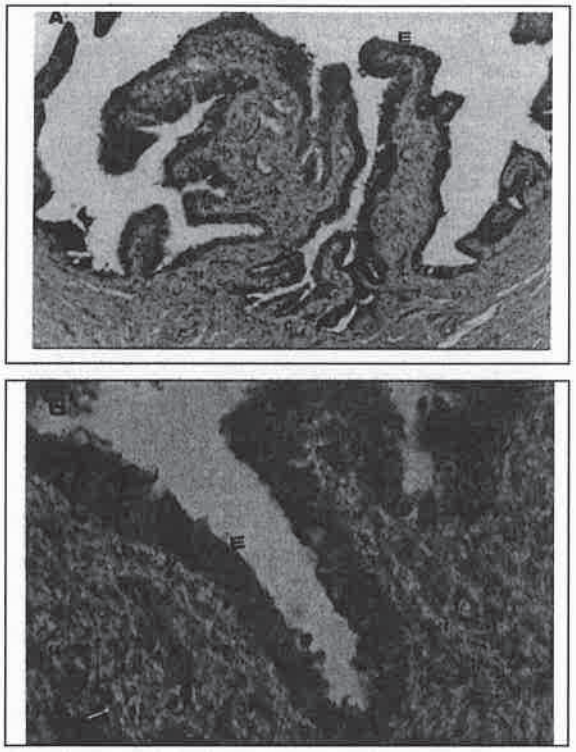

FIGURE 1:

A, B: Immunohistochemical localization of HLA-DR in oviduct during proliferatory phase. Immunoperoxidase staining for HLA-DR in the oviduct, showing strong staining in the tube epithelium. There were increased HLA-DR+ cells in the lamina propria in the proliferatory phase of the menstrual cycle. Immunoreactivity was detected especially in epithelial cell cytoplasm and under the epithelial layer. Immunoreactivity was observed better in higher magnification during the proliferatory phase. (A) X100, (B) X400 (Original magnification).

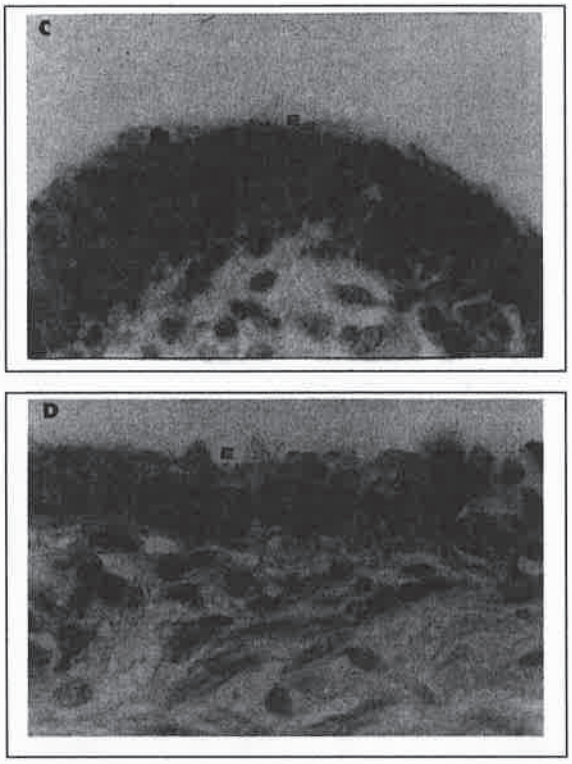

FIGURE 1:

C, D: Immunohistochemical localization of HLA-DR in human oviduct during secretory phase. Immunoreactivity decreased after ovulation, but HLA-DR+ cells were seen in the intraepithelial region in the beginning of this phase (C). Weak immunoreactivity was still detected in the cytoplasm of epithelial cells during the secretory phase (D). (C,D) X1000 (Original magnification).

FIGURE 1:

E: Immunohistochemical localization of HLA-DR in oviduct during early pregnancy. Decreased immunoreactivity was detected in the epithelial layer, and this staining was observed in the cytoplasm of epithelial cells . X1000 (Original magnification).

FIGURE 1:

F: Control immunostaining for HLA-DR. In the control section, there was no immune staining. X1000 (Original magnification). E: Oviduct epithelium

epithelium in the proliferatory phase, this immunoreactivity was withdrawn in the secretory phase.

In addition to its role in transporting and supporting the nutrition of the fertilized ovum, the oviduct plays an important role in the early division and differentiation of the embryo. This study also extends the characterization of the local immune system in the oviduct. Detection of MHC-class II cells in normal non-lymphoid tissues such as breast and endometrial epithelium suggests that the expression and secretion of these cells during the menstrual cycle may be under hormonal control (6). A number of studies on different animals species also indicated that immune cells played an important role in cyclical ovarian activity (7-11). These studies also emphasized that HLA-DR+ cells can show changes throughout the menstrual cycle. Bulmer and Earl previously described MHC-class II antigen expression in the columnar epithelium of the human oviduct (6). Hormonally mediated regula-

tion was suggested, based on the differences in class II antigen expression in the epithelium of oviducts in pregnant and non-pregnant women. Edelstam et al demonstrated that cyclical MHC-class II antigen variation in the oviduct may indicate hormonal regulation of class II antigen synthesis (5).

Strong HLA-DR immunoreactivity in the proliferatory phase may be required for the response against foreign infective microorganisms. In contrast, decreased HLA-DR immunoreactivity in the secretory phase might provide more optimal conditions for the immunologically foreign spermatozoa and fertilized ovum to escape from potential MHC-class II cells that are restricted by the mother's immune system.

The reduction in HLA-DR immunoreactivity in the secretory phase would be reflected in an impaired ability to recognize and respond to pathogens. As a result, Chlamydial infections, the major cause of acute salpingitis, usually start in the secretory phase (12). Strong MHC-class II antigen expressions in the secretory phase could lead to various kinds of inflammatory diseases. This would lead to the secretion of inflammatory mediators in the local environment, such as (-IF, which might counteract the normal secretory reduction of class II antigen expressions in the oviduct (5).

While there were no specific changes of Ig-positive cells in the non-infected oviduct in the different menstrual cycle phases, intraepithelial lymphocytes were detected (13). The lymphocytes in the human oviduct consist exclusively of T suppressors (CD8+). The secretion of these T lymphocytes is unresponsive to sperm antigens, although Ig-positive cells, especially IgA and IgG, are detectable against sperm antigens (13). These results suggested that intraepithelial lymphocytes may normally function in assisting in the induction of immune tolerance to sperm antigens in the oviduct. In addition, HLA-DR immunoreactivity at the fimbrial portion of the oviduct was found with T lymphocytes in fertile cases. In infertile cases, immunoreactivity was strongly stained in the epithelium, and T lymphocytes increased proportionally. These results lead us to believe that infertility occurs when there is high immunological response. In another study that aimed to investigate the protective effects of the oviduct against microorganisms, E Coli was injected into the oviduct lumen. The results showed that the human oviduct epithelium exhibits endocytic properties towards luminal soluble and particle antigens that are unrelated to MHC-class II expression and menstrual cycle phase (2). Oviducts prohibit the transportation of microorganisms from the vagina to the abdomen, which also provides protection against foreign bodies through immune-system reactivity of antigen-presenting cells.

In addition to the observation of MHC-class II cells in the oviduct epithelium during the menstrual cycle, macrophages were also detected using specific antibodies for human macrophages (PM-1K and PM-2K) (12). When human macrophage immunoreactivity was observed to be +++, well-developed macrophages were also detected (14). It is possible that such macrophages might be involved in the physiological functions of the tubes during the reproductive period. While large numbers of macrophages were present in both ectopic and intrauterine pregnancy tissue, occasional macrophages were identified in the oviduct walls of non-pregnant women. Local immune modulation of maternal cytotoxicity, in response to foreign fetal antigens, may revolve around the production of soluble suppressor factors by macrophages that down-regulate the activities of other immuno-component cells. Oviduct epithelium does not show morphological changes in ectopic pregnancy, nor is there loss of MHC-class I surface antigens, although changes in expression of HLA-D locus products have been described (15).

Bulmer and Earl observed that oviducts of non-pregnant women had a variable number of epithelial cells labeled for HLA-DR (6). The relative proportions of DR-Positive and DR-Negative epithelium showed no obvious relation to the stage of the menstrual cycle. In the early pregnancy stage, the oviduct epithelium showed uniform intense reactivity for HLA-DR. These results

suggest differential regulation of class II- MHC immunoreactivity in tube epithelial cells, possibly mediated by hormones and/or a trophoblast product.

Oviduct and endometrium epithelial cells are capable of different responses to a given stimulus; in intrauterine pregnancy, the oviduct epithelium is uniformly class II- MHC+, whereas endometrial gland epithelium is essentially negative for HLA-D locus antigens (6). Thus, a difference in HLA-DR distribution in the oviduct epithelium during the menstrual cycle and/or early pregnancy stage may be caused by differences in hormonal regulation and/or secreted proteins. However, the role of MHC-class II antigens is still unclear.

Based on our own and previous findings, we can suggest that if immune cells and their products are involved in cyclical differences, there must be a trigger that activates the immune cells. The MHC-class II cells play a central role in regulating the immune response, the effect of which is limited by lymphocytes and antigen-presenting cells such as macrophages and B cells. Their positive immunoreactivity indicates increased immune response in the oviduct. These results show that the oviduct, with the help of its antigen-presenting cells, has the important role of producing an immune response against microorganisms before ovulation, supplying the necessary local immunocompetence for the survival and post-ovulation protection of the embryo, which is regarded as a foreign body before implantation.

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A HISTOPATPOLOGICAL STUDY OF HODGKIN'S LYMPHOMA AND ITS ASSOCIATION WITH EPSTEIN-BARR VIRUS IN TURKISH PATIENTS

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SUMMARY

We evaluated 25 cases of Hodgkin's lymphoma for the presence of Epstein-Barr virus in Reed-Sternberg cells and Hodgkin cells. We also compared the epidemiological features of our patients with previous reports on Hodgkin's lymphoma in industrialized and developing nations. Among our cases, the most common subtype of Hodgkin's lymphoma was mixed cellularity (10 cases, 40%), followed by nodular sclerosis (9 cases, 36%). In 11 of 25 cases (44%), immunohistochemical studies demonstrated that Reed-Sternberg cells were positive for LMP-1 (four out of five in the lymphocyte-rich classic group, three of nine in the nodular sclerosis group and four out of 10 in the mixed cellularity group). The prevalence of Epstein-Barr virus and the high incidence of mixed cellularity in Turkey are similar to findings in developing countries. These findings further support the hypothesis that the prevalence of Epstein-Barr virus in Hodgkin's lymphoma and the epidemiological features of Hodgkin's lymphoma may be linked with socioeconomic conditions and geographic location.

Key Words: *Hodgkin's Lymphoma, EBV, LMP-1*

ÖZET

HODGKİN LENFOMA VE TÜRK HASTA POPÜLASYONUNDA EPSTEİN-BARR VİRÜSÜ İLE İLİŞKİSİNİ ARAŞTIRAN HİSTOPATOLOJİK BİR ÇALIŞMA

Bu çalışmada 25 Hodgkin lenfoma olgusunda Reed-Sternberg hücreleri ve Hodgkin hücrelerinde Epstein-Barr virüsü varlığını araştırdık. Ayrıca kendi hasta popülasyonumuzun epidemiyolojik özelliklerini daha önce gelişmiş ve gelişmekte olan ülkeler için bildirilmiş olan epidemiyolojik özelliklerle karşılaştırdık. Bu olgular arasında, 10 vaka (%40) ile en sık saptanan subtipin mikst sellüler suptip olduğu ve bunu 9 vaka (%36) ile nodüler sklerozan suptipin izlediği tesbit edilmiştir. Beş lenfositten zengin klasik subtipin 4'ünde, 9 nodüler sklerozan subtipin 3'ünde ve 10 mikst sellüler subtipin 4'ünde olacak şekilde toplam yirmibeş olgunun 11'inde (%44) immünhistokimyasal olarak Reed-Sternberg hücrelerinde LMP-1 ekspresyonu saptanmıştır. Türkiye'de Epstein-Barr virüs prevalansı ve mikst sellüler subtipin yüksek insidansı gelişmekte olan ülkeler için daha önce bildirilmiş olan oranlara benzer olarak saptanmıştır. Daha da ötesi, bu bulgular Hodgkin lenfoma olgularında Epstein-Barr virüs prevalansının ve Hodgkin lenfoma epidemiyolojik özelliklerinin sosyoekonomik özellikler ve jeografik lokalizasyonla ilişkili olabileceği hipotezini desteklemektedir.

Anahtar Sözcükler: *Hodgkin Lenfoma, EBV, LMP-1*

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Received: Jan 23,2001 Accepted: March 26,2001

The oncogenic potential of the Epstein-Barr virus (EBV), a lymphotropic herpesvirus, has been widely researched since the virus was first isolated by Epstein et al in 1964 (1). EBV has been detected in infectious mononucleosis (2), nasopharyngeal carcinoma (3) and certain types of non-Hodgkin's lymphomas (4-6). It has been detected in 95% of Endemic Burkitt's Lymphomas, 20% of Sporadic Burkitt's Lymphomas, 40% of lymphomas arising in patients with HIV infection and some subgroups of T-cell lymphomas (7). However, the association between EBV and Hodgkin's lymphoma (HL) remained speculative until Weiss et al showed monoclonal EBV-DNA in some HL tissues and the virus was localised in Reed-Sternberg (R-S) cells (8) using molecular genetic techniques (9). Data obtained from immunohistochemical studies also supports the association between HL and EBV by demonstrating LMP expression in R-S cells and variants (10,11), the neoplastic cell population of HL. LMP-1 is an EBV-gene product expressed from EBV-transformed B lymphocytes. The oncogenic potential of LMP-1 seems to act by preventing differentiation and immortalization of B lymphocytes (12).

HL has a bimodal age distribution in industrialized countries; however, in developing countries, it peaks in young adults (13-15). The association of HD and EBV seems to correlate with certain histological and geographic features. EBV is generally detected in R-S cells of mixed cellularity (MC) or lymphocyte depletion (LD) subtypes, in contrast to its rare detection in the nodular sclerosis (NS) subtype and still more rare detection in remaining subtypes (13). There are also some reports concerning the effects of geographic features on the prevalence of EBV. In developed countries, EBV is detected in 50% of HL cases, in contrast to developing countries, where EBV is detected in large numbers of HL cases together with a high incidence of MC subtype (14,15).

The aim of the present study is to investigate the association of EBV and HL in Turkish patients in terms of EBV prevalence among those subtypes seen in children and adults and also to observe the age distribution of HL subtypes.

MATERIALS AND METHODS

Patient Population

This study included 25 cases of HL diagnosed at the Ankara University Medical School, Department of Pathology between 1989 and 1992.

Histopathologic Studies

Routinely fixed, paraffin embedded, 5(-thick sections were stained with H&E, examined by a pathologist (AE) and subtyped according to WHO classification (16) as follows:

1. Nodular Lymphocyte Predominance (NLP)
2. Lymphocyte-rich classic (LRC)
3. Nodular Sclerosis (Grade I and Grade II) (NS)
4. Mixed Cellularity (MC)
5. Lymphocytic Depletion

Cases were evaluated according to histopathological subtype, sex and age distribution.

Immunohistochemical Studies

5μ-thick sections obtained from paraffin blocks were used to detect LMP-1 expression using the Streptavidin-Biotin Complex-Alkaline Phosphatase technique. Following antigen retrieval by microwave treatment using the monoclonal anti-LMP-1 antibody CS1-4, LMP-1 expression was evaluated (17, 18). The results were scored between (+) and (+++) according to the intensity of staining in R-S cells.

Statistical Analysis:

Patient groups were compared for LMP-1 expression in HL subtypes using the Chi-square test. A p value of less than 0.05 was considered significant.

RESULTS

Clinicopathological Findings:

Patient age ranged from 3.5 to 57 years, with a mean of 24.5 years. There were 17 males (68%) and eight females (32%), for an overall male to

female ratio of 2.1:1. However, the male to female ratio was found to be higher in children (5.5:1; 11 boys and two girls).

Overall, there were 10 cases (40%) of MC subtype, 9 cases (36%) of NS subtype, five cases (20%) of LRC subtype, and one case (4%) of NLP subtype. Thirteen of the cases (52%) were in the pediatric age group (17 years) and 12 of the cases (48%) were in the adult group. In the adult age group, 11 of the patients were between 18-49 years of age and one patient was over 50 years of age. Mean ages according to HL subtypes were as follows: MC: 25.7; NS: 25; LRC: 22.

Immunohistochemical Findings:

LMP-1 expression was detected in 11 (44%) of the cases overall. Immunohistochemical reaction was restricted to R-S cells, and no positivity was detected in the background small lymphocytes. LMP-1 positivity was slightly higher in the pediatric age group (46.1%) than in the adult age group (41.6%), although the difference was not statistically significant. When subtypes were evaluated, the LMP-1 expression rate was found to be 40% in the MC subtype, 30% in the NS subtype and 80% in the LRC subtype ($p>0.05$). Interestingly, vascular elements showed weak positivity in eight of 25 cases (32%), suggesting nonspecific staining.

DISCUSSION

Previous studies have shown significant geographic differences in both the prevalence of EBV infection and the epidemiological properties of HL. In industrialized countries, HL incidence shows bimodal distribution, with one age peak at 15-35 years of age and another at over 50 years of age (19,20). These peaks correlate with the prevalence of the NS subtype among adolescents and young adults and the MC subtype among adults over 50 years of age. However, in developing countries, the incidence of NS subtype is lower compared with MC subtype, which comprises the majority of HD cases (21-23). EBV prevalence in HL seems to correlate with the incidence of MC subtype in the pediatric age group (14).

In our study, the incidence of HL subtypes differed from that of industrialized countries, but was similar to that of developing countries (18). MC was the commonest subtype (40%) among all age groups, and NS and LRC subtypes were less frequently observed. There was no detection of any age-related distribution of subtypes. Overall, there were more patients in the pediatric age group (52%) than in the adult age group (48%). Gender distribution of the cases showed the NS subtype to be slightly higher in female patients (55%), while the MC and LRC subtypes were common among male patients. (80% and 100%, respectively). Our observation regarding children with a higher incidence of HL parallels the age distribution of HL in developing countries (18). Moreover, the distribution by gender of particular subtypes is in line with previously reported rates (24,25).

R-S cells are known to be the most common type of HL cells. When the results of LMP-1 expression were examined, expression was found to be restricted to R-S cells, with no reaction detected in the small lymphocytes in the background. These findings support the role of EBV in oncogenesis. In the present study, weak, non-specific vascular staining was also detected (32%) with LMP-1, which seems to be due to biotin cross-reacting with the vessel wall.

Previous studies have reported a correlation between MC subtype and EBV prevalence. Our study detected a 40% incidence of MC and LMP-1 expression in 11 of 25 cases, a rate of 44%. Overall, the age, gender, subtype and LMP-1 expression rates of HL seem to show similarities with previous reports in the literature (14,18-25). Despite this similarity and the reliability of immunohistochemical techniques in EBV detection, immunohistochemistry should not be used as the sole criteria to establish association. Other EBV-gene products that are also expressed by the EBV-transformed cells should be demonstrated by PCR and in-situ hybridization techniques, and this data should be combined with the immunohistochemical data. Our future goal is to combine immunohistochemistry with PCR and in-situ hybridization techniques in order to obtain more reliable data.

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IRON NEED DURING STIMULATED ERYTHROPOIESIS IN VERY LOW BIRTH WEIGHT PRETERMS: CAN SERUM CONCENTRATION OF TRANSFERRIN RECEPTOR BE USED AS AN ADDITIONAL MEASURE?

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SUMMARY

Serum transferrin receptor (sTfR) levels reflect both iron need and the rate of erythropoiesis. The objective of this study was to investigate iron requirements in simultaneously transfused very low birth weight (VLBW) preterms when erythropoiesis is stimulated by standard doses of human recombinant erytropoietin (rHuEPO). Included in the study were 23 VLBW infants. All of the infants received rHuEPO 200 IU/kg subcutaneously three times a week, starting by the end of the 1st week and continuing until the end of the 7th week. Fourteen of the infants were randomly selected to be supplemented with 3mg/kg/day of iron at the start of rHuEPO therapy (Group I). In the remaining nine infants, iron supplementation began after the end of the 6th week or when serum ferritin concentrations fell below 150 ng/ml (Group II). All of the infants were transfused according to the same transfusion policy. Haematocrit, reticulocyte, ferritin and sTfR levels were evaluated and compared at the beginning, during, and at the end of the study. Baseline parameters were similar in both groups. Haematocrit concentrations remained similar during and at the end of the study. Serum ferritin level was higher in Group I than in Group II during (day 28) and at the end of the study (day 42), but the differences were not significant (422.78(122.98 versus 227(33.47 and 204.5(61 versus 111.2(16.4 mg/dl, respectively). STfR concentrations were higher in Group I than in Group II during the study (Days 14 and 28) but lower at the end (Day 42) of the study, but the differences were again not significant (53.87(22.38 versus 23.17(1.09 on Day 14; 46.3(14.27 versus 24.84(2.66 on Day 28; and 26.1(3.01 versus 34.21(3.36 on Day 42, respectively). Reticulocyte counts in Group I were significantly higher than in Group II at the end of the study (6.1 (0.6 versus 2.9 (0.5; p=0.006).

Serum ferritin levels are high and remain stably high during rHuEPO treatment in VLBW infants, if they are transfused heavily, due to large volumes of phlebotomy during clinical follow-up; thus, the timing and dose of iron supplementation is questionable in this group. Erytropoiesis and iron need is slightly better met when iron supplementation is started simultaneously with rHuEPO. STfR levels are unpredictable, as the preterms were given heavy transfusions.

Key Words: *Anemia of Prematurity, Erythropoietin, Iron, Serum Transferrin Receptor.*

ÖZET

ÇOK DÜŞÜK DOĞUM AĞIRLIKLI PRETEMLERİN UYARILMIŞ ERİTROPOEZİNDE DEMİR GEREKSİNİMİ

Serum transferrin reseptör (sTfR) konsantrasyonu eritropoez ve doku demir gereksinimini göstermektedir. Bu çalışmanın amacı; sıklıkla kan transfüzyonu uygulanan çok düşük doğum ağırlıklı pretermlerde (ÇDDA) standart doz eritropoetin (rHuEPO) tedavisi ile uyarılmış eritropoez sırasında demir gereksiniminin belirlenmesidir. Çalışmaya alınan 23 ÇDDA'lı preterme 200 U/kg/doz, haftada 3 gün, subkütan postnatal 1. haftanın sonundan 7.hafta sonuna dek rHuEPO uygulandı. Grup I'i oluşturan 14 hastaya rHuEPO tedavisi ile eş zamanlı 3 mg/kg/gün dozunda demir verildi. Grup II'i oluşturan 9 hastaya ise serum ferritin düzeyleri 150 ng/ml'nin altına düştüğünde veya tedavi sonunda demir başlandı. Hastalara transfüzyon aynı kriterler göre uygulandı. Tedavi başlangıcı, tedavi sırasında ve sonunda hematokrit, retikülosit, ferritin, ve sTfR düzeyleri değerlendirildi ve gruplar karşılaştırıldı. Uygulanan benzer transfüzyon politikasına bağlı olarak her iki grupta hematokrit düzeyleri tedavi sırasında ve sonunda farklı değildi. Serum ferritin düzeyleri tedavi sırasında (28.gün) Grup I'de 441.78 (122.98 mg/ml Grup II'de 227(33.47 mg/ml, tedavi sonunda (42.gün) Grup I'de 204.5(61 Grup II'de 111.2(16.4 mg/ml bulundu. Grupların ferritin düzeyleri arasındaki fark anlamlı değildi. STfR düzeyleri Grup I'de 14.gün, 28.gün ve tedavi sonunda sırasıyla 53.87(22.8, 46.3 (14.27, 26.1(3.01 nmol/L; GrupII'de ise 14.gün, 28.gün ve tedavi sonunda sırasıyla 23.17(1.09, 24.84(2.66, 34.21(3.36 nmol/L idi. Gruplarda günlere göre sTfR düzeyleri arasındaki fark anlamlı değildi. Tedavi sonu retikülosit değerleri Grup I'de 6.1.(0.6 iken Grup II'de 2.9.(0.5 bulundu. Bu fark istatistiksel olarak anlamlı idi (p=0.0006). ÇDDA'lı pretermlerde aneminin eritropoetin ile tedavisi sırasında flebotomi kayıplarının yerine koymak ve klinik takip için uygulanan transfüzyonlara bağlı olarak serum ferritin düzeyleri yüksektir. Bu hastalarda demir suplemantasyonunun dozu ve zamanlaması tartışmalıdır. Eritropoetin tedavisi ile eş zamanlı demir başlanan Grup I'de demir gereksiniminin biraz daha iyi karşılandığı ve ferritin düzeylerinin yüksek bulunduğu erken dönemde sTfR'nin yükselmesinin uyarılmış eritropoezde artmış demir gereksinimine bağlı olabileceği düşünülse de yapılan çoklu transfüzyonlar nedeniyle sTfR düzeylerinin değerlendirilmesi güçtür.

Anahtar Kelimeler: *Demir, Eritropoetin, Preterm Anemisi, Serum Transferrin Resptörü*

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Received: Feb 19,2001 Accepted: May 24,2001

Abbreviations: sTfR: serum transferrin receptor

rHuEPO: recombinant human erytropoietin

VLBW: Very Low Birth Weight

AOP: Anemia of Prematurity

AGA: Appropriate for Gestational

Erythropoiesis in premature babies is limited by a relatively inadequate production of erytropoietin. This is partly due to dependence on the hepatic production of erytropoietin and an incomplete switchover to renal production. This model of neonatal erytropoiesis suggests that the use of exogenous erythropoietin should correct the early anemia developed as a result of frequent blood sampling and prevent the late anemia of prematurity (1,2). Investigations into the safety and efficacy of recombinant human erythropoietin treatment in anemia of prematurity have demonstrated a rise in haematocrit and reticulocyte counts, fewer blood transfusions, reduced transfused volume of blood per kilo of body weight and a decrease in bioavailable iron. RHuEPO treatment is more efficient when premature infants are older and in stable condition (3). Severity of illness and iron consumption represent the major limiting factors of the stimulated erytropoiesis of VLBW prematures (4).

STfR concentration is a recent sensitive measure of iron status, identifying iron deficient erythropoiesis. The serum levels increase soon after signs of iron deficiency appear, and this reflects the depletion of available tissue iron(4). Unlike serum ferritin concentrations sTfR concentrations are not influenced by infections and chronic inflammation. Not much information is available on the role of sTfR revealing iron depletion in stimulated erytropoiesis(5).

Timing of iron supplementation may theoretically create a problem in cases, where multiple transfusions cannot be withheld due to large volumes of phlebotomy during rHuEPO treatment. Packed red cells supply 0.75 mg/ml iron in each transfusion, whereas blood collected during phlebotomies contains less than half of this value per milliliter. As a result, serum ferritin levels are usually above normal in heavily transfused patients. The question is whether these levels suffice to meet the need of iron in stimulated erythropoiesis with rHuEPO in VLBW preterms.

This study was undertaken to assess the roles of sTfR and ferritin concentrations in the evaluation of iron need in transfused VLBW prematures when erythropoiesis is stimulated by standard doses of rHuEPO.

MATERIALS AND METHODS

Twenty-three VLBW infants were administered rHuEPO 200 IU/kg subcutaneously thrice a week, starting by the end of the first week until the end of the seventh week. All of the infants were AGA (birth weights within ±2 SD for gestational age). They received erythrocyte transfusions with a haematocrit of less than 0.30 and when sign and symptoms attributed to anemia - including persistent tachicardia, frequent apnea with bradicardia and weight gain less than 10g/kg/day, despite an optimal caloric and protein intake (120 kcal/kg/day and 3.5g/kg/day) - were present with a haemotocrit of less than 0.35. Fourteen of the infants were randomised to be supplemented with oral iron (ferroglycine sulphate) doses of 3mg/kg/day simultaneously with the start of rHuEPO treatment (Group I), while nine of them were started on oral iron supplementation after the end of the sixth week, when their serum concentration of ferritin fell below 150 ng/ml (Group II).

Blood samples were collected weekly from the first to sixth weeks of age for haemoglobin, haemotocrit, reticulocyte, white blood cell, ferritin, and sTfR measurements. The serum used for measuring sTfR concentrations were stored at -20°C until assayed.

Haemotocrit counts were measured with an automatic counter. Reticulocyte and granulocyte counts were determined via peripheral blood smear. Serum concentrations of ferritin were measured with RIA using commercial reagents (Ferritin Ria Kit, Kodak Clinical Diagnostics, UK)

ELISA method (Quantikine TM, IVD TM sTfR ELISA, R&D System Inc. Minneapolis MN 55413,

USA) was used to measure the serum concentration of TfR.

Mann-Whitney U test was used for statistical analysis. A p value of <0.05 was considered significant. (Mean (SEM values are used unless otherwise noted.)

RESULTS

There were no significant differences between the groups with regard to birth weight (1258.71 ± 16.05 versus 1244.44 ± 52.70 g), gestational age (30 ± 0.45 versus 30.55± 0.60 weeks), total phlebotomy (76.85 ±11.37 versus 54 ±6.82 ml) and transfusion volumes (48.14 ±13.18 versus 33.22 ±7.8 ml) (Table 1).

Haemotocrit, reticulocyte, ferritin and TfR were evaluated and compared between the groups at the beginning, during and at the end of the study (Tables 2 and 3).

All of the baseline parameters were similar in both groups. Haematocrit concentrations remained similar during and at the end of the study. In both groups, reticulocyte counts decreased following the first week of life and remained stably low until the end of erytropoietin therapy. By the completion of the study, the reticulocyte count in Group I was significantly higher than in Group II (6.1 ± 0.6 versus 2.9 ± 0.5; p=0.006) (Table 2).

Ferritin levels remained high in both groups through the first three weeks of erythropoietin therapy, probably due to heavy transfusions. However, from the end of the fourth week until the completion of the study, a decrease in ferritin levels was observed, and the serum ferritin level

Table 1: Clinical charachteristics of the infants*

	Group I	Group II
Number of the infants	14	9
Girls : Boys	7:7	4:5
Birth weight (g)	1258.71±16.05	1244.44±52.70
Gestational age (week)	30±0.45	30.55±0.60
Total phlebotomy (ml/kg)	76.85±11.37	54±6.82
Total transfusion (ml/kg)	48.14±13.18	33.22±7.8

*Mean ± SEM

Table 2: Comparison of haematocrit and reticulocyte counts at the beginning, during and at the end of the therapy

Age	Haematocrit (%)		Reticulocyte (%)	
(day)	Group 1	Group 2	Group 1	Group 2
0	48.70±1.22	49.28±1.94	5.35±1.36	3.65±0.44
7	37.58±1.24	40.25±1.75	2.22±0.40	1.86±0.45
14	34.95±1.47	36.61±1.33	2.25±0.25	3.21±0.61
28	34.03±1.47	34.48±1.29	2.80±0.28	4.32±0.97
42	30.55±0.72	34.65±2.37	6.07±0.64*	2.94±0.59*

*p<0.05

Table 3: Comparison of ferritin and sTfR levels at the beginning, during and at the end of the therapy

Age (day)	Ferritin (ng/ml)		sTfR (nmol/L)	
	Group I	Group II	Group I	Group II
7	339.28±31.91	305±11.45	26.17±2.47	20.91±1.21
14	380.21±92.07	304.55±33.77	53.87±22.38	23.17±1.09
28	422.78±122.98	227±33.47	46.30±14.27	24.84±2.66
42	204.46±61.3	111.22±16.4	26.10±3.01	34.21±3.36

was higher in Group I compared to Group II both during and at the end of the study (422.78±122.98 versus 227±33.47 and 204.46 ± 61.30 versus 111.22 ± 16.40 mg/dl, respectively). (Table 3 and Figure 1)

The concentrations of sTfR were higher in Group I compared to Group II during the study period (53.87 ± 22.38 versus 23.17±1.09 on Day 14 and 46.3±14.27 versus 24.84±2.66 nmol/L on Day 28, respectively). Serum TfR concentrations were lower in Group I compared to Group II at the end of the study (26.10 ±3.01 versus 34.21 ± 3.36 nmol/L, respectively (Table 3 and Figure 2). None of the differences was significant.

DISCUSSION

VLBW infants are likely to receive multiple blood transfusions in order to replace blood drawn during their medical course or to treat clinical symptoms attributed to AOP (1,2). Concerns over the large amounts of blood given to the average preterm infant lead to a search for an alternative therapy. Although rHuEPO levels are relatively low in infants with AOP compared to the degree of their anemia, it has been shown that there are sufficient erythroid precursor cells in the bone marrow to respond to rHuEPO stimulation (2,6). Several studies show that rHuEPO in doses of 300-1200 u/kg/week, with iron supplementation of 2-4mg/kg/day, induces erythropoiesis, resulting in an increase in the haematocrit and reticulocyte count and a reduction in the number of blood transfusions (7,3,8). In this study haematocrit concentrations remained similar in Groups I and II at the beginning, during and at the end of treatment because of the same trans-

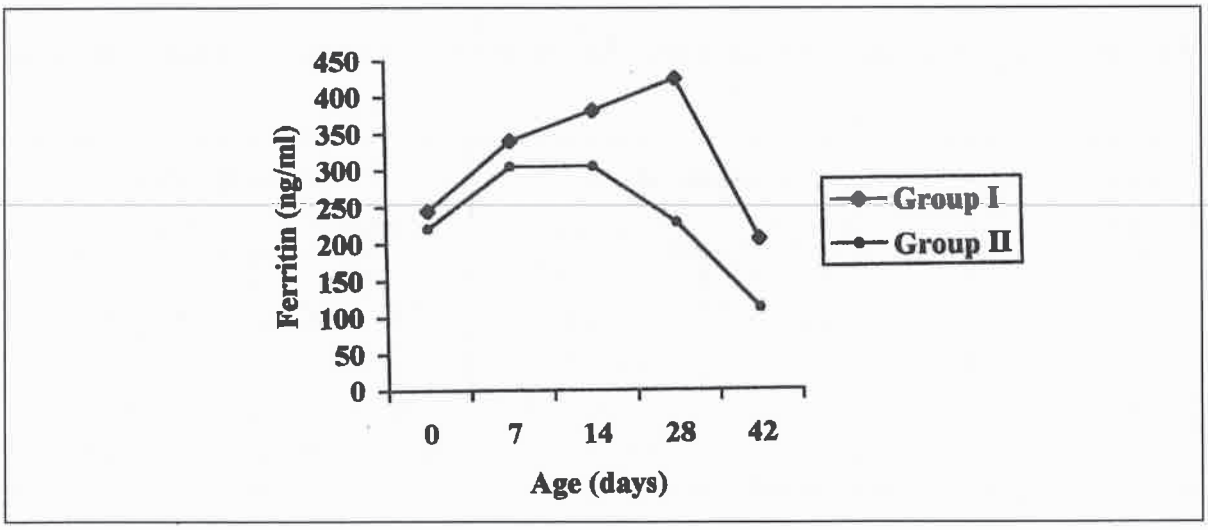

Figure 1: The concentrations of ferritin in group I and II

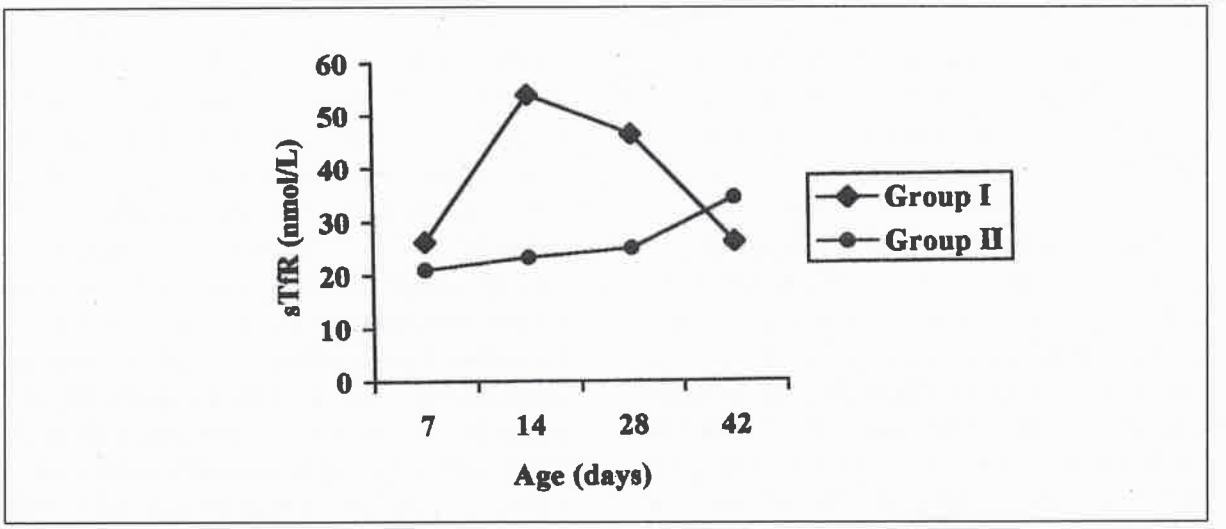

Figure 2: The concentrations of sTfR in group I and II

fusion policy. However, the significantly higher reticulocyte count in Group I compared to Group II (6.1 ± 0.6 versus 2.9 ± 0.5, p=0.006) at the end of the study may indicate a later but better-stimulated erytropoiesis in the simultaneously iron-supplemented group.

Severity of illness and iron consumption represent the major limiting factors of stimulated erytropoiesis (9,10,11). Preterm infants have low iron stores due to their short gestation and low iron intake during their prolonged medical course. Functional iron deficiency has been shown to develop in infants during rHuEPO therapy, placing limits on its efficacy. In order to prevent iron depletion during rHuEPO therapy, Carnielli et al, were among the first to supplement preterm infants with 20mg/kg/week of iron early in the course of rHuEPO therapy without complications (12). The European Multicenter Erytropoietin Study Group concluded that 2mg/kg/day caused depletion of iron stores, as reflected by decreased serum ferritin levels (13). Bader et al showed that iron at a dose of 6mg/kg/day in conjunction with rHuEPO 900 u/kg/week is effective in inducing erythropoiesis in stable growing preterms (2,13). Bechansteen et al reported an improved response to rHuEPO therapy by using even higher doses of iron (18-36mg/kg/day) with no apparent side effects. They also found that even with moderate doses of erythropoietin, higher doses of iron and protein intake improved the erythropoietic response to rHuEPo treatment (14,15). The decrease in serum ferritin levels and increase in sTfR levels in transfused preterms during the course of rHuEPO therapy raises the possibility that increased iron supplementation could further enhance the efficacy of rHuEPO(13,14,15). In our study, as a result of heavy transfusion, serum ferritin levels remained stably high in both of the groups until the end of the study. Although the iron stores were not depleted, functional iron deficiency may limit the erytropoiesis. The early rise of sTfR in Group I, when serum ferritin levels are relatively high, may be due to better-stimulated erytropoiesis with the simultaneous start of oral iron.

It should be taken into consideration that the amount of iron needed will depend on the volumes of blood removed and transfused (4). At collection, blood contains 3.4mg of iron per gram of haemoglobin (at a haemoglobin concentration of 10g%, this equals 0.34 mg /ml) whereas each milliliter of packed cells supplies 0.75mg iron (10). Most of our patients received transfusions during the study period, receiving extra iron simultaneously with the iron lost due to phle-

botomy. Thus, iron status of this group of infants were exposed to considerable changes. On the other hand, the use of rHuEPO therapy in more seriously ill preterm infants in the first weeks of life before enteral feedings are established makes oral iron supplementation even more difficult.

Functional iron deficiency during rHuEPO treatment has been frequently reported (16,5,17). It has been seen in the presence of increased as well as normal concentrations of stored iron. This may be because of the diminished rate of supply of available iron for the expansion of stimulated erythroid mass. This state in the human corresponds to what is seen in neonatal rabbit, in which parenteral iron increases red cell production to such an extent that the animals experience no early anemia. This effect of parenteral iron occurs even though the animals have abundant storage iron (14).

In our study, when rHuEPO therapy was initiated in unstable VLCBW infants at about seven days of age, despite the stimulation of erythropoiesis, the number of transfusions could not be reduced. Serum ferritin levels are high and remain stably high during rHuEPo treatment, due to heavy transfusions. Thus, the timing of iron supplementation in this group is questionable. Although serum ferritin levels of both groups were similar at the beginning of the study, they were lower in Group II compared to Group I during and at completion of the study. Due to the differences in iron intake among the groups, there was a notable decrease in serum ferritin level in Group II; however, the difference was not significant. Iatrogenic iron deficiency developed in Group II, raising the possibility that increasing iron supplementation irregardless of high serum ferritin levels could further enhance erythropoiesis. Erythropoiesis and iron need seem to be better met when iron supplementation is started simultaneously with rHuEPO.

Serum levels of TfR may reflect both iron status and rate of erytropoiesis. An early increase in TfR of more than 20% in adults has been regarded as an early indicator of successful rHuEPO treatment (15). High doses of rHuEPO in preterm infants also significantly induces elevated TfR levels. In our study, the early increase (Days14 and 28) in TfR in Group I, at a time when ferritin levels indicated no iron deficiency, may reflect stimulated erytropoiesis in the presence of orally supplemented iron. Similarly, the lack of oral supplementation in Group II may explain why this group showed no increase in TfR during the treatment. When iron supplementation was begun in Group II at the end of treatment, a slight increment of sTfR levels was also observed in this group.

The elevated sTfR levels may also be explained by an increase in the erythroid precursor cell mass. This relationship of transferrin receptor number to erythropoiesis exists only when there is sufficient iron-bearing transferrin to saturate receptors. When an iron deficiency exists, both erythroid and non-erythroid receptors increase, distorting the relationship between receptor number and erytropoiesis (16,5,18,19).

In conclusion, we suggest that early rHuEPO therapy given at a dose of 600 U/kg/week in conjunction with 3mg/kg/day of iron supplementation may stimulate erythropoiesis better in sick VLBW infants. In that unstable population, iatrogenic blood loss contributed to greater transfusion and a lower level of erytropoiesis. Based on this and other studies, if VLBW infants are at risk of greater phlebotomy losses, the use of vigorous iron supplementation is promising.

In stimulated erytropoiesis of VLBW infants, the rise in sTfR levels with a small decline in serum ferritin concentrations may explain a potential functional iron deficiency. Heavy transfusions lead to unpredictable sTfR levels, which should be further investigated.

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ANALYSIS OF FOUR-HOUR GROWTH HORMONE PROFILES AFTER ONSET OF SLEEP IN NORMAL AND GH-DEFICIENT CHILDREN: A STANDARDIZATION OF THIS SIMPLE TEST FOR CLINICAL USE

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Sema Akçurin** ✣ Nihal Memişoğlu***

SUMMARY

The study comprised three groups of 131 prepubertal children. Group A included 55 healthy children and Group B included 66 children with GHD. Group C included 10 children with growth retardation but normal GH response to pharmacological tests with flat GH-sleep testing compared with those of Group A.

All children except Group A underwent two pharmacological stimulation test for selection of patients. Spontaneous GH secretion was estimated by taking integrated 30 minutes blood samples for the first 4 - hour period of nocturnal sleep. The mean 4 - hour integrated GH concentration (ICGH), number of peaks (>5ng/ml) and the peak amplitude were calculated on 1048 occasions of sampling. The sensitivity of sleep test was defined by the percentage of true positive results.

The mean ICGH, peak number and peak amplitude in the control group were 5.77+1.22, 3.98 + 1.37 and 15.91 + 2.2 ng/ml respectively. The mean ICGH was 2.14+0.26 ng/ml in Group B and 3.12+0.6 ng/ml for Group C. There was concordance between GH response to pharmacological stimulation and short time sleep testing in 58 of 66 GHD patients. Thus the sensitivity of the test is 87.7 %.

In conclusion short time sleep test is safe, reliable and practical for clinical use. Four-hour sleep study should be especially indicated when GH responses to provocative testing are inconsistent with observed growth pattern. Long term sleep testing can be considered as a research tool rather than clinical one.

Key Words: *Neorosecretuary Dysfunction; Short Time Sleep Test.*

ÖZET

Derin Uykunun İlk 4 saatinde Büyüme Hormonu Profilinin Normal Çocuklarda ve Büyüme Hormonu Eksik olan Hastalarda Karşılaştırmalı Olarak Değerlendirilmesi: Uygulanımı Kolay ve Pratik Olan Bu Testin Klinik Kullanım İçin Standardizasyonu

Bu çalişmada normal çocuklarda derin uykunun ilk 4 saatinde büyüme hormonu (BH) profilinin değerlendirilmesi ve bu verilerin BH eksikliği olan hastalar ve nörosekretuvar disfonksiyonu olan hasta grubu ile karşılaştırılarak testin standardizasyonunu yapmak amaçlandı.

Çalışmada toplam 131 prepubertal olgu 3 grup halinde değerlendirildi. Grup A'da 55 sağlıklı çocuk, Grup B'de BH eksikliği tanısı alan 55 olgu ve Grup C'de boy kısalığı olan ancak farmakolojik uyarı testlerine yeterli yanıt veren 10 olgu değerlendirildi. Grup A dışındaki tüm olguların en az iki farmakolojik uyarı testiyle BH yanıtları değerlendirildi. Uyku profili sırasında 30 dakikalık aralarla 4 saat boyunca örnekler alındı. Ortalama 4 saatlik BH yoğunluğu (ICGH) pik sayısı (>5ng/ml) ve pik amplitüd toplanılan 1048 örnek üzerinden hesaplandı. Testin sensitivitesi Grup B'nin sonuçları ile karşılaştırılarak gerçek pozitif sonuçların yüzdesi ile hesaplandı.

Kontrol grubunu oluşturan Grup A'da sırası ile ICGH, pik sayısı ve pik amplitüdü 5.77(1,22, 3.98(1,37 ve 15.91(2,2ng/ml olarak bulundu. Grup B ve C'de elde edilen sonuçlar daha düşüktü. Ortalama ICGH Grup B'de 2,14(0,26 iken Grup C'de 3.12(0.6ng/ml idi.BH eksikliği olan 66 olgudan 58'nin uyku profili sonuçları farmakolojik uyarı testi sonuçlarıyla uyumluydu (sensitivite %87.7).

Kısa süreli üyku testi güvenilir, uygulanımı kolay pratik bir testtir. Büyüme yanıtları ile farmakolojik uyarı testi yanıtları uyumsuz olan olgularda 4 saatlik uyku profili özellikle denetlenmelidir. Uzun süreli uyku testleri kullanım zorlukları açısından günlük klinik uygulamadan çok araştırmalarda tercih edilmelidir.

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Received: Feb 19,2001 Accepted: June 19,2001

The accurate diagnosis of growth hormone deficiency (GHD) is essential before a patient is committed to long-term growth hormone (GH) therapy. Since the use of solely auxological criteria to GHD is unreliable, laboratory assessment of GH secretion induced by pharmacological and physiological stimuli has facilitated the diagnosis of GHD. Physiological impairment of GH secretion may well be more important than pharmacological deficiency (1,2). Investigations performed in the assessment of GH secretion in short children should be safe, reliable and easy to be carried out. Most of the investigators agree that the sampling for 12-24 hours is not practical and routine for evaluation of short stature (3,4,5,6). Night time profiles of GH secretion have been evaluated as investigative tools for the assessment of endogenous secretion (1-22). There is no any agreement for standardization of method for evaluation of GH sleep profiles (duration of sleep, with or without EEG monitoring, sampling intervals and cut off points) (1-24, 25, 26). In this study, GH secretory capacity of prepubertal healthy Turkish children with normal statural growth was investigated in the first 4 hours of nocturnal sleep in order to standardize this diagnostic procedure, and results were compared with those of GH-deficient children in the same age group.

SUBJECTS AND METHODS

Study Groups: The study comprised three groups of 131 prepubertal children.

Group A: A total of 55 healthy children (24 girls, 31 boys) with no statural retardation aged 8.60 ± 2.10 years. Their bone ages were 8.90 ± 1.00 years.

Group B (Idiopathic GH deficient group): A total of 66 (19 girls, 47 boys) growth retarded euthyroid children (height below -2.5 SD for chronological age) with subnormal height velocity (HV below 25th percentile for age); delayed bone age (above 2 years) and subnormal GH (below 7 ng/ml for complete GHD (n:58); 7-10 ng/ml for partial GHD (n:8)(response to two pharmacological tests. Organic brain disorders were excluded by the MRI and/or CT scan of the central nervous system.

Group C: Ten children (3 girls, 7 boys) with growth retardation just like those of Group B but normal GH (above 10 ng/ml) response to two pharmacological tests. Selection of this group was made after the results of sleep testing of group B were obtained (ICGH < 3.33ng/ml and/or peak amplitude < 11.5). Twenty seven patients with concordant response to pharmacological and sleep testing (idiopathic short stature or Kowarski syndrome) were excluded. Informed consent were obtained from all of the patient's parents for this study.

All children of the three groups were well nourished, euthyroid (with total and free thyroxine, basal TSH and TRH stimulated TSH levels) and had no hepatic, renal, cardiopulmonary, and gastrointestinal problems. None had skeletal dysplasia or dysmorphic syndromes. The height of each child was expressed as the SD score, i.e. height in relation to the sex and age matched Turkish standards (27). Bone age was estimated according to the method of Greulich and Pyle (28).

All the children except Group A (control group) underwent provocative tests for GH secretion with at least two pharmacological stimuli [insulin induced hypoglycemia (IIH 0,1 U/kg) and L-dopa (20 mg/kg)]. Complete GHD was diagnosed if the peak GH concentration was below 7ng/ml; partial deficiency if the peak concentration was 7-10 ng/ml and normal if the peak concentration above 10 ng/ml (24).

Spontaneous GH secretion was estimated by taking integrated 30-minutes blood samples for the first 4-hour of period nocturnal sleep without EEG monitoring. All of the subjects were evaluated for sleep-induced GH secretion on 1048 occasions of sampling. Children stayed at the hospital at least one night. They went to sleep at their own choxn times. Samples were drawn from an indwelling venous catheter, which was inserted prior to the onset of sleep. Nursing personnel

recorded each subject's status as awake or as sleep. Serum was separated within one hour of blood collection and stored at 40 C until being sent to laboratory the following morning. Plasma GH concentrations were measured by double antibody RIA, using DPC kits (1 ng/ml = 2mU/L). Intra- and interassay coefficients of variations were 5 and 7 %, respectively. The sensitivity of the GH assay varried between 0.5-1ng/ml.

Pulse Analyses: The mean 4-hour integrated GH concentration, number of peaks (GH > 5ng/ml) and the peak amplitude (highest nocturnal peak) were calculated for the first 4 hours of nocturnal sleep on 1048 occasions of sampling. Values of below 1 ng/ml were assigned as 1ng/ml (3).

Statistics: Results were expressed as mean + SD. Lower and upper normal limits are defined as + 2SD from mean. Compression of the mean concentration of GH, peak amplitude and peak frequency of groups were made using Student's "t" tests.

The sensitivity of sleep test was defined by the percentage of true positive result obtained when test was applied to patients known to have GHD confirmed by IIH and L-dopa testing in group B.

RESULTS

Evaluation of the first 4-hours GH profiles in the control group (Group A): The mean concentration of GH (ICGH), GH peak amplitude and peak number were 5.77 + 1.22 ng/ml, 15.91 + 2.20 ng/ml, and 3.89 + 1.37, respectively (Table 1). The lower normal limits of GH secretory profiles were 3.27 ng/ml for the GH concentration, 11.50 ng/ml for the peak amplitude, 1.15 for the peak number. The upper normal limits were 8.26 ng/ml for the GH concentration, 20.30 ng/ml for the peak amplitude, 6.63 for the peak number among 8 measurement. None in the Group A had the mean sleep GH concentration below 3 ng/ml . While only two subjects, (3.63 % of subjects) had the mean GH concentration above 8 ng/ml, most (96.37 % of subjects) had values between 3 and 8 ng/ml.

Evaluation of GH reserve in Group B: On baseline data the mean peak GH values obtained after L-dopa and IIH were 4.46 +1.50 ng/ml and 5.70 +0.85 ng/ml, respectively, in selection of GHD children. Pulsatile GH in this group was blunted and found to be significantly different from that of the control group as expected. The mean GH concentration was 2.14 +0.60 ng/ml, and significantly different from that of control group ($p<0.01$). Maximum value of integrated concentration of GH of Group B was below the lower limit of the control groups. This characteristic was also valid for other parameters of pulsatile GH secretion such as peak amplitude (6.16 +1.10 ng/ml) and peak number above 5 ng/ml (1.00 + 0.26). All of the 66 GH-deficient patients except 8 had concordant results from pharmacological tests and nocturnal sampling. So that short time sleeping test is diagnostic for GHD just as pharmacological testing in this group. The sensitivity of sleeping testing was 87.7 %.

Growth hormone reserve in Group C: The peak values of L-dopa and IIH tests were always >10 ng/ml, 14.02 +3.30 ng/ml for the first test and 12.72 +4.40 ng/ml for the second test, in selection of this group patients. The mean GH concentration, GH peak amplitude and peak

Table 1: The clinical characteristics and GH-reserve of groups.

Group A: Control

Group B: Growth retarded patients with blunted GH response to pharmacological stimuli

Group C: Growth retarded patients with normal GH response to pharmacological stimuli

ICGH * : Integrated concentration of GH

( )** : Lower normal limit - Upper normal limit

number were low: at 3.12 +0.63 ng/ml/4hr, 6.60 +1.23 ng/ml and 2.14 +1.06 respectively, similar to those of GH deficient children. This group of patients could be diagnosed as GH neurosecretory dysfunction (GH-NSD) by short time GH sleep testing.

DISCUSSION

In this study a practical method of sampling and analysis of 4 hour nocturnal GH profiles in prepubertal children was reevaluated for standardization of the method. Analysis of test performance was confined to the 55 healthy prepubertal children without growth retardation, in comparison with 76 prepubertal patients with short stature (66 GH deficiency, 10 growth retardation with normal GH response to pharmacological stimuli). Normal GH response to pharmacological stimuli may not always prove that spontaneous GH secretion is adequate (1,2,12). Children with GH-NSD may bear a clinical resemblance to subject with GHD but their response to provocative tests are normal. However, spontaneous GH secretory profiles in these children are abnormally low (2).

GH profiles have been widely studied in the last years and in this paper only few reports have been quoted among the references (1-23, 25, 29, 30,31). There is no any agreement for standardization of method for evaluation of pulsatile GH secretion. Some investigators have advocated the frequent sampling of GH during 24 or 12 hours period, but the clinical utility of this approach is controversial (2,3,20,25). The test with EEG recording of the deep sleep in children has been reported to be clinically useful (9,10,11,16,17). These studies showed that GH peaks occur with the onset and recurrence of slow wave stages of sleep (16). Previously clinical tests with sleep have concerned either single sampling after onset of sleep as screening procedure or greater sophistication than practicable in routine work such as EEG monitoring use of sleep laboratories (7,9,10,11,13,14,16,17). Frasier regarded sleep testing as a screening procedure in his review of GH testing in children, but he cited authors who had taken on one or two samples 60-90 minutes after sleep (31). King and Price sampled every 15 minutes from 30 to 120 minutes after clinical sleep onset, without EEG monitoring (14). They found low false-negative rate for the sleep test (5%) compared with arginine infusion (29%). Ward and Savage compared the peak serum GH concentration during first 5 hours of sleep with EEG monitoring with the serum GH response to insulin-induced hypoglycemia and arginine stimulation in 23 short children (16). Bierich described abnormalities in number of clinical condition associated with short stature by measuring serum GH concentration every 30 minutes during the first 5 1/2 hours of deep sleep (18). Most investigators agree that sampling for 12-24 hour is not practical and adequate for routine evaluation of short stature (6). As shown there is no any consensus about duration of short time sleep testing for evaluation of pulsatile GH secretion.

Also little information exist about the universal cutoff points of the mean GH concentration in physiological secretion for diagnosis of GHD and especially GH-NSD. Are the reported cutoff values for 24 hours or 12 hours of pulsation tests valid for short time sleep testing? Hypopituitary range of 24-hour integrated GH concentration was given as below 3.20 ng/ml (2,12).Costin et al assessed 24-hour GH secretion and GH response to provocative tests in 50 short statured children (none of them had complete GHD). They thought that those who failed to achieve a stimulated or sleep-induced GH peak of over 15 ng/ml and also had a 24 hour GH concentration less than 3 ng/ml may have some dysregulation in GH secretion (3). Lanes et al reported that normal values of GH during 9 hour sleep are 13 ng/ml for peak amplitude, 4.40 ng/ml for ICGH (5). Mori diagnosed GH-NSD in 6 short children with a mean GH concentration less than 5 ng/ml (22). Ward and Savage found that in those children who had normal GH reserve, the mean peak amplitude of serum GH during 5 hour sleep was 33.58 mU/ml (16.79 ng/ml). It has been reported that GH concentration during the first 3 hours of nocturnal sleep can be used instead of the 24 hour concen-

tration to assess spontaneous GH secretion (22,23). A cutoff point of 4 ng/ml for mean sleep GH was calculated from the correlation between mean GH values during 3 hours of nocturnal sleep and the mean value over 24 hours (The Japanese Criteria by the Foundation for Growth Science)(22,23). In this standardization, the children with idiopathic short stature were used as control group.

In our study control group for standardization of 4 hour sleep test include healthy, euthyroid, prepubertal Turkish children without growth retardation and bone age delay. The results of the control group were compared with those of GHD patients diagnosed by provocative testing.

Rose et al. questioned the diagnostic usefulness of spontaneous GH testing because they observed an overlap in mean 24 hr plasma concentrations of normal, short normal and GH-deficient children (20). In our study 66 GH-deficient patients (diagnosed with pharmacological tests) had very low GH levels compare to our results of the control group in sleep testing. In this group pharmacological stimulation tests and short time sleep test showed significant concordant results and the sensitivity was 87.7 %. There was a significant discordance between the results of pharmacological tests and nocturnal sampling in the group C. The diagnosis of growth hormone neurosecretory dysfunction could be obtained by short time sleep testing in this group.

In conclusion, short time sleep test is safe, reliable and practical for clinical use (sensitivity 87.7 %). Four- hour sleep study should be especially indicated when GH responses to provocative testing are inconsistent with observed growth pattern. On the other hand long term sleep testing can be considered as a research tool rather than clinical one.

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JOURNAL OF ANKARA MEDICAL SCHOOL Vol 23, No 3, 2001 201-204

FIXED R-R INTERVALS IN BRAIN DEATH

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SUMMARY

Diagnosis of brain death is one of the most challenging problems in medical practice. Reliable tests must be used in the assessment of brain death because the diagnosis must be 100 percent correct.

The variation of R-R intervals in brain death has not been evaluated systematically. In this study, we attempted to find a correlation between the diagnosis of brain death and the variation of R-R intervals. We observed that R-R intervals in our intensive care unit patients were becoming stationary by the time of brain death diagnosis. In our study, electrocardiogram and Holter recordings were taken for six patients (age: 16-66 years) with the clinical criteria of brain death. No change in heart rate was observed during carotid massage and artificial ventilation. We suggest that this finding, which we have termed "fixation of R-R intervals", might be useful in evaluating brainstem damage and in diagnosing brain death.

Key Words: *Brain Death, R-R Intervals*

ÖZET

R-R İNTERVALLERİ BEYİN ÖLÜMÜNDE SABİTLEŞİR

Beyin ölümü tanısı tıbbi pratikte en karmaşık problemlerden birisidir. Tanının %100 doğru olması gerektiği için beyin ölümünün değerlendirilmesinde güvenilir testlerin kullanılması gereklidir.

Beyin ölümünde R-R intervallerinin değişkenliği henüz sistematik olarak incelenmemiş bulunuyor. Bu çalışmada beyin ölümü tanısı ile R-R intervallerinin değişkenliği arasında korelasyon olup olmadığını incelemeyi planladık. Yoğun bakım ünitemizde beyin ölümü tanısı konduğunda R-R intervallerinin sabitleştiğini gözlemledik. Beyin ölümü klinik kriterlerine sahip 6 beyin zedelenmesi olan hastanın (yaşları 16 ile 66 arasında) elektrokardiogram ve Holter kayıtları alındı. Karotis masajı ve mekanik solunum sırasında kalp hızında değişiklik olmadığını gözlemledik. Bu durumu "R-R intervallerinin sabitleşmesi" olarak adlandırdık. Bu bulgunun beyinsapı hasarının değerlendirilmesi ve beyin ölümü tanısında yararlı olduğunu düşünüyoruz.

Anahtar Kelimeler: *Beyin Ölümü, R-R İntervalleri*

The main findings in brain death are coma, absence of brainstem reflexes and the demonstration of apnea. Persistence of these clinical signs determines the diagnosis of brain death. Clinical examination for the diagnosis of brain death includes investigation of cerebral hemisphere functions and brainstem reflexes as well as laboratory tests.

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Received: April 25,2001 Accepted: May 30,2001

The clinical diagnosis of brain death is not generally a difficult problem; however, it carries a great responsibility, because it must be 100 percent correct. Several committees and reviewers have proposed clinical criteria for brain death (1,2,3). Most brain death codes allow for the use of electroencephalography (EEG), which must demonstrate electrocortical silence over a certain period. Cerebral evoked potentials (EP) can also be used to demonstrate the successive loss of activity of various pathways, and other neurophysiological tests demonstrating the loss of cerebral perfusion can also be implemented. Brain scintigraphy can confirm the loss of isotope uptake into the brain, and Doppler sonography can be used to demonstrate cessation of brain perfusion. In essence, brain death is diagnosed when there is no discernible evidence of either cerebral hemisphere or brainstem function for an extended period of time, usually 12 hours or more, and when the loss of brain functions is the result of a structural brain disease and not of a reversible metabolic disease or of depression caused by drugs.

We observed that the R-R intervals of patients in our intensive care unit (ICU) were becoming fixed along with the diagnosis of brain death. As variation of R-R intervals have not been evaluated systematically in brain death, we aimed to focus on this issue using our patient data.

METHOD

Six cases of brain death (ages: 16-66 years) with fixed R-R intervals were observed in our ICU. Brain death was diagnosed according to the existing criteria, based on clinical examination results supported by EEG, EP and cranial computed tomography. After admission to the ICU, patients' pulse rates, blood pressure (by arterial catheter), temperature, saturation of oxygen, etc. were monitored. The patients were all in unresponsive comas, they were not breathing spontaneously, and they had absent cephalic reflexes with non-reactive fixed pupils. Spinal segmental reflexes were occasionally observed.

The etiologies leading to brain death included cerebrovascular event, head injury due to traffic accident, respiratory arrest due to acute respiratory distress syndrome and bullet-induced head injury (Table 1). Patients were ventilated using 10/min inspiration and expiration cycles, generally with a 500 cc volume. The respiratory supply was turned off only in the event of spontaneous cardiac arrest. None of the patients' relatives gave their consent for organ transplantation after the declaration of brain death.

In all cases, 12-lead electrocardiograms (ECG) were obtained at least twice a day, with at least 50 beats per second, and continuous ECG monitoring was carried out for at least two hours with the aid of a Holter system. R-R intervals and heart rate variations were measured from the records in 25 consecutive beats. Heart rates were measured during carotid massage, applied to either carotid for two minutes, with 10 minutes intervals and during artificial ventilation.

Table 1: Age, sex, etiology, tests and follow up distribution of cases.

No	Age	Sex	Cause of the Brain Death	Duration of artificial ventilation (day)	EEG	Cephalic reflexes	BAER	Apnea test	Follow up	R-R Intervals
1	66	Female	Cerebrovascular accident	6	Flat	Negative	No response	No response	Exitus	Fixed
2	45	Male	Cerebrovascular accident	5	Flat	Negative	No response	No response	Exitus	Fixed
3	62	Male	Traffic accident, severe head trauma	5	Flat	Negative	No response	No response	Exitus	Fixed
4	38	Male	Acute respiratory distress syndrome and respiratory arrest	3	Flat	Negative	No response	Not done	Exitus	Fixed
5	22	Male	Bullet injury, head trauma	5	Flat	Negative	No response	No response	Exitus	Fixed
6	16	Female	Traffic accident, severe head trauma	12	Flat	Negative	No response	No response	Exitus	Fixed

RESULTS

R-R intervals were found to be fixed during artificial ventilation over a determined period of time (minimum: 1 minute) (Figure 1). Heart rate was observed to change with increased body temperature and rate of dopamine infusion. However if the vital signs were stable, the R-R intervals were fixed for at least one minute; in other words, there was no change in R-R intervals. We referred to this condition as "fixation of R-R intervals" (Figure 2). No change in heart rate was observed during carotid massage applied to either carotid or during the application of painful stimulus (Figure 3). We also observed by Holter monitoring that the heart rate was fixed if there were no external factors.

DISCUSSION

Cardiac activity is normally under the antagonistic influence of the intracranial parasympathetic (vagal dorsal nucleus) and the extracranial sympathetic systems (4). Three vagal areas participate in cardiac control. The principal parasympathetic afferent input via the nodosa ganglion is to the nucleus of the solitary tract. Cardiac motor efferent sources are the nucleus ambiguus and dorsal vagal nucleus. The principal medullary site of sympathetic cardiac control resides in the rostral ventrolateral medulla. In addition, supramedullary areas such as the parabrachial nucleus in pons, hypothalamus, amygdala, insular cortex, medial medullary region, temporal lobe and cingulate gyrus have important functions in cardiac control mechanisms.

All of the effects of CNS elements on cardiac functions disappear in brain death (5,6). The effects of the parasympathetic system disappear directly. The sympathetic system is affected indirectly with the disappearance of the effects of afferent impulses on the central and cervicothoracal systems. As a result of all these changes, cardiac pacemakers are released from the effects of central regulating systems. They gain autonomy, and start to work with their own rhythms.

Since the variation of R-R intervals are influenced by the respiratory center in the medulla and by parasympathetic nervous system activity associated with the vagal nerve circuit, this variation might be lost in brain death, resulting in fixed R-R intervals.

The coefficient of variation of R-R intervals has been reported to decrease in some conditions such as peripheral neuropathy, barbiturate intoxication and presence of severe brainstem lesions (7,8,9). There are only a few reports examining the relationship between R-R intervals or heart rate variation and brain death. Neru et al reported on four children with brain death among a group of patients, in which they observed an extremely low coefficient of variation of R-R intervals (1.00-1.29%) with brain death (7).

We also observed fixed R-R intervals in our adult patients with brain death. We believe that this is a result of autonomic dysfunction due to severe brain damage. Therefore, we suggest that the investigation of R-R interval variations might be useful in evaluating and diagnosing brain death. We conclude that "R-R interval fixation" is a confirmatory test and could be included in the protocol for assessment of brain death.

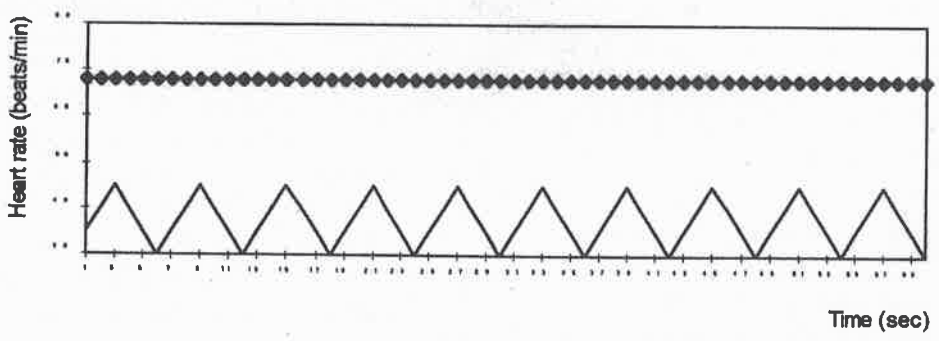

Figure 1: No change in heart rate with artificial ventilation.

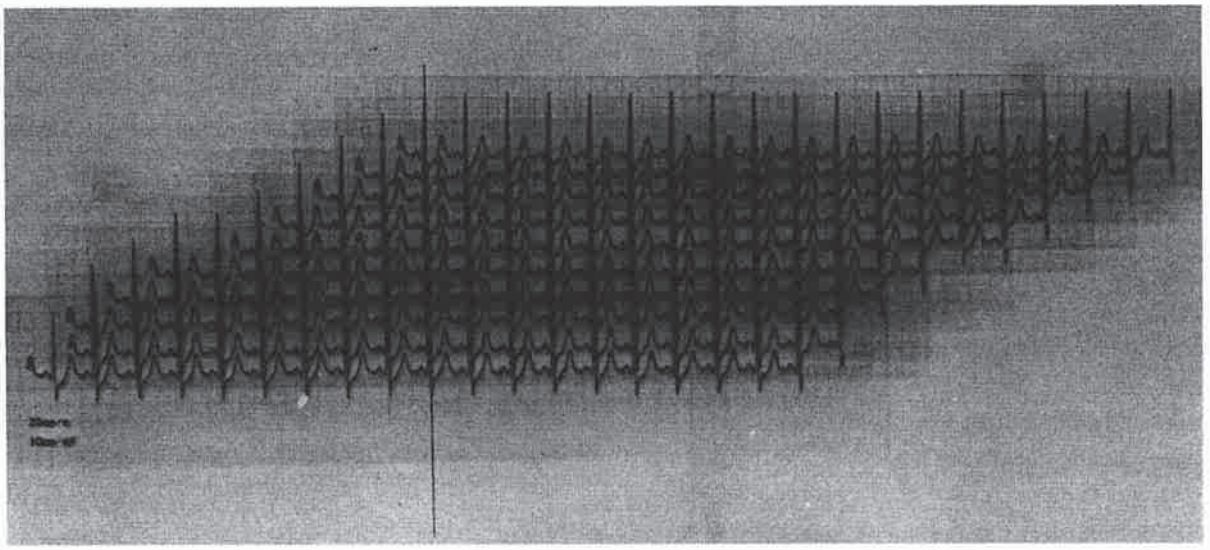

Figure 2: Heart rate stability - "fixation of R-R intervals".

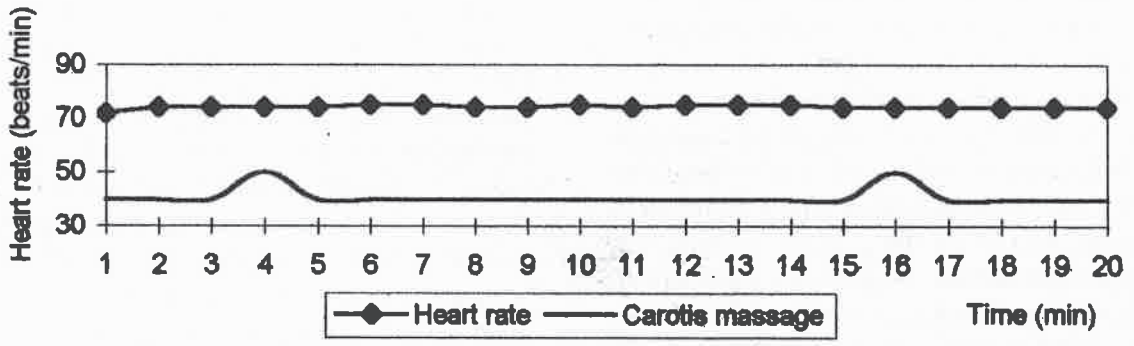

Figure 1: No variation in heart rate during carotis massage

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IS DOPPLER TISSUE EARLY LEFT VENTRICULAR FILLING VELOCITY PRELOAD INDEPENDENT OF PRELOAD ALTERING MANEUVERS?

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SUMMARY

Objective. Transmitral Doppler flow indices are used to evaluate diastolic function. Recently, Doppler tissue velocities have been used as an index of left ventricular relaxation. The aim of this study was to determine whether Doppler tissue velocities are influenced by preload alterations.

Methods. We altered left ventricular preloads in 17 patients (all men, 49±8 years) while recording echocardiographic measurements of left ventricular end-diastolic volume, left atrial maximal area, peak early Doppler filling velocity and left ventricular myocardial velocities during early filling. Preload altering maneuvers included Trendelenburg (Stage 1), reverse Trendelenburg (Stage 2) and amyl nitrate (Stage 3). Systolic blood pressures were taken at every stage.

Results. Compared to baseline left ventricular end diastolic volume (p=0,001), left atrial area (p=0,003), peak early mitral Doppler filling velocity (p=0,01) and systolic blood pressures (p=0,001) were changed as a result of preload altering maneuvers. Only left ventricular myocardial velocities during early filling were significantly unaffected by preload altering maneuvers.

Conclusion. In contrast to standard transmitral Doppler filling indices, Doppler tissue early diastolic velocities are not significantly affected by physiological preload altering maneuvers. Thus, Doppler tissue velocities during early left ventricular diastole may be more useful as an index of diastolic function by providing a preload-independent assessment of left ventricular filling.

Key Words: *Doppler Tissue Echocardiography, Preload Alterations*

ÖZET

DOPPLER DOKU SOL VENTRİKÜL ERKEN DOLMA HIZI ARDYÜK DEĞİŞTİRİCİ MANEVRALAR KULLANILDIĞINDA ARDYÜKTEN ETKİLENİR Mİ?

Amaç: *Transmitral Doppler akım verileri diyastolik fonksiyonu değerlendirmek için kullanılmıştır. Son zamanlarda, Doppler doku hızları sol ventrikül gevşemesinin bir göstergesi gibi kullanılmıştır. Bu çalışmada Doppler doku hızlarının ön yük değiştirici manevralardan etkilenip etkilenmediğinin saptanması amaçlandı.*

Yöntem: *17 hastada (tümü erkek, 49 ± 8) ekokardiyografik sol ventrikül miyokardiyum hızları ölçümlerini yaparken sol ventrikül ön yükünü değiştirdik Trendelenburg (1. basamak), ters Trendelenburg (2. basamak) and amil nitrat (3. basamak) ön yük değiştirici manevralar olarak kullanıldı. Her basamak için sistolik kan basıncı ölçüldü.*

Sonuçlar: *Başlangıç ile karşılaştırılınca, sol ventrikül diyastol hacmi (p=0,001), sol atriyum alanı (p=0,003), pik erken mitral Doppler dolum hızı (p=0,001) ve sistolik kan basınçları (p=0,001) ön yük değiştirici manevralar ile değişti, ancak sol ventrikül miyokardı hızı erken dolumda ön yük değiştirici manevralar ile anlamlı değişmedi.*

Yorum: *Mitral seviyeden Doppler dolum verilerinin tersine, Doppler doku erken diyastol hızları fizyolojik ön yük değiştirici manevralardan önemli derecede etkilenmez. Böylece erken sol ventrikül diyastolu sırasında Doppler doku hızları sol ventrikül diyastolu sırasında Doppler doku hızları sol ventrikül dolumunun ön yükden bağımsız değerlendirilmesini sağlayan daha yararlı bir gösterge olabilir.*

Anahtar Kelimeler: *Doppler Doku Ekokardiyografi, Ön Yük Değiştirme*

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Received: Dec 06,2000 Accepted: July 25,2001

Diastolic dysfunction is the primary mechanism responsible for dyspnea in patients with heart failure, irrespective of the presence or severity of systolic dysfunction (1-3). Left ventricular (LV) diastolic dysfunction usually precedes systolic dysfunction (4,5) and abnormal relaxation is observed at its earliest stage (2-6). Conventional clinical evaluation of LV relaxation involves determining the time constant of pressure decay during isovolumic diastole as calculated from the LV pressure curve (7). Doppler echocardiography has become the noninvasive technique of choice for the evaluation of diastolic function (8,9). LV filling and, more recently, pulmonary venous Doppler flow indices have been used to evaluate different parameters of diastolic function, including LV filling, pressure, relaxation and stiffness (8,10-12). Unfortunately, because there are several physiologic variables - including volume status, left atrial pressure and rate of myocardial relaxation (13-15) - it is often difficult to determine which individual variable is responsible for a specific Doppler pattern unless other relevant clinical and/or invasive information is available (6,16-18).

Doppler tissue imaging (DTI) is a new ultrasound modality that records systolic and diastolic velocities within the myocardium (19-23) and at the corner of the annulus (24-26). Recently, the early diastolic velocity recorded at the lateral corner of the annulus (EDTV) has been demonstrated to decline progressively with age and to be reduced in pathologic LV hypertrophy (25) as well as in patients with restrictive cardiomyopathy (26). These findings suggest that EDTV is an index of LV relaxation that may not be influenced by left atrial pressure. Therefore, this study was designed to assess whether EDTV as recorded by DTI is a preload-independent index of LV relaxation that will differentiate pseudonormalized mitral flow from a normal pattern.

Methods

PATIENTS

The study group consisted of 17 patients (all men, 49±8 years of age) with stable forms of chronic ischemic syndrome. All patients underwent echocardiographic evaluation in our laboratory for assessment of cardiac structure and function. Criteria for inclusion were presence of sinus rhythm and absence of any congestive heart failure, valvular heart disease, primary myocardial heart disease, secondary hypertrophy (hypertension, aortic stenosis, etc.) or endocrinological and renal diseases. All subjects gave written informed consent before participation.

ECHOCARDIOGRAPHY

Subjects studied in the echocardiography laboratory were first imaged with a commercially available echocardiography machine (Acuson model 128, Mt. View, California, or Hewlett-Packard model 72020A, Andover, Maryland) equipped with a multifrequency transducer as well as the DTI program. Images were taken in the left lateral decubitus position, and complete echocardiographic studies were performed using standard views and techniques. Two-dimensional studies were recorded from the parasternal long and short axis and the apical four- and two-chamber views. End-diastolic volumes and left atrial area were obtained from the apical four-chamber view. All Doppler echocardiographic and DTI recordings were obtained during normal respiration.

PULSED DOPPPLER ECHOCARDIOGRAPHY

The sample volume was set at the mitral valve orifice in the long axis view of the left ventricle or the four-chamber view recorded from the cardiac apex, and transmitral flow velocity patterns were recorded. Early diastolic wave velocity was then obtained.

Doppler Tissue Imaging: In the apical view of the left ventricle, sample volumes were set at the lateral portion of the mitral annulus. Motion velocity patterns for each patient were recorded using the pulsed Doppler method. After baseline parameters were obtained, the studies were repeated on the same subjects using the preload-altering maneuvers. These stages were defined as Baseline, Stage 1 (Trendelenburg position), Stage 2 (reverse Trendelenburg position) and Stage 3

Table 1: Changes in Echocardiographic Measurements with Preload Altering Maneuvers

Variables	Baseline	Stage 1	Stage 2	Stage 3
LVEDV (ml)*	199 ± 132	242 ± 186	212 ± 163	188 ± 133
LA Area (mm2)**	22 ± 9	24 ± 10	22 ± 10	20 ± 8
E Wave (cm/sec)¶	82 ± 33	85 ± 32	72 ± 26	73 ± 20
EDTV (cm/sec) #	10 ± 4	11 ± 3	11 ± 3	11 ± 4
BP*	126 ± 19	123 ± 17	118 ± 21	103 ± 22

*p=0,001; **p=0,003; ¶p=0,01; #p= NS (stage 1,2,3 parameters comparing baseline)

(amyl nitrate inhalation). Blood pressure was recorded continuously at all stages.

STATISTICAL ANALYSIS

Data was presented as mean value ± SD. Analysis of variance and t tests were used to compare differences between the stages, with a value of p< 0,05 considered significant.

Results

Normal values for left ventricular end diastolic volume (LVEDV), left atrial (LA) area, left ventricular early mitral flow velocity (E) and EDTV were obtained from the annulus for the Baseline (Table 1). In Stage 1, early diastolic mitral inflow velocity profile increased (p=0,01) with the Trendelenburg maneuver, which is known to increase preload. LVEDV (p=0,001) and LA (p=0,003) area increased significantly in Stage 1, but EDTV did not change significantly (p<0,05). In Stage 2 (reverse Trendelenburg) and Stage 3 (amyl nitrate inhalation), EDTV was unchanged, but other variables changed significantly (Table 1).

Discussion

The results of this study suggest that peak Doppler tissue early left ventricular velocities are not affected by varying preload conditions. In spite of the changes in hemodynamic parameters, EDTV did not change significantly.

Mitral flow variables are load-dependent, and patients with a relaxation abnormality may show a normal pattern with elevated atrial pressure. This pattern may occur because mitral flow variables are velocity data determined by the pressure difference between the left atrium and left ventricle during diastole. The effect of volume changes on Doppler tissue velocities has the theoretical advantage of being less preload-dependent than mitral flow variables. Garcia et al (26) observed that peak EDTV correlates poorly with peak E velocity, suggesting the relative preload-independence of peak EDTV. In this study, we demonstrated that peak early mitral annulus velocity, in contrast to mitral inflow velocity, did not change significantly after preload alteration by Trendelenburg or reverse Trendelenburg maneuvers or amyl nitrate inhalation. Some findings were previously demonstrated by Sohn et al (27) by alteration of the preload with infusion of saline or nitroglycerin. Aranda et al (28) also showed that during routine examination of heart transplant cases, peak EDTV did not change as a result of preload alteration with nitroglycerin.

In contrast to standard Doppler echocardiography, DTI is capable of measuring myocardial tissue velocity, which directly reflects the contractile and relaxation properties of the myocardium. All of our subjects had evidence of normal left ventricular systolic function, no evidence of coronary artery disease as detected by coronary angiography, and no pericardial effusion. Both Trendelenburg maneuvers and inhalation of amyl nitrate affected the blood pressure response (p=0,001), LVEDV (p=0,001) and LA area change

(p=0,003). Changes in the hemodynamic profile such as increase in heart rate and decrease in blood pressure, LVEDV and LA area following inhalation of amyl nitrite are well known and probably mediated by venous and arterial dilatation via reflex sympathetic stimulation by baroreceptors (29). Relaxation velocities by Doppler tissue echo with different preload conditions did not change significantly, suggesting that myocardial relaxation is independent of preload. This observation is consistent with the findings of Stoddard et al (30). Impaired relaxation is a common denominator in patients with heart failure, with or without systolic dysfunction (1-3). During heart failure, left atrial pressure increases in response to a reduction in LV compliance. This increase masks the influence of impaired relaxation on the transmitral velocity, producing a pseudonormal pattern with an E/A ratio >1 and shortening the isovolemic relaxation time and deceleration time (9, 10, 31). However, abnormal myocardial relaxation still exists in these patients and can be demonstrated with invasive measurements of the time constant of relaxation and with the flow propagation velocity of LV inflow assessed by color M-mode echocardiography (32-34).

EDTV as a preload-independent index of LV relaxation is significant in that it may permit differentiation between pseudonormal mitral flow patterns and normal patterns. Diagnosis and management of this group of patients seem to be more confidently carried out by a combination of clinical and echocardiographic variables of the pulmonary vein velocity as well as EDTV as a new high technologic tool. Recently, greater emphasis has been given to the possibility of using EDTV as a pre-load independent method of detecting left ventricular relaxation abnormalities in practical cardiology.

In conclusion, in contrast to standard diastolic transmitral Doppler filling indices, Doppler tissue early diastolic velocities are not significantly affected by physiological preload altering maneuvers. Thus, EDTV during early LV diastole may be a more useful index of diastolic function by providing preload independent assessment of LV filling.

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THE ADMINISTRATION OF GRANULOCYTE COLONY-STIMULATING FACTOR TO HEALTHY DONORS FOR ALLOGENEIC PERIPHERAL BLOOD PROGENITOR CELL COLLECTION MAY INDUCE THE TISSUE FACTOR DEPENDENT PATHWAY

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SUMMARY

The hypercoagulable state caused by the use of rhG-CSF has been cited in several case reports. Since tissue factor (TF) is the main initiator of the coagulation protease cascade, we examined whether or not rhG-CSF had an inductive effect on the TF-dependent pathway. We measured plasma TF antigen (TF Ag) and TF procoagulant activity (TF PCA), TF expression on peripheral blood monocytes and neutrophils and plasma coagulation factor activities in 18 healthy donors (8F/10M; aged 17-52) receiving 10μg/kg/day rhG-CSF for five days in the aim of peripheral blood progenitor cell mobilization. Blood samples were collected before starting G-CSF and on the first day of stem cell apheresis. There were significant Increases in TF Ag ($p<0.05$) and TF PCA ($p=0.06$) levels. Flow cytometric evaluation revealed a significant increase in TF expression on CD33 (+) cells ($p=0.04$). Mean plasma FVIII activity and vWF activity also increased significantly. Thrombin time was slightly prolonged ($p=0.06$) due to significant increases in plasma D-dimer levels ($p<0.05$). In addition, while FIX activity remained stable, there were marked decreases in mean plasma FX and FII activities and a slight decrease in FVII activity that resulted in significant prolongation of prothrombin time. The administration of rhG-CSF in healthy stem cell donors increased the mean TF Ag and TF PCA in plasma and TF expression on cells, decreased extrinsic pathway factor activity, increased D-dimer levels and endothelial markers and prolonged PT. In conclusion, the administration of rhG-CSF led to a 'prothrombotic state' via stimulation of TF and increased endothelial markers such as F VIII and vWF. In light of these findings, the use of rhG-CSF for stem cell mobilization should be undertaken cautiously in healthy donors with underlying thrombotic risk factors.

Key Words: *Coagulation, G-CSF, Healthy Donor, Tissue Factor*

ÖZET

ALLOJENEİK PERİFERİK KÖK HÜCRE TOPLANAN SAĞLIKLI VERİCİLERDE GRANÜLOSİT KOLONİ STİMÜLE EDİCİ FAKTÖR KULLANIMI DOKU FAKTÖRÜ BAĞIMLI YOLU UYARABİLİR

Granülosit koloni stimule edici faktör (rhG-CSF) kullanımının neden olduğu hiperkoagulabiliteden değişik vaka sunularında söz edilmektedir. Doku faktörünün (DF) koagulasyon-proteaz kaskadının esas başlatıcısı olduğundan dolayı, rhG-CSF'nin DF bağımlı yol üzerine uyarıcı bir etkisi olup olmadığı çalışıldı. Periferik kan kök hücre mobilizasyonu amacı ile 10μg/kg/gün rhG-CSF 5 gün süre ile kullanan 18 sağlıklı (8K/10E, yaş 17-52) vericide, plazma DF antijen (DF:Ag), DF prokoagulan aktivite (DF:PKA), periferik kandaki monosit ve nötrofillerde DF:Ag ekspresyonu ve plazma koagulasyon faktör aktivite düzeyleri çalışıldı. Kan örnekleri G-CSF başlamadan önce ve kök hücre aferezinin ilk günü toplandı. DF:Ag ($p<0,05$) ve DF:PKA'da ($p=0,06$) önemli artış vardı. Akım sitometrik değerlendirme CD33 (+) hücrelerde DF ekspresyonunda anlamlı bir artış ortaya kondu ($p=0,04$). Beraberinde plasma FVIII ve vWF aktivitesi de anlamlı olarak arttı. Trombin zamanı, plazma D-Dimer seviyesindeki anlamlı artış ($p<0,05$) nedeni ile hafif uzadı ($p=0,06$). Ek olarak FIX aktivitesi sabit kalırken, ortalama plazma FX ve FII aktivitelerinde ise belirgin azalma ve protrombin zamanında (PTZ) anlamlı uzamaya neden olan FVII aktivitesinde hafif bir azalma vardı. Sağlıklı kök hücre vericilerinde rhG-CSF kullanımı ortalama plazma DF:Ag ve DF:PKA ve hücrelerde DF ekspresyonunu arttırdı, ekstrensek yol faktör aktivitesini azalttı D-Dimer düzeyleri ve endotelyal belirleyicileri arttırdı ve PTZ'yi uzattı. Sonuç olarak rhG-CSF kullanımı DF uyarımı ve, FVIII ve vWF gibi endotelyal belirleyicileri arttırarak bir 'protrombotik durum''a neden olmaktadır. Bu bulguların ışığında kök hücre mobilizasyonu için rhG-CSF kullanırken sağlıklı vericilerde altta yatabilecek trombotik risk faktörleri dikkate alınmalıdır. Biz G-CSF kullanımından önce sağlıklı vericilerin altta yatan trombotik risk faktörleri yönünden değerlendirilmelerini öneriyoruz.

Anahtar Kelimeler: *Koagulasyon, G-CSF, Sağlıklı Verici, Doku Faktörü*

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Received: July 19,2001 Accepted: Aug 28,2001

Granulocyte-colony stimulating factor (G-CSF) is a hematopoietic cytokine that stimulates neutrophil production and the release of mature granulocytes from bone marrow and enhances neutrophil function (1). Because of its ability to induce mobilization of CD34+ hematopoietic progenitor cells (HPC) into circulating blood, G-CSF has recently been used in normal subjects to mobilize and collect HPC for allogeneic transplantation (1) The most commonly used protocol for stem-cell mobilisation is the administration of G-CSF to donors at a moderate-high dose such as 10μg/kg/d for four or five days and subsequent leukapheresis (1,2).

The safety of G-CSF administration in healthy donors has been investigated by several authors (1,2). However, there is limited knowledge about the effects of G-CSF on hemostasis. Thrombosis was the first event attributable to G-CSF (3), and two cases of healthy donors with acute arterial thrombosis, which might be related to G-CSF, have been documented (2). In a meta-analysis of studies investigating the use of G-CSF with chemotherapy, 1.2% of cancer patients were reported to have encountered thrombotic complications (4). Whether G-CSF has a direct role on thrombus formation is still unknown. Following preliminary studies that showed the presence of a receptor for G-CSF on platelets and megakaryocytes (5), it was claimed that G-CSF caused thrombosis by increasing platelet aggregation (6). Tissue factor (TF) is a main physiological initiator of blood coagulation-protease cascade in normal hemostasis (7). It has a cofactor role for factor VII/factor VIIa (FVII/FVIIa) during activation of factor IX (FIX) and factor X (FX) by FVII (4). TF is constitutively expressed on cells separated from the blood stream (8). Normally, peripheral blood cells (monocytes/macrophages) and endothelial cells do not express TF. However, recent studies have shown that monocytes and macrophages also contributed to thrombin generation under various pathological conditions in which TF activation on these cells increased procoagulant activity (9,10).

To date, there is a lack of knowledge about the role of TF in the hypercoagulable state induced by the use of G-CSF for allogeneic HPC mobilisation in healthy donors. This study investigated the effect of G-CSF on plasma TF antigen and TF procoagulant activity as well as TF antigen expression on peripheral blood cells, the factors involved in intrinsic and extrinsic coagulation pathways, naturally occurring anticoagulants and contribution of endothelium-derived factors in healthy donors.

MATERIAL AND METHOD

Eighteen HLA-identical healthy sibling donors who were candidates for allogeneic stem cell collection (8 female, 10 male; mean age: 32) and 18 normal control cases (no G-CSF) were studied. Donors received G-CSF (Filgrastim; Amgen, Roche) 5μg/kg subcutaneously twice daily for nine consecutive doses. Following the ninth dose on Day 5, leukapheresis was performed using a continuous-flow cell separator (COBE Spectra or Baxter CS 3000 plus).

Blood Collection: Thirty-five mLs of blood BF were collected from each donor before the first dose and after the ninth dose of G-CSF. EDTA anticoagulated tubes were used for complete blood count (CBC), differential leukocyte count (DLC) and detection of TF antigen (TF:Ag) expression. Citrated (3.8%) tubes were used for coagulation tests and of TF:Ag and procoagulant analysis activity (TF:PCA). Plasma samples obtained by centrifugation of citrated tubes (3000G, 10 min, 22°C) were processed immediately or stored at -80°C as aliquots until tests could be performed.

Assays: Plasma TF:Ag was determined using an Imubind Tissue Factor ELISA Kit (#845, BF American Diagnostic, Greenwich, USA). The kit employs a murine anti-human tissue factor monoclonal antibody for antigen capture. Prediluted plasma samples (1/10 in sample buffer) were incubated in micro test wells precoated with capture antibody. Captured TF was detected with a biotinylated antibody fragment that specifically recognised bound TF. After an incubation step using streptavidin conjugated horseradish peroxidase (HRP), the addition of TMB substrate creat-

ed a blue colour in the wells. Addition of 0.05 M sulphuric acid top solution caused it to turn yellow and stabilise. TF levels were determined by measuring absorbency at 405 nm and comparing the values with those of the standard curve.

Plasma TF:PCA was determined using an Actichrome Tissue Factor Activity Kit (#846, American Diagnostic, Greenwich, USA), which measures intact TF as well as TF/Factor VII (TF/FVII) and TF/factor VIIa (TF/FVIIa) complexes. Peptidyl activity of TF present in plasma was measured to form a complex with FVII. Plasma samples diluted 1/10 with 5% TF/Tissue Factor Pathway Inhibitor depleted plasma, mixed with human factor VIIa and incubated, allowing formation of the TF/FVII complex. The complex was allosterically activated, and its activity was directly measured by its ability to cleave to a highly specific chromogenic substrate for TF/FVIIa complexes (Spectrozyme FVIIa) that was added to the reaction solution. The cleavage of the substrate was terminated by releasing a paranitroaniline (pNA) chromophore into the reaction solution. The absorbency of the solution was read at 450 nm and compared to the values obtained from a standard curve generated using known amounts of lipidated TF. To obtain the actual TF concentration (ng/mL), results were multiplied by 10 (dilution factor) and 42 (conversion factor, 1 ng/mL TF:PCA is equal to 42 nM).

TF:Ag expression was detected using a Fluorescein IsothioCyanete (FITC) conjugated murine monoclonal antibody specific to human tissue factor (#4508CJ, American Diagnostica, Greenwich, USA). Whole blood was diluted with PBS to obtain 0.5x106 WBC/mL. In order to identify specific bounding to monocyte/neutrophils, monoclonal antibodies (moAbs) specific to CD14, CD45 and CD33 and an isotypic control were used simultaneously (Becten Dickinson Immunologic System = BDIS, San Jose, USA). Five µl of anti-TF Mo.Ab. and 7 µL of other MoAbs were added to prelabeled tubes. Monoclonal antibodies were incubated with 100 µL diluted blood for 30 min at 4(C. Following red cell lyses procedure, cells were washed twice with PBS and fixed with 500 mcL 1% paraformaldehyde solution (Cellfix, BDIS, Sana Jose, USA). Data was collected within 24 hour by counting 10.000 cells per tube in a flow cytometer (FacSort, BDIS, San Jose, USA). Data was analysed using Cell Quest Software (BDIS). Monocytes and neutrophils were gated according to forward- and side-scatter characteristics and CD33 or CD14 expression intensity. CD14+/CD33bright cells and CD33dim cells were evaluated respectively as monocytes and neutrophils. Isotypic control tubes and lymphocyte gates were used to set a marker for calculating TF expreszing cell percentage.

Other Parameters

Complete blood counts (CBC) and white blood cell (WBC) differential counts were obtained by processing anticoagulated blood specimens through a properly calibrated and quality-controlled automated hematology analyser (STKS, Coulter Beckman).

Coagulation parameters including prothrombin time (PT), partial thromboplastin time (PTT), thrombin time (TT), factor II (FII), FVII, FVIII, FIX, FX and plasma fibrinogen level were measured nephelometrically in an automated and quality-controlled coagulometer (ACL, Futura, IL) using reagents purchased by the same manufacturer. The level of D-Dimer was measured using a latex-enhanced turbidometric immunoassay in the same coagulometer. vWF Ri:CoF activity was measured semi-quantitatively using von Willebrand reagent (Dade Behring). Natural anticoagulants including protein C, protein S and antithrombin III (ATIII) were measured functionally.

RESULTS

Baseline laboratory parameters (Day 0) of all subjects were found to be within normal sauges (Table 1 and Table 2). Significant increases in WBC, neutrophil and monocyte counts were observed in all donors administered rhG-CSF (Day 5 vs Day 0, $p<0.001$). There were no changes in red blood cell (RBC) and platelet counts at Day 5 vs Day 0 (Table I).

Hemostatic parameters

As shown in Table I, PT measures were slightly but statistically prolonged from 11.5±0.6 sec to 12.0±0.6 sec after G-CSF ($p<0.05$). We observed a significant rise in D-Dimer levels, with a slightly prolonged TT at Day 5. G-CSF caused no change on PTT and fibrinogen levels.

As shown in Table 2, a significant rise was observed in mean activity of vWF:Ri CoF and VIII, from 70±24% to 90±20% ($p<.05$) and from 131±47% to 198±63% ($p<0.05$), respectively. However, mean activity of FII and FX decreased significantly after G-CSF, from 108±14% to 97±16% ($p<0.05$) and from 118±20% to 103±15 ($p<0.05$), respectively. The mean activity of natural anticoagulants protein C, protein S and ATIII decreased after G-CSF; however, only the decrease in protein C activity was slightly significant. There was no observable change in APCR-R after G-CSF.

Table 1: Laboratory parameters before G-SCF (Day 0) and after G-CSF (Day 5)

Variables	Day 0 (mean±SD)	Day 5 (mean±SD)	p
Leukocyte (10^9/L)	7.3±1.0	47.4±13.8	<0.001
Neutrophil (10^9/L)	4.3±0.9	40.5±13.2	<0.001
Monocyte (10^9/L)	0.4±0.1	2.1±1.7	<0.001
Red blood cell (101^2/L)	4.9±0.5	4.7±0.5	0.35
Platelet (10^9/L)	273±86	271±56	0.94
PT (second)	11.5±0.6	12.0±3.7	0.01
PTT (second)	31.5±3.7	31.4±8.1	0.96
TT (second)	12±1.0	14±2.4	0.06
Fibrinogen (mg/dL)	339±90.1	377±72.2	0.17
D-Dimer (ng/mL)	125±92.1	188±88.0	0.04

Table 2: Plasma factor activities and natural anticoagulants of healthy donors before (Day 0) and after G-CSF (Day 5)

Variables	Day 0 (mean±SD)	Day 5 (mean±SD)	P
Factor II (%)	108±14.1	97±16.0	0.03
Factor VII (%)	126±32.7	114±63.7	0.49
Factor VIII (%)	131±47.6	198±63.7	0.001
Factor IX (%)	136±44.8	157±75.0	0.32
Factor X (%)	118±20.8	103±15.5	0.02
vWF:Ri CoF (%)	70±24.0	90±20.4	0.01
ATIII (%)	108±20.5	99±19.4	0.17
Protein C (%)	106±28.4	95±8.6	0.09
Protein S (%)	100±30.4	92±28.2	0.42
APC-R	2.1±0.3	2.0±0.3	0.68

Tissue factor antigen (ELISA assay)

There was a significant increase in mean plasma TF:Ag level from 34.3±52 pg/mL (0-185 pg/mL) to 100.4±90 pg/mL (0-287 pg/mL), ($p<0.01$) while mean TF:Ag level was 100.8±78 pg/mLin the control group (Figure 1). When evaluated according to gender, the increase in mean TF:Ag level was significantly higher in males (from 37.2±58 pg/mL to 122.8±105 pg/mL, $p<0.04$) than in females (from 30.4±47 pg/mL to 70.9±72 pg/mL, $p=0.2$) (Table 3).

Tissue factor procoagulant activity (ELISA assay)

Plasma TF:PCA level increased from 9.1±20 ng/mL to 48.9±81ng/mL after G-CSF, with a tendency to be significant ($p=0.06$) (Figure 2). Although this increase was more pronounced in males, there were no significant changes in either gender (12.5±27 ng/mL to 62.3±92 ng/mL, $p=0.14$ in males; 5.2±7 ng/mL to 33.9±71 ng/mL, $p=0.27$ in females) (Table 3).

No correlation between TF:Ag and TF:PCA either before or after G-CSF could be detected.

Flow cytometrical data (Figure 3)

There was no change in TF:Ag expression on CD14+ (monocytic) cells at Day 5 vs Day 0 (16.1±23% v.s.12.8±18%, $p=0.37$). However, a significant increase in the level of TF:Ag expression on CD33+ cells after G-CSF (from 9.3±13% to 15.6±18%, $p=0.04$) was observed. This increase was detected on both CD33bright cells and CD33dim cells, from 13.2±22% to 30.1±32% ($p=0.04$) and from 3.9±8% to 19.3±25% ($p=0.01$), respectively.

DISCUSSION

To provide further insight into the action of G-CSF on hemostasis and thrombosis, we evaluated coagulation parameters of 18 healthy stem-cell donors and compared them with a control group. The mean activities of FII and FX were reduced by a ratio of 11-13% following G-CSF. Mean FVII activity also decreased slightly, resulting in prolonged PT. However, there was no change in mean FIX activity or mean PTT on Day 5. This indicates that the coagulation factors in the extrinsic pathway are consumed rather than vitamin K-dependent factors. If G-CSF had affected vitamin K-dependent factors, we would also have observed a decrease in FIX activity and prolonged PTT. To date, there has been insufficient data about the effect of G-CSF on plasma coagulation factors. G-CSF has short- and long-term effects on hemostatic parameters, other than an increase in FVIII levels following G-CSF reported by LeBlanc (11). Following intravenous G-CSF administration to healthy donors, platelet aggregation response to adenosine diphosphate (ADP) and collagen increased while D-dimer levels remained unchanged (12). In our study, we found that the level of D-dimer, an indicator of an activated coagulation system, increased significantly from 125 ng/mL to 188 ng/mL ($p<0.05$), although values were within the normal control range. Additionally, TT was slightly prolonged ($p=0.06$), possibly as a result of increased fibrinogen

Table 3: Plasma TF:Ag and TF:PCA levels before G-CSF (Day 0) and after G- CSF (Day 5)

Variables	Day 0 (mean±SD)	Day 5 (mean±SD)	P
TF:Ag (pg/mL) (n=18)	34.3±52	100.4±92	0.01
Female (pg/mL) (n=8)	30.4±47	70.9±72	0.20
Male (pg/mL) (n=10)	37.2±58	122.8±105	0.04
TF:PCA (ng/mL) (n=18)	9.1±20	48.9±81	0.06
Female (ng/mL) (n=8)	5.2±7	33.9±71	0.27
Male (ng/mL) (n=10)	12.5±27	62.3±91	0.14

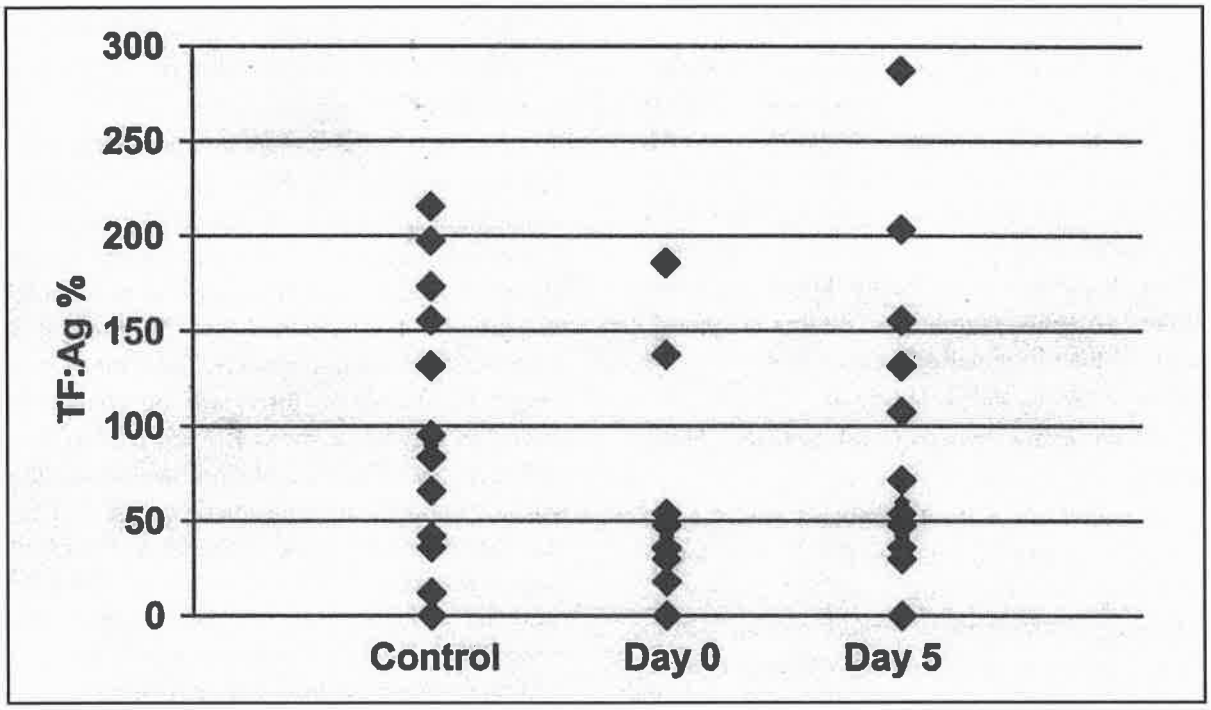

Figure 1: TF:Ag (pg/mL) levels in control group and healthy donors

turnover. Falanga et al reported a similar increase in D-dimer levels, although within normal control range, at the first day of apheresis, using a higher dose of G-CSF (15μg/kg/day) (10). Our study presents the first line of evidence that the coagulation cascade is triggered following G-CSF

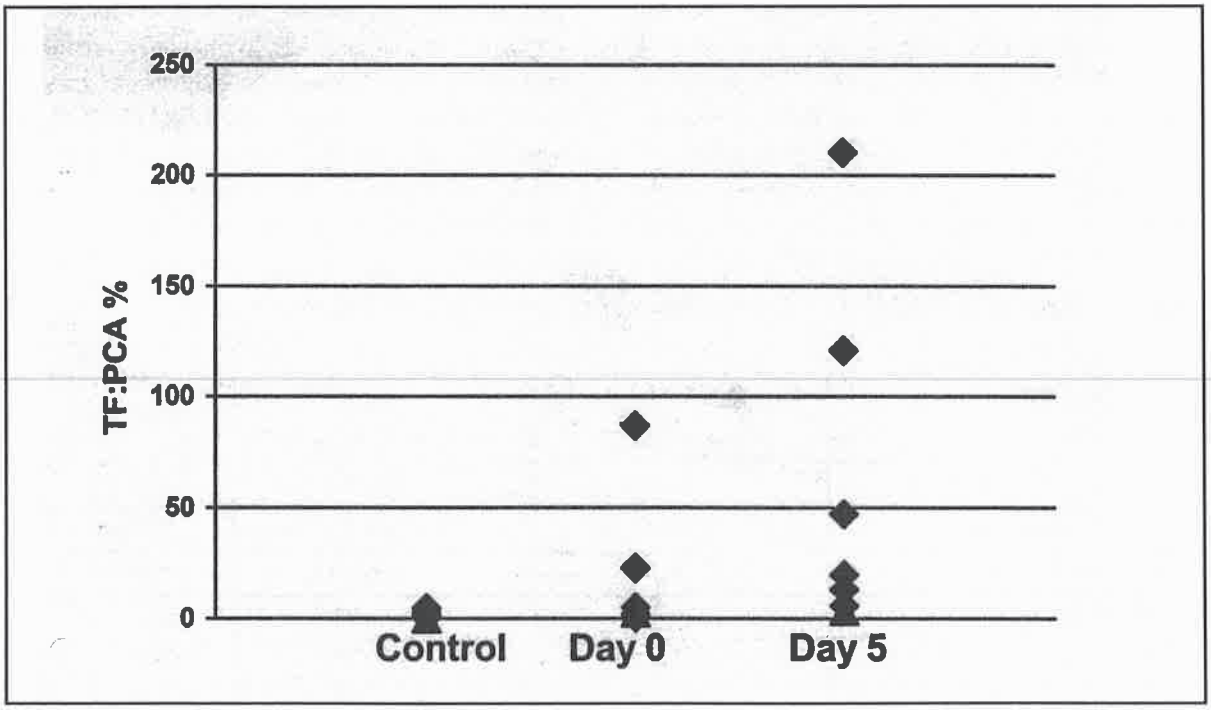

Figure 2: TF:PCA (ng/mL) levels in control group and healthy donors

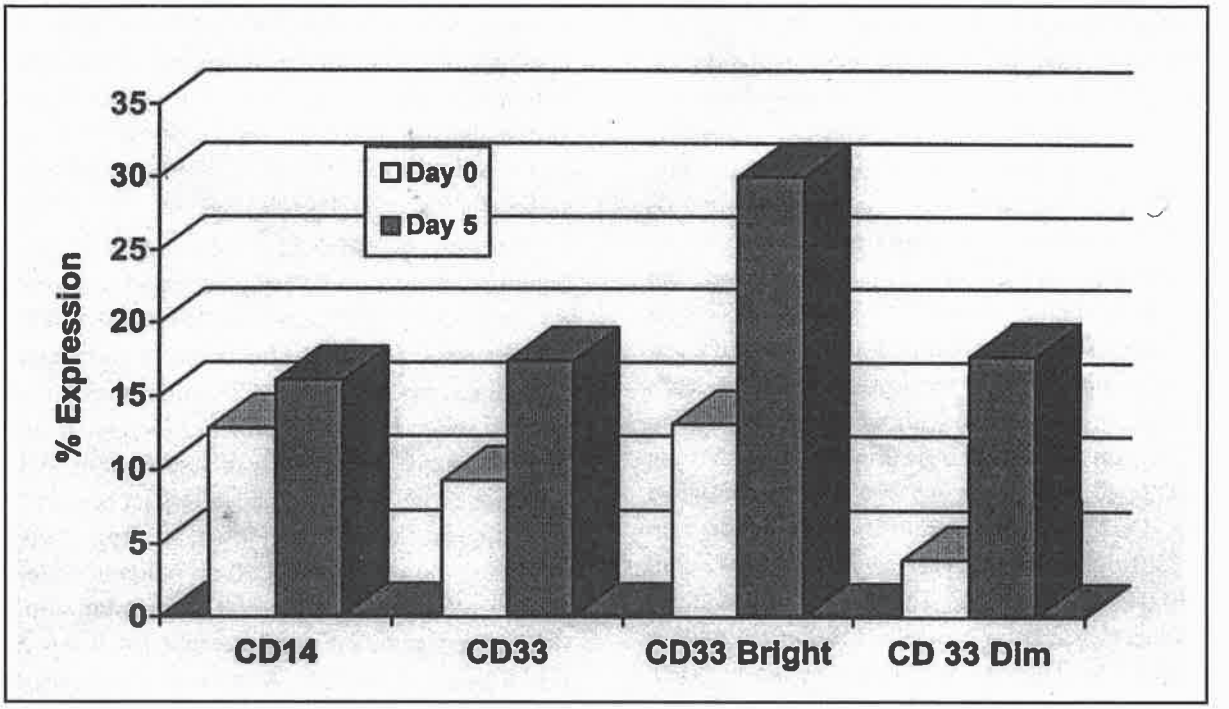

Figure 3: Comparison of mean TF:Ag expression (flow cytometric) on peripheral blood monocytes and neutrophils before rhG-CSF (Day 0) and after rhG-CSF (Day 5)

administration by demonstrating a decrease in coagulation factor activity of extrinsic pathway, prolongation of PT and elevation of D-dimer levels.

At present, our knowledge about the contribution of growth factors on TF and/or TF-dependent pathway of coagulation is limited. We observed significant increases in the mean levels of both TF:Ag and TF:PCA in plasma after G-CSF ($p=0.01$ and $p=0.06$, respectively). However, there was no correlation between TF:Ag and TF:PCA at either Day 0 or Day 5. One of the possible reasons for this conflict may be the incomplete activation of TF:Ag. It has been suggested that with respect to procoagulant activity, the majority of cell surface TF activity is normally "encrypted", in which state it is capable of binding antibody and FVII/VIIa, but not fully expressing antigenic activity. To become fully activated, it must be "de-encrypted" by processes involving plasma membrane phospholipids and monomerization of TF (13). In previous reports, G-CSF was shown to increase monocyte procoagulant activity (10). No influence of age and gender on plasma level of TF:Ag has been demonstrated in normal individuals (14). In our study, the elevation of TF:Ag after G-CSF tended to be more pronounced in males (from 37pg/mL to 123pg/mL, $p<0.05$) than females (from 30.4pg/mL to 71pg/mL, $p=0.2$) (Table 3). Data related to the role of TF in the development of atherosclerosis and an increased incidence in men must be evaluated within this context.

In normal individuals, TF:PCA in the circulation is very low (14). What is the source of TF:Ag and TF:PCA after G-CSF is a question that should be answered. While there is debate over the contribution of neutrophils to TF:PCA (15,16), the main sources of TF in the circulation are considered to be monocytes and macrophages (9). It has been put forward that circulating endothelial cells might have been contributing to TF:PCA during the aplasia period of stem-cell transplantation because of the absence of mature myelo-

monocytes (17). However, Özcan et al followed TF:PCA of stem cell transplant recipients during the peritransplant period and detected a considerable amount of remaining TF:PCA originating neither from myelo-monocytes nor circulating endothelial cells (18). The rise of endogenous G-CSF levels during the aplasia period of stem-cell transplantation has been previously shown, but the effect of endogenous G-CSF levels on TF:PCA remains to be elucidated (19). We observed a significant increase in TF expression on CD33+ cells (mature myeloid cells) without a major change in TF:Ag on CD14+ cells (monocytes). Among CD33+ cells, the increase was more pronounced on CD33dim (neutrophil) cells than on CD33bright (monocyte) cells ($p=0.01$ and $p=0.04$, respectively). These two observations lead us to suspect a possible contribution of neutrophils to TF:PCA. While an increase of TF expression has been shown on G-CSF-treated neutrophils in vitro (20), a clear correlation between the number of monocytes and increased TF expression has been demonstrated in vivo (16,21).

The acquisition of TF:Ag by neutrophils may have contributed to the increased TF levels in our study, and monocytes are likely to have played a role in this mechanism.

G-CSF exerts an important effect on endothelial cells by inducing their migration and proliferation (22). vWF and thrombomodulin (TM) are not only released from endothelial cells, but also accepted as surrogate markers of stimulation and toxicity of the endothelium (23). There have been some studies reporting that G-CSF increases the levels of vWF antigen, vWF activity and soluble TM (10,11). We found a significant elevation of plasma vWF:Ri CoF and FVIII activity after G-CSF. The mechanism by which G-CSF exerts its effects on the endothelium has not been fully explained. Upon activation, neutrophils release reactive oxygen species and intracellular protease that perform several activities on endothelial cells and platelets and may modify the hemostatic balance towards a prothrombotic state (10,24), to which a series of leukocyte-mediated events may have contributed, in addition to direct toxicity. As we did not investigate the relationship between levels of neutrophil-derived proteases and activation markers, we were not able to demonstrate the role of leukocyte-mediated endothelial toxicity.

The most common factor causing prothrombotic tendency is hereditarily activated protein C resistance due to FV-Leiden mutation (25). G-CSF did not result in APCR phenotype in our study. Data about the effect of G-CSF administration on natural anticoagulants is still insufficient. It has been reported that the use of G-CSF after HSC transplantation had no negative impact on natural anticoagulants (26). However, one study observing the effect of G-CSF on patients undergoing allogeneic stem-cell transplantation showed a significant decrease in ATIII levels at Day 5 versus Day 0 (27). Another study of cancer patients showed that administration of G-CSF at different dosages (1, 3, 10, 30 and 60 mcg/kg/day, iv) for 14 days did not affect ATIII levels (28). After G-CSF, we observed a mild reduction of natural anticoagulants with a marked tendency of protein C levels. In contrast, a study using higher doses of G-CSF (12.5mcg/kg/day) on 25 healthy donors showed a significant increase in protein C and protein S levels and a slight decrease in ATIII levels (29). Moreover, leukocyte-derived proteases such as elastase, which is vulnerable to G-CSF, have been known to suppress protein C, protein S and ATIII activities via proteolysis (10). The increase in activated neutrophils as a result of G-CSF might have contributed to the reduction in the activities of natural anticoagulants.

The prothrombotic state seldom encountered with the administration of G-CSF to both healthy donors and patients still needs to be clarified. Our study has provided the first evidence demonstrating that G-CSF increases the consumption of extrinsic pathway factors by stimulating coagulation by TF and the production of D-dimer, enhances vWF and FVIII levels by stimulating endothelial cells and reduces the activity of natural anticoagulants. Therefore, we propose that

before administration of G-CSF to both donors and patients with known risk factors, a survey should be undertaken for evidence of inherited thrombophilia, including FV Leiden mutation, which has a quite frequent incidence of 3-10% in the population. In individuals with underlying risk factors (hereditary and/or acquired thrombophilia), the responsible physician should take the adverse effects of G-CSF on hemostasis into consideration.

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JOURNAL OF ANKARA MEDICAL SCHOOL Vol 23, No 3, 2001 221-226

THE RELATIONSHIP POSTTHERAPEUTIC SERUM PROSTATE-SPECIFIC ANTIGEN LEVELS AND OVERALL SURVICAL IN PATIENTS WITH HORMONE-REFRACTORY PROSTATE CANCER

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SUMMARY

The aim of this study was to determine the relation between survival and variations in prostate-specific antigen levels, an objective criterion for estimation of survival in patients with hormone-refractory prostate cancer. Twenty-four patients with hormone-refractory prostate cancer were administered epirubicine 30mg/m2 intravenously weekly for eight weeks and then monthly for four to six months, along with oral administration of 560mg estramustine.

The mean length of survival was 9.3 months in the nine patients (37,5%) whose prostate-specific antigen levels increased during the treatment, 13 months in the eight patients (33,5%) whose prostate-specific antigen levels decreased by 0-50% and 19 months in the seven patients (29%) whose prostate-specific antigen levels decreased by 50% or more. The mean length of survival was significantly longer in patients whose prostate-specific antigen levels decreased by more than 50% when compared to those whose prostate-specific antigen levels decreased by less than 50% or increased ($p<0.001$). It can be concluded that prostate-specific antigen levels can be considered a reliable indicator for prognosis during treatment of hormone-refractory prostate cancer.

Key words: *Hormone-Refractory Prostate Cancer, Prostate-Specific Antigen.*

ÖZET

HORMON REZİSTAN PROSTAT KANSERLİ HASTALARDA TEDAVİ SONRASI PSA DEĞİŞİMİNİN YAŞAM SÜRESİ İLE İLİŞKİSİ

Bu çalışma, hormona rezistan prostat kanserli (HRPK) hastaların yaşam sürelerinin tahmininde objektif bir kriter olan PSA değerlerinin değişimi ile yaşam süresi arasındaki ilişkiyi belirlemek amacıyla planlandı. Hormona rezistan prostat kanserli 24 hastaya sistemik epirubisin tedavisi ile birlikte oral estramustin tedavisi uygulandı. Epirubisin 30 mg /m2 dozunda haftada bir kez sekiz hafta süreyle verildi ve daha sonra 4- 6 ay kadar süre ile ayda bir kez tekrarlandı. İlave olarak 560 mg dozda ve oral yolla estramustin fosfat uygulandı.

Tedavi sırasında PSA sı yükselen 9 hastanın (% 37,5) ortalama yaşam süresi 9,3 aydı. PSA değeri % 0-50 arasında azalan 8 hastanın (%33,5) ortalama yaşam süresi 13 ay olarak tesbit edildi. Buna karşılık PSA sı % 50 den fazla azalan 7 (%29) hastada ise 19 aylık bir yaşam süresi elde edildi.

PSA sı % 50 en fazla azalan hastaların ortalama yaşam süresi daha az azalan veya artan hastalara göre anlamlı olarak yüksek bulundu ($P< 0,001$). PSA hormona refrakter prostat kanserinin tedavisi sırasında prognoz göstergesi olarak güvenilebilir bir parametre olarak kabul edilebilir.

Anahtar Kelimeler: *Hormona Dirençli Prostat Kanseri, Prostat Spesifik Antijen*

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Received: June 19,2001 Accepted: July 17,2001

It has been suggested that prostate-specific antigen (PSA), employed as an objective parameter in diagnosing and evaluating treatment of prostate cancer, can also be used in assessing the response to treatment of hormone-resistant prostate cancer (HRPC). Due to the difficulties and costs involved in assessing the response to treatment according to WHO criteria (1), various studies have attempted to determine the reliability of PSA results by themselves, both for estimating prognosis and for demonstrating the efficacy of drugs used in treatment (2,3).

The aim of the present study was to determine the changes in PSA levels after the administration of epirubicine and estramustine phosphate in HRPC cases and the effect of these changes on prognosis.

MATERIALS AND METHODS

The study comprised 24 patients diagnosed with HRPC between August 1998-January 2001. Data on the pre-chemotherapy status of the patients is outlined in (Table 1).

Patient age ranged from 49-79 years (mean: 66 years). Initially, 13 patients underwent bilateral orchiectomy and 11 patients were administered one of the LHRH analogues. All patients were exposed to maximal androgen blockage, using flutamid or biculamid. Patients with increases in PSA levels and progression in pain

Table 1: Characteristics of Patients Before Treatment

CHARACTERISTICS	
Number of Patients	24
Mean Age	66 years (49-79years)
Previous Treatment	
-Orchiectomy+M.A.B.*	13
-M.A.B. with LHRH analogues	11
Mean Time of Progression	17 months (6-40months)
Measurable Lesions	
-LAP	4
-Liver Metastasis	2
Urinary Obstruction	3
Pain Score	
0	3
1	14
2	7
Mean Pain Score	1.27
Performance Score	
0	13
1	9
2	2
Mean Performance Score	0.52
Mean PSA Value on Diagnosis of HRPC	110 (9-520) (g/ml

* M.A.B. = Maximal androgen blockage

and performance scores during regular controls requested once-a-month visits for PSA analyses and bone scintigraphy. Patients with significant increases in at least two PSA measurements and increases in lesions in bone scintigraphy after the initiation of treatment were considered to have HRPC. Patients treated with LHRH analogues underwent bilateral orchiectomy after being diagnosed with HRPC.

Disease progression ranged from 6 to 40 months (mean 17 months). Patients with a pain score greater than 3 and a performance score greater than 2 according to WHO criteria were not included in the study (1). Patients taking medication for obvious heart disease or serious disorders in renal functions were also excluded, as were six patients whose medication was administered irregularly due to estramustine-related gastrointestinal problems. At the baseline of the study, the mean pain score was found to be 1.27 and the mean performance score 0.52.

Prior to treatment, all patients underwent routine blood analysis, ultrasonography, IVP, lung radiography, PSA and alkaline phosphatase analyses, EKG and bone scintigraphy. Cardiology consultation was requested due to the possibility of cardio-respiratory dysfunction as a result of drug administration.

During treatment, 30 mg/m2 epuribicine was administered weekly for eight weeks and then monthly for four to six months. In conjunction with epirubicine, 560 mg estramustine phosphate was administered orally. Patients were informed about the possible effects of these drugs. A CBC was performed every two weeks during treatment. PSA analysis was repeated every month. Pain and performance scores and PSA results were evaluated within the first month of treatment and subsequently every three months. Estramustine was continued in patients who completed weekly and monthly epirubicine therapy until general performance decreased to a level at which patients could not tolerate oral nutrition. After this stage, therapy was limited to symptom management. The same protocol was applied to patients with increases in PSA levels.

The mean follow-up time was 15.2 months for all patients. Results were evaluated using 't' statistical analysis.

RESULTS

Three months after initiation of treatment, pain and performance scores improved at least one point in all patients. Pain scores decreased from 1.27 to 0.22 ($p<0.001$) and performance scores decreased from 0.52 to 0.08 ($p< 0.001$) (Figure 1).

However, PSA values were observed to increase in nine patients (37.5%), although their subjective complaints improved by at least one point (Table 2). The mean length of survival of these patients was 9.3 months (Figure 2).

PSA level decreased by 0-50% in eight patients (33.5%) and continued to decrease for a mean of five months (2-12 months). The mean length of survival of these patients was 13 months (Figure 2).

PSA level decreased by more than 50% in seven patients (29%). Low levels of PSA were observed to be maintained for a mean of 10 months (7-30 months). It decreased by more than 80% in four patients. Those patients who are still alive have been followed for 12, 15, 18 and 30 months, respectively, and low PSA levels are still maintained in two of these patients. The mean survival of this group is 19 months (12-30 months) (Figure 2).

There was no significant difference in the mean survival between patients whose PSA levels increased and those whose PSA levels decreased by less than 50%. However, there was a significant difference between patients whose PSA levels decreased by more than 50% and those whose PSA levels increased ($p<0.001$).

At the end of six months, pain and performance scores increased in seven patients. Of these, five had high PSA levels and two had PSA levels that had decreased by 0-50%. One patient from each group died at the end of six months. At the end of nine months, 55% of patients had increased pain and performance scores. Two patients with increased PSA levels died. At the

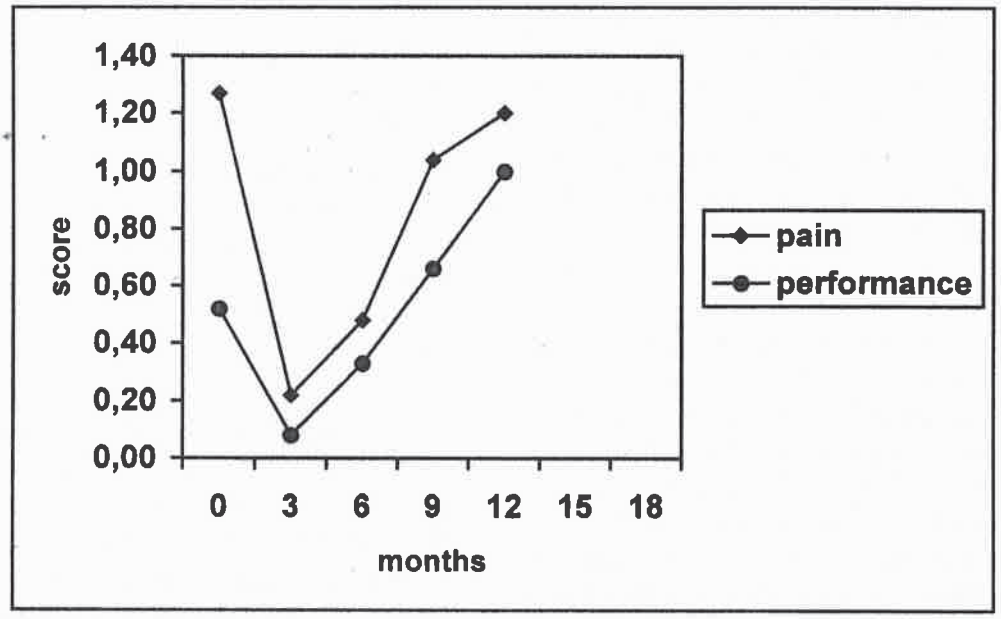

Figure 1: Distribution of Pain and Performance Scores of Patients During Treatment According to Months

end of one year, it was established that subjective complaints increased from a mean of 8.4 months on. Four patients whose PSA levels fell by more than 50% are still alive, and their mean follow-up is 19 months as of the present. PSA levels of two of these patients continue to remain within a normal range, while the other two have normal pain and performance scores but a slight increase in PSA. One of these patients with a decrease of 0-50% in PSA levels in the 15th month of follow-up was found to have normal pain and performance scores but a significant increase in PSA. The average survey of the 19 patients who died due to the disease was 12.7 months.

The decrease in PSA continued for a mean of five months in patients with 0-50% PSA suppression and 10 months in patients with greater than 50% PSA suppression. However, the number of patients was not high enough to perform a statistical analysis. The duration of PSA supression was longer in patients with more than a 50% decrease in PSA levels, particularly in those with more than an 80% decrease.

Four patients whose PSA levels increased or decreased by 0-50%. had feet oedema due to ultrasonographically detected LAP; this regressed for up to six months but recurred later. Two

Table 2: Changes in PSA During Treatment and Mean Survival

Changes in PSA	Number of Patients	Length of Time Changes Sustained	Mean Survival
Increase in PSA	9 (37.5%)	-	9.3 (6-24) months
Decrease in PSA by 0-50%	8 (33.5%)	5 months (mean)	13 (6-24) months
Decrease in PSA by more than 50%	7 (29%)	10 months (mean)	19 (12-30) months

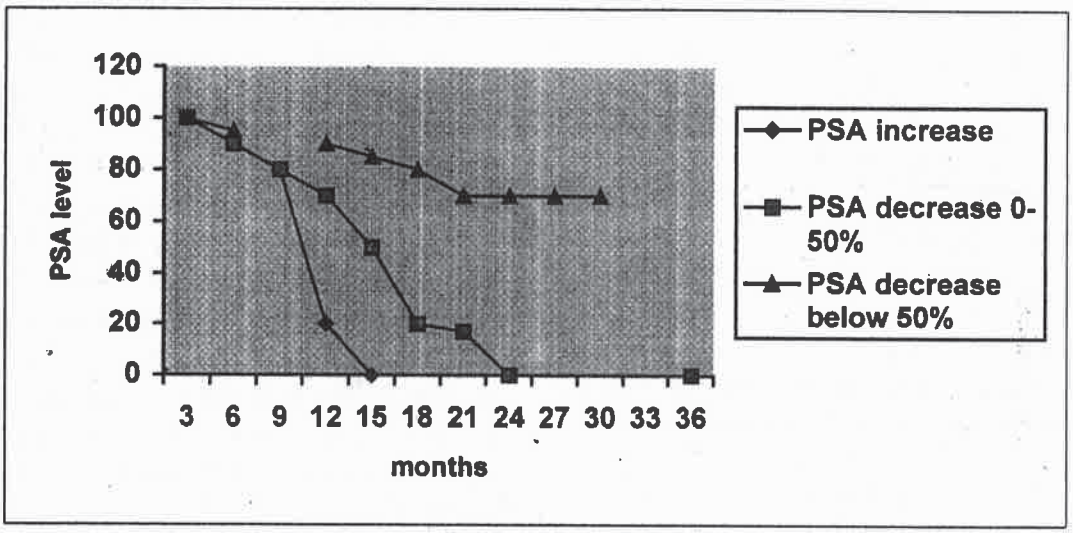

Figure 2: PSA Changes and Survival of Patients

patients whose PSA levels decreased by 0-50% had liver metastasis that did not regress during therapy and died after 12 and 16 months, respectively. Three patients underwent percutaneus nephrostomy due to urinary obstruction. The nephrostomy catheter was removed after 45 days from one of these patients, whose PSA level decreased by more than 50% due to regression of obstruction in antegrad pyelography. The PSA levels of the other two patients increased despite the therapy, with no change in obstruction.

During therapy, two patients experienced cardiotoxic side effects and died as a result of heart failure. Gastrointestinal side effects due to estramustine were observed in eight patients, whose therapy was discontinued for short intervals. Four patients received blood transfusions for anemia. We observed changes in pigmentation in one patient and painless gynecomastie in five patients caused by epirubicine.

DISCUSSION

Since the recognition of the significance of PSA in prostate cancer, PSA has been used in the evaluation of cytotoxic treatment administered in HRPC (4). Nevertheless, the interpretation of changes in PSA levels has not been standardized to date.

Culine et al. observed a decrease in PSA levels of 50% of their 31 patients treated with estramustine and 20 mg/m2 doxorubicine weekly. The decrease continued for three months, and the mean length of survival was 12 months (2). Hermes et al. maintained a decrease in PSA levels of 50% in 54% of their 24 HRPC patients treated with 100 mg epirubicin/m2 and estramustine. The decrease continued for three months, and the mean length of survival was 13.2 months (3). In their 110 HRPC cases, Kelly et al determined that those patients with a minimum 50% decrease in PSA levels at 60 days after initiation of treatment had longer survival rates (5). In his study using estramustine phosphate and vinblastine, Hudes obtained similar results (6). In a Phase II study evaluating 114 patients treated with estramustine phosphate and etoposide, a decrease of more than 50% in PSA levels in the eighth month of treatment when compared to baseline values was associated with an increase in survival length (7). The mean length of survival of patients whose PSA levels decreased by more than 50% was 91 weeks, compared to 38 weeks in the remaining patients in the study. Schultz et al suggest that a decrease of 80% in PSA levels is more valuable than a 50% decrease in determining survival lengths (8).

In the present study, a significant difference was found between patients whose PSA levels increased and those whose PSA levels decreased more than 50%. Moreover, four patients whose PSA levels decreased more than 80% could be followed with normal PSA levels. Thus, consistent with the literature, we can also assert that a decrease of more than 80% in PSA levels is related to higher survival.

The mechanism by which PSA levels decrease is not clearly understood (9). However, PSA is known to be released under the control of hormones. Androgen receptors regulate the expression of PSA by linking with androgen-response components. Other factors that regulate PSA expression include Vitamin D, Transforming Growth Factor, Basic Fibroblast Growth Factor and Proteinchinase C. Any component that influences these agents can affect PSA level, irrespective of cell death. Thus, in order to evaluate the results, changes in PSA level must be investigated in terms of three criteria: the rate of decrease in PSA levels; the time to decrease in PSA levels; and the length of time over which the decrease is maintained. As stated, previously published articles have asserted that a reduction of over 80% in PSA levels has positive impact on survival. Another study showed that a decrease in PSA levels of more than 50% that is maintained for over one year is more significant in terms of survival than a decrease of 80% maintained for two months (9).

In conclusion, PSA may be used as an important objective parameter in evaluating treatment of HRPC.

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CLINICAL EVALUATION INTRAHEPATIC CHOLESTASIS OF PREGNANCY: IMPROVED OBSTETRIC OUTCOMES WITH URSODEOXYCHOLIC ACID THERAPY

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SUMMARY

Aim: *Intrahepatic cholestasis of pregnancy (ICP) is a condition that manifests during the second or third trimester as pruritus, with or without jaundice. Although it is a disease that alters maternal well-being, no severe maternal morbidity or mortality is attributed to it. The purpose of this study was to focus on the clinical features of ICP and to determine the influence of ursodeoxycholic acid (UDCA) on the prognosis of pregnancy in terms of symptomatic relief and obstetric outcome.*

Material-Methods: *Forty-seven patients diagnosed with ICP were enrolled in this study. Of these, 35 received cholestyramine (4gr/day) and 12 received UDCA (750 mg/day). The severity of pruritus was graded before treatment and once a week following treatment.*

Results: *UDCA administration significantly improved fetal prognosis and maternal symptoms ($p<0,05$).*

Conclusion: *Early and accurate detection of ICP is important in order to provide sufficient obstetric surveillance and fetal monitoring. The results of this study suggest that UDCA alters the pathogenesis of the disease and prevents adverse effects on pregnancy.*

Key Words: *Intrahepatic Cholestasis, Pregnancy, Cholestyramine, Ursodeoxycholic Acid*

ÖZET

GEBELİĞİN İNTRAHEPATİK KOLESTAZINDA KLİNİK DEĞERLENDİRME: URSODEOKSİKOLİK ASİD (UDCA) TEDAVİSİNİN GEBELİĞİN PROGNOZUNA ETKKİSİ

Amaç: *Gebeliğin intrahepatik kolestazı, gebeliğin ikinci veya üçüncü trimestirinde kaşıntı ve /veya sarılık ile ortaya çıkan bir hastalıktır. Annenin iyilik halini etkilese de bu hastalığa bağlı ciddi maternal morbidite veya mortalite bildirilmemiştir. Bu çalışma ile hastalığın klinik özelliklerini ortaya koymayı ve UDCA tedavisinin gebelik prognozu ile maternal semptomatik rahatlama üzerine olan etkisini araştırmayı amaçladık.*

Materyal-Metod: *Çalışmaya gebeliğin intrahepatik kolestazı tanısını almış 47 hasta dahil edildi. 35 hastaya kolesteramin (4 gr/gün) ve 12 hastaya da UDCA (750 mg/gün) doğum gerçekleşinceye kadar verildi. Tüm hastalarda tedaviye başlanmadan ve tedavi sonrası da her hafta tekrarlanmak üzere kaşıntının şiddeti derecelendirildi.*

Bulgular: *UDCA tedavisinin maternal semptomları ve fötal prognozu belirgin düzelttiği saptandı ($p< 0,05$).*

Sonuç: *Yeterli obstetrik takip ve fötal monitörizasyon hastalığın erken ve kesin tanısı önemlidir. Bu sonuçlar, UDCA'nın, hastalığın patogenezi üzerine etki ederek gebelik seyirindeki olumsuzlukları önlediğini düşündürmektedir.*

Anahtar Kelimeler: *İntrahepatik Kolestaz, Gebelik, Kolesteramin ,Ursodeoksikolik Asid*

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Received: Sep 14,2001 Accepted: Sept 20,2001

Intrahepatic cholestasis of pregnancy (ICP) is characterised by pruritus in almost every patient with the disease, a minority of which have signs of jaundice. The symptoms usually appear late during pregnancy and disappear after delivery. The disease was first described by Alvar Svanborg and other Scandinavian clinicians as 'Jaundice in late pregnancy'. Later, when it was understood that pruritus rather than jaundice was the most common clinical symptom of this disease, those names that emphasised jaundice were abandoned, and 'intrahepatic cholestasis of pregnancy' or 'cholestasis of pregnancy' became the accepted terms with which to define the disease. High rates of ICP have been reported in Sweden and Chile (3% and 15%, respectively). Although the cause of ICP is unknown, the pathogenesis of the disease seems to be multifactoral, including hereditary predisposition, abnormal hepatic metabolism due to elevated hormones, environmental, and, possibly, dietary factors.

The main consequence of this disease is a high rate of perinatal morbidity and mortality. Serious fetal monitoring should be considered in order to prevent adverse obstetric outcomes. The disease affects the mother mildly, and no maternal mortality has been attributed to it.

Several drugs have been used to relieve maternal symptoms and to correct cholestasis. The most promising results have been reported in connection with the use of UDCA, due to its influence on both prognosis of pregnancy and severity of pruritus. However, delivery provides the only cure for ICP.

Materials and Methods

This study comprised 47 patients with ICP admitted to the Department of Gynecology and Obstetrics at Ankara University School of Medicine (mean patient age: 26.9 years; age range: 19-39). Improvement of symptoms and obstetric outcomes were analysed according to patients' preferred drug therapy.

A diagnosis of ICP was made if the prominent symptom of skin pruritus, with no skin lesions except those caused by scratching, appeared during pregnancy. In addition to routine biochemical and urine examinations, liver function tests (SGOT, SGPT, GGT, Alkaline phosphates, serum proteins, prothrombin time, 5 nucleotidase), hepatitis markers and thyroid functions were checked, and all patients underwent ultrasonographic examination to exclude any hepatobiliary abnormality.

Patients were divided into two groups, according to their choice of cholestyramine or ursodeoxycholic acid (UDCA) for drug therapy. Patient histories are summarised in Tables 1 and 2.

Patients in Group 1 were administered cholestyramine (4gr/day) and those in Group 2 UDCA (750 mg/day) orally up until delivery. The severity of pruritus was graded before treatment and once a week following treatment, according to Ribalta et al (Table 3; Figure 1) (1).

Obstetric prognosis and newborn status of the two groups were also compared, and the differences between the groups were evaluated using student's t test or ANOVA analysis.

Results

Values of biochemical parameters did not differ significantly between the two groups, except in those cases whose dominant symptom was jaundice. However, there was a statistical difference between the two groups in terms of symptomatic relief and obstetric prognosis ($p<0,05$).

Maximum levels of laboratory results for the ichteric form of ICP cases are shown in Table 5. Four patients received cholestyramine and two patients received UDCA treatment. Significant symptomatic relief was observed in those patients treated with UDCA. Due to the limited number of patients in the study, no statistical parameters are provided.

Symptoms in patients in both groups showed improvement, but the improvement in pruritus was higher, according to Ribalta's scale, in the UDCA group (1). All the newborns had apgar scores greater than six at five minutes, and their weights were appropriate for their gestational age. No fetal distress was detected during ante-

Table 1: Patient Histories

	Mean	(n)	Range
Age	26,9		19-39
Parity			1-5
Family History of ICP		5	
History of ICP in previous pregnancy (Table 3)		5	
Twin Pregnancy		2	

Table 2: Distribution of Patients According to Trimester

Trimester	Gestational week	n (total 47)
I	(4-14)	1
II	(15-27)	22
III	(28-40)	24

natal monitoring using a non-stress test, and Caesarean sections in the UDCA group were performed only as a result of cephalopelvic disproportion or patient preference.

Obstetric complications in the cholestyramine group were: premature delivery (n=5), intrauterine growth retardation (n=2), fetal distress (n=8), twin pregnancy (n=1), antenatal urinary infection (n=4), presence of meconium (n=13) and intrauterine exitus (n=1).

Mean newborn weights for Groups 1 and 2 were 2600 gr and 3300 gr, respectively ($p<0.05$).

Table 3: Obstetric History of the Patients with Recurrent ICP

No	Parity	Onset of Pruritus (Week)	Delivery	Newborn Weight (gram)	Presence of Meconium
1 UDCA	2G1P Current P. Previous P.	 36 30	 Vaginal Vaginal	 3600 3000	 + -
2	2G1P Current P. Previous P.	 28 20	 Sectio/Cesarian Intrauterin exitus (IUEx)	 3040 2200	 +
3	2G1P Current P. Previous P.	 30 34	 Vaginal Vaginal	 3350 2750	 - -
4 UDCA	2G1P Current P. Previous P.	 31 28	 Vaginal Vaginal	 3660 2300	 - -
5	5G4P Current P. Previous P.	 28 36	 Vaginal IU Ex	 3570 3600	 + -

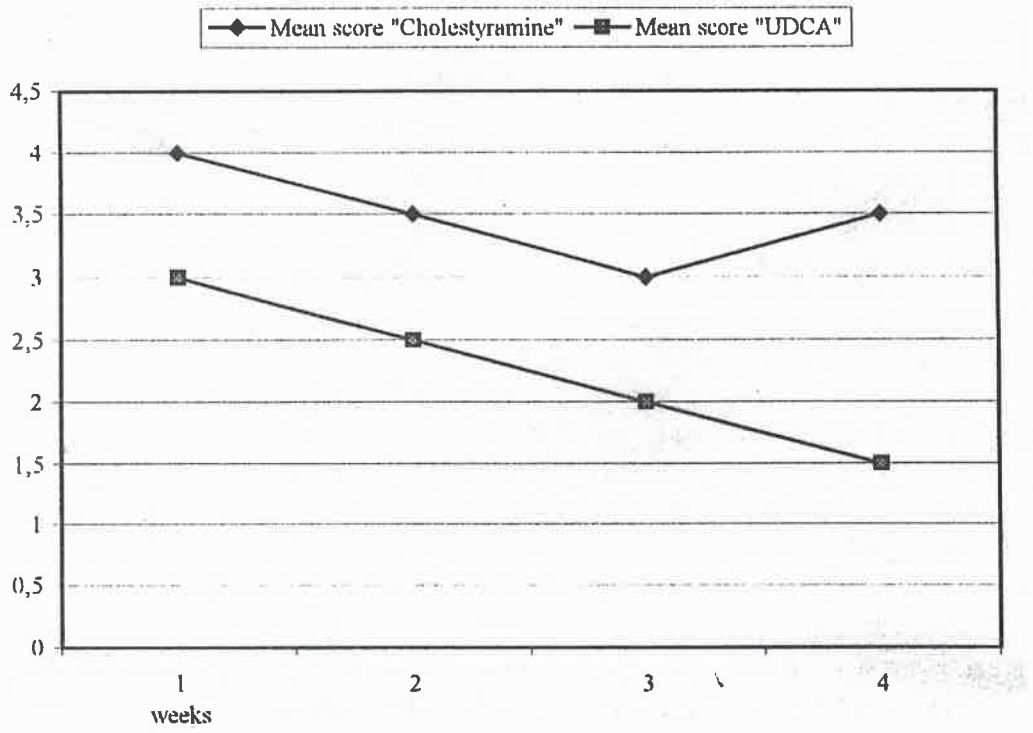

Figure 1: Comparison between cholestyramine and UDCA effects in patinets with ICP

There was no significant difference between the groups in onset of symptoms in terms of mean gestational age or patient age or parity. No correlation was observed between the trimester of pregnancy in which the disease was first diagnosed and the rate of adverse obstetric outcome.

Discussion

ICP is among the hepatic disorders that occur during pregnancy in normal healthy women and resolve after delivery. ICP is also known as recurrent intrahepatic cholestasis of pregnancy, obstetric hepatosis and pruritus gravidarum (2). It is characterised by pruritus, with or without jaundice, and increased levels of serum bile acids. ICP is often associated with premature delivery, fetal distress and perinatal mortality. There have been many studies whose main aim was to show the effect of UDCA treatment on the bile acid pool (3). Although the effect of UDCA on serum

Table 4: Severity of Pruritus

Score	Severity of Pruritus
0	Absence of pruritus
1	Occasional pruritus
2	Discontinuous pruritus every day, prevailing asymptomatic lapses.
3	Discontinuous pruritus every day, prevailing symptomatic lapses.
4	Permanent pruritus, day and night.

Table 5: Maximum Values of Biochemical Cholestatic Criteria of Ichteric ICP Cases (n=6)

Lab Parameters	Normal Ranges	Unit	Maximum Values
SGOT	0-40	U/L	140
SGPT	0-38	U/L	180
T. Bilurubin	0-1,4	mg/dl	2,9
Direct Bilurubin	0-0,25	mg/dl	1,34
Alkaline phosphatase	25-100	IU/L	341
5' Nucleotidase	2-10	U/L	24

steroid sulphate profiles in patients with ICP has been shown, the mechanism behind it is still unclear. UDCA has been found to stimulate biliary excretion of sulphated progesterone metabolites and restore the placenta's ability to carry out vectorial bile acid transfer (4). The impairment in bile acid transport across the placenta during ICP has been shown to reverse with UDCA, accompanied by the apparent clinical improvement in symptoms such as the relief of pruritus (5). Our study showed compatible results. We also found statistically significant improvement in symptoms of patients using UDCA ($P<0.05$) (Figure 1). Because the prognosis of obstetric outcome was significantly better in this treatment group, we believe that there must be an increase in placental bile acid transfer in patients using UDCA. Similar findings have been reported by other authors, who observed that 'deliveries occurred at or near term in all mothers who received UDCA, while in the placebo group, they occurred before 36 weeks of pregnancy, including one intrauterine excitus' (6,7).

Although cholestyramine is used to increase the excretion of bile acids into feces by reducing their enterohepatic circulation, it has not been fully satisfactory in ICP (8). The reported risk of hemorrhage due to hypoprothrombinemia, a result of malabsorbtion of fat and fat-soluble vitamins, was not observed in our patients. We evaluated the prothrombin time of Group 1 at weekly intervals. In spite of the administration of 4gr/day cholestyramine for at least two weeks, we did not observe any abnormal prothrombin time; however, we also administered vitamin K parenterally at weekly intervals to patients receiving cholestyramine for more than two weeks. No hemorrhagic obstetric complication was seen in this group. In Group 2, UDCA was administered at a dose of 750 mg/day (3x1) for at least one week, and significant clinical improvement was observed in almost every patient. No adverse effect was reported on the fetus due to long-term administration of UDCA, which some patients received for as long as four weeks. A previous study showed administration of UDCA for up to seven weeks had no side effects on the newborn, with an apgar score of 9 (9). We believe that by decreasing the passage of bile salts to the fetus, UDCA may improve the outcome of pregnancy (9). Although further investigation is required to establish the usefulness of UDCA in patients with ICP, the clinical improvement in symptoms and fetal prognosis should be taken seriously. In conclusion, although there is currently no specific treatment for ICP, therapeutic measures using UDCA may be of benefit to mother, fetal-placental unit and baby.

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MEDICAL APPROACH TO ECTOPIC PREGNANCY

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SUMMARY

The management of ectopic pregnancy has changed dramatically over the years and a conservative approach now predominates. Many women with ectopic pregnancies are now treated with methotrexate instead of surgery. Randomized studies have demonstrated that in selected cases medical treatment with methotrexate is as effective as laparoscopic surgery. However, medical treatment might have a more negative impact of patients health-related quality of life than surgical treatment. In general, medical treatment with methotrexate is less costly than the surgical approach. All patients treated with methotrexate should be followed up closely until hCG is no longer detectable in the serum. The initial serum hCG concentration is the best prognostic indicator of treatment success in women with ectopic pregnancies who are treated according to methotrexate protocol.

Key Words: *Ectopic Pregnancy, Medical Management, Methotrexate.*

ÖZET

EKTOPİK GEBELİKTE MEDİKAL YAKLAŞIM

Günümüzde ektopik gebeliğin tedavisi değişmiş, konservatif yaklaşım ön plana geçmiştir. Ektopik gebeliği olan pek çok kadın cerrahi yerine metotreksat ile tedavi edilmektedir. Randomize çalışmalar uygun vakalarda metotreksat ile tedavinin laparoskopik cerrahi kadar etkili olduğunu göstermektedir. Bununla birlikte medikal ve cerrahi tedavinin yaşam kalitesi üzerine olan etkileri karşılaştırılmalıdır. Genel olarak metotreksat ile medikal tedavi cerrahi yaklaşıma göre daha ekonomiktir. Metotreksat ile tedavi edilen tüm hastalar serumda hCG değerleri negatifleşene kadar yakından takip edilmelidir. Başlangıç serum hCG konsantrasyonu metotreksat protokolüyle tedavi edilen hastalarda tedavi başarısını gösteren en iyi prognostik faktördür.

Anahtar Kelimeler: *Ektopik Gebelik, Medikal Tedavi, Metotreksat*

Ectopic pregnancy is a life-threatening condition. Recent reports affirm that ectopic pregnancy is becoming a medical disease. This evolution is driven first by increasingly reliable nonsurgical diagnosis. Algorithms using combinations of hormone measurements and gynecologic ultrasound facilitate timely diagnosis and eliminate need for surgical visualization. Second, the evolution is driven by lower costs. Third and finally, medical therapy virtually eliminates surgical complications from treatment.1

Epidemiology:

The incidence of ectopic pregnancy is approximately 0.5-1.0% of all pregnancies but

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Received: Jan 10,2001 Accepted: May 28,2001

this rises to about 5% after assisted conception therapies and 20-30% in women with tubal damage after tubal surgery or a past history of ectopic pregnancy. A past history of pelvic infection accounts for about 40% of ectopic pregnancies and it is argued that women with significant tubal damage should be sterilized before they commence IVF. It is therefore important to understand the modern management of ectopic pregnancy in order to minimize any compromise of future fertility.2

Pathogenesis and Risk Factors:

As previously noted, ectopic pregnancy occurs when the blastocyst implants in a location other than the endometrial lining of the uterus. These pregnancies are generally the result of factors that delay or prevent passage of the fertilized egg into the uterine cavity or factors inherent in the embryo that result in premature implantation.3 Over 98% of ectopic pregnancies occur in the fallopian tube itself. Sites for ectopic pregnancy other than the fallopian tube include the cervix (0.1%), ovary (0.5%), and abdominal cavity (0.03%). Of those ectopic pregnancies confined to the fallopian tube, approximately 93% occur in the ampullary portion, 4% in the isthmic portion, and 2.5% in the interstitial or cornual portion of the tube.4

Numerous risk factors for the development of an ectopic pregnancy have been proposed. The more commonly cited risks include prior pelvic inflammatory disease (PID), previous tubal surgery, intrauterine contraceptive device (IUD) use, previous ectopic pregnancy, in vitro fertilization (IVF), progestin-containing contraceptives, smoking, previous abdominal surgery and induced abortion.5,6

Diagnosis:

Patients with normal intrauterine pregnancies can present with the same symptoms encountered in patients with unruptured ectopic pregnancies. The best way to diagnose ectopic pregnancy is to be highly suspicious and sensitive to its possibility, and to utilize the new tools of diagnosis the quantitative measurement of (-hCG and transvaginal ultrasonography.7 Laparoscopy is necessary only when the diagnosis is in doubt, or when laparoscopy is the technique selected for surgical treatment.8

Classically, the most common presenting symptoms seen with an ectopic pregnancy were pain, vaginal bleeding and amenorrhea. Abdominal pain has been reported to occur in 90% to 100% of ectopic pregnancies and frequently begins far in advance of tubal rupture. Other classical symptoms reported in association with ectopic pregnancy were dizziness, pregnancy symptoms, and vaginal passage of tissue.9 The most common classical finding on physical examination is adnexal tenderness. This finding has been reported to occur in 75% to 90% of symptomatic patients.9 Adnexal mass, uterine enlargement, orthostatic changes and fever are other classical findings.

The production of (-hCG by the trophoblast usually starts 6 days after fertilization, and 3-5 days later, traces of (-hCG can be detected in the circulation. In normally growing intrauterine early gestation, the quickly expanding trofoblast is responsible for a rapid increase in the plasma (-hCG level at a doubling rate approximately every 48-72 hours. The dynamics of (-hCG production may vary in ectopic pregnancy.10 It was reported that 49% of women with ectopic pregnancy had decreasing levels of serum (-hCG and in another 44% of women the rice of the (-hCG level was lower than expressed or there was no increase.11 Only 7% of the subjects in their study had a normal increase of the (-hCG level, which was defined as not less than a 66% when tested every 48 hours or not less than a 114% increase every 72 hours. An abnormal pattern of (-hCG secretion in ectopic pregnancy cannot be distinguished from the one observed in a failing intrauterine pregnancy. On rare occasions, an ectopic pregnancy is found in a woman in whom (-hCG cannot be detected in the serum or urine.12 The (-hCG level decreased rapidly after salpingostomy, and on postoperative day 12 it usually decreased to less than 10% of the preoperative value.13 Although an increasing level or a

plateau of (-hCG levels are obvious indicators of persistent ectopic pregnancy, a slowly decreasing (-hCG level poses a more challenging diagnostic issue.

The highest (-hCG level at which an intrauterine pregnancy could not be seen by a 5-MHz transvaginal sonography was 2,600 mIU/ml, and 800mIU/ml was the lowest level at which an intrauterine pregnancy was detected.14 The sonographic signs of a normally developing pregnancy have been correlated with the serum (-hCG levels. Such a correlation is helpful in distinguishing a normal intrauterine pregnancy from an abnormal pregnancy. It should be noted that the (-hCG levels corresponding to the sonographic findings are much higher if transabdominal sonography is used.

A single measurement of the serum progesterone level may be helpful in identifying a normally developing pregnancy. A level exceeding 25 ng/ml is associated with a viable intrauterine pregnancy, whereas values of less than 5 ng/ml are highly suggestive of a nonviable pregnancy. Progesterone levels between 5-25 ng/ml are inconclusive and thus not helpful in making a diagnosis.15

Recently several other endocrine markers have been used to distinguish a normal pregnancy from an abnormally developing ectopic pregnancy. Inhibin levels have been found to be significantly lower in the serum of women diagnosed with ectopic pregnancy when compared with women who had a confirmed single intrauterine pregnancy.16 Recent interest in creatine kinase as a potential marker in the diagnosis of ectopic pregnancy has come from a finding that the tubal muscularis penetration and damage by the expanding trophoblast may increase the presence of creatine kinase in the serum.17 Saha et al evaluated serum creatine kinase levels and found that the levels in women with ectopic pregnancy were higher than in those with intrauterine pregnancy.18 Whether it can be used to distinguish ectopic pregnancy from non-viable intrauterine pregnancy remains to be seen. Others studied fetal fibronectin levels from cervicovaginal swabs19, and serum vascular endothelial growth factor levels.20 They found that the levels in women with ectopic pregnancy were higher than in the other groups.

Laparoscopy is considered as a "gold standard" in the diagnosis of ectopic pregnancy, and at the same time it enables surgical treatment.

Surgery remains the preferred therapy for ruptured ectopic pregnancy. Although operative laparoscopy has significantly decreased complications compared with laparotomy, there remains an irreducible minimum of morbidity, patient discomfort, and expense intrinsic to surgery and anesthesia. Because nonsurgical treatment bypasses these problems, medical approaches now are preferred primary treatment in many centers.21

Methotrexate:

A folic acid antagonist, methotrexate (MTX) inhibits de novo synthesis of purines and pyrimidines, interfering with DNA synthesis and cell division.22

MTX may be given orally, intramuscularly, or by continuous infusion. When large doses of MTX are needed, leucovorin rescue should be used to salvage any normal cells and prevent toxicity to them. When administrating large doses of MTX intravenously, a large volume of alkaline urine output must be maintained to avoid precipitation of the drug in acidic urine. MTX has been shown to be absorbed from the gastrointestinal tract at doses less than 25 mg/m2, whereas larger doses are usually administered intravenously.23

The two most common methods of administering methotrexate to patients with ectopic pregnancy are the single dose method, based on body surface area, employing 50 mg/m2 without the need for leukovorin rescue, and the multidose regimen of 1 mg/kg of MTX, alternating with 0.1 mg/kg of leucovorin rescue for up to four daily doses of each drug.

Use in Ectopic Pregnancy:

Many uncontrolled studies report that systemic intramuscular MTX therapy and laparoscopic salpingostomy have similar outcomes with

respect to success rates, tubal patency, and reproductive outcome.24-26 Other studies demonstrated that MTX can be administered by intratubal injection or intramuscularly with similar result.27

Multi-dose administration: MTX at a dose of 1 mg/kg is administered as the sodium salt intramuscularly, followed by leucovorin in a dose of 0.1 mg/kg as a calcium salt intramuscularly 24 hours later (Table 1). One injection is given daily. This regimen continued until the hCG level decreases by at least 15% on 2 consecutive days.28 Using this multidose regimen, a success rate of 96% with 100 patients was obtained. None of these 96 patients required more than four doses.28

Single-dose administration: It is less expensive, has fewer side effects, requires less intensive patient monitoring, and has greater patient acceptance.29 Success rate is approximately 94%.30 This success rate is similar to that obtained when a multidose protocol was followed. This method carries a higher risk of persistence, requiring more than one course. MTX 50 mg/m2 is administered intramuscularly. A second dose should be administered if the (-hCG is greater on day 7 than on day 4. Seven studies, one cohort and six case control studies, involving 393 patients were evaluated.21 Although overall success of treatment, measured as no surgical intervention, was 87%, 8.0% of patients required more than one course of MTX. Of the patients considered to be treated successfully (either with one or more doses), tubal patency was found in 81% of the 75 women evaluated. Subsequent intrauterine pregnancies were 61% and ectopic pregnancies were 8% in the 64 patients desiring future fertility in the group treated with either one or more doses of MTX, rates comparable with the variable dose regimen.21

The initial serum hCG concentration is the best prognostic indicator of treatment success in women with ectopic pregnancies who are treated according to MTX protocol.31

However, medical treatment with MTX might have a more negative impact on patients' health-related quality of life than surgical treatment. This is partly because of the long resolution time after MTX treatment. New evidence suggests that combining MTX and mifepristone can shorten this resolution time.32,33

MTX by direct injection: Direct injection delivers MTX to the site of implantation at higher concentrations than can be achieved with systemic administration. Less systemic distribution of the drug decreases toxicity. This approach, however, has the substantial disadvantage of requiring laparoscopic or ultrasonographic nee-

Table 1: Multiple dose MTX protocol8

Day1:	Baseline studies, MTX	1.0 mg/kg im.
Day2:	Citrovorum factor	0.1 mg/kg im.
Day3:	MTX	1.0 mg/kg im.
Day4:	Citrovorum factor, hCG titer	0.1 mg/kg im.
Day5:	MTX, hCG titer	1.0 mg/kg im.
Day6:	Citrovorum factor, hCG titer	0.1 mg/kg im.
Day7:	MTX, hCG titer	1.0 mg/kg im.
Day8:	Citrovorum factor, hCG titer	0.1 mg/kg im.
	Complete blood and platelet counts	
	Renal and liver function tests	
Weekly:	hCG titer until negative.	

Table 2: Single dose MTX protocol

Day1:	Baseline studies, MTX	50 mg/m2 im.
Day4:	hCG titer	
Day7:	hCG titer, complete blood and platelet count.	
	Liver and renal function tests.	
Weekly:	hCG titer until negative.	

dle guidance. Between 1989 and 1997 of 660 cases of ectopic pregnancy treated with MTX by direct injection, only 76% were treated successfully, and some patients required more than one injection of MTX.18 Treatment success rates are still unacceptably low for direct injection of MTX. It has not become a standard treatment for ectopic pregnancy.

MTX by tubal cannulation: This instillation of MTX by hysteroscopically directed tubal cannulation has been described. Risquez et al34 reported resolution of 27 of 31 cases by this method, with the remaining 4 ultimately requiring surgery. Although these results are encouraging this approach seems to have no major advantage over other methods.

Indications and contraindications to MTX therapy:

For carefully selected patients, medical management may avoid a surgical procedure with success similar to the outcome of linear salpingostomy. Before one chooses to use single dose or multidose therapy, a definitive diagnosis of ectopic pregnancy must be made, and it must be determined that the patient desires and eligible for medical management. Because the medical management of ectopic pregnancy is relatively new and because there are many published protocols, there is no absolute consensus on the indication for therapy. Medical management should most likely be reserved for hemodynamically stable patients who have been definitively diagnosed with a small unruptured ectopic pregnancy and who will be compliant with rigorous outpatient follow up. MTX should not be administered to patients with a suspected ruptured ectopic pregnancy or to patients who will not be compliant with frequent office visits to check hCG values.35 The resolution of hCG to a negative value averages about 35 days but may take up to 7 weeks.

A modified version of the American College of Obstetricians and Gynecologists criteria for receiving MTX is listed in (Table 3)23 Relative contraindications to medical therapy include parameters that suggest that a woman is at high risk for treatment failure. These parameters include a high initial hCG level, the presence of fetal cardiac activity, or an adnexal mass (the entire mass, not just the gestational sac) of 3.5 cm. The treatment of women with these characteristics is not absolutely contraindicated, but the patient should understand that the success rate is expected to be lower. Approximately 33% to 40% of patients diagnosed with an ectopic pregnancy are eligible to receive medical management.28,35

Contraindications to medical therapy are listed in Table-IV. MTX is contraindicated if there is evidence of immunocompromision, if there is damage to organs that metabolize MTX, or if the patient is effected by a condition that may be screened with a complete blood. count, liver function tests, and serum creatinine. If the woman has a history of pulmonary disease, she should also be screened with chest radiography. Cases of fatal interstitial pneumonitis have occurred in patients with underlying pulmonary disease after MTX administration.

Table 3: Criteria for receiving MTX23

Absolute indications
Hemodynamically stable without active bleeding or signs of hemoperitoneum
Nonlaparoscopic diagnosis
General anesthesia poses a significant risk
Patient can return for follow-up care
Patient has no contraindications to MTX
Relative indications
Unruptured mass (3.5 cm in greatest dimension
No fetal cardiac motion detected
(-hCG level does not exceed a predetermined value (6000-15000 mIU/ml)

Complications of Methotrexate Therapy

A - Side Effects of MTX Therapy:

The most common side effect observed with the single dose MTX protocol is excessive flatulence and bloating caused by intestinal gas formation. This problem is usually self limiting and handled as previously described. Transient mild elevation of liver function values can occur but rarely exceeds twice the upper limits of normal. These values invariably return to normal within 2 weeks. Stomatitis generally only occurs in patients receiving more than one MTX injection. Viscous lidocaine can be used as needed for symptomatic relief in patients with stomatitis. High doses MTX can cause bone marrow suppression, pulmonary fibrosis, alopecia and photosensitivity.36 Life-threatening neutropenia and febrile morbidity were reported after a single dose

Table 4: Contraindications to medical therapy

Absolute contraindications
Breast-feeding
Overt or laboratory evidence of immunodeficiency
Alcoholism, alcoholic liver disease, or other chronic liver disease
Preexisting blood dyscrasia, such as bone marrow hypoplasia, leukopenia, thrombocytopenia, or significant anemia
Known sensitivity to MTX
Active pulmonary disease
Peptic ulcer disease
Hepatic, renal or hematologic dysfunction
Relative contraindications
Gestational sac (3.5 cm
Embryonic cardiac motion

and three doses of intramuscular MTX requiring hospitalization for 1 month and 13 days respectively.37 Two cases of transient pneumonitis were reported from MTX therapy for ectopic pregnancy.38,39

B - Separation Pain:

Approximately 75% of patients will experience an episode of increased abdominal pain during treatment Although the etiology of this pain is unknown, the most logical explanation is that the pain results of hematoma formation.3

C - Hematoma Formation:

Following treatment with MTX, 56% of ectopic masses will increase in size if followed by ultrasound. Most of these patients are asymptomatic. These masses will frequently persist after the disappearance of hCG, with the longest documented time to resolution being 180 days.40

Conclusion:

When ectopic pregnancy is managed in a correct way it will become a medical disease where surgical treatment is necessary only when there is a delay in diagnosis and complication is present.

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JOURNAL OF ANKARA MEDICAL SCHOOL Vol 23, No 3, 2001 241-245

TWO SIBLINGS WITH FRAGILE X SYNDROME

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SUMMARY

The fragile X syndrome is the most common specific cause of familial mental retardation. It is associated with fragility at Xq27.3. Because of variations in route of inheritance and clinical features, it can only be diagnosed through genetic studies. However, cytogenetic expression levels of fragility in lymphocytes show variation, and it is not always possible to identify affected males with a lower degree of fragility expression using cytogenetic techniques.

In this study, two siblings with Martin-Bell phenotype are reported. Although they had typical clinical findings and positive family history, fragility at Xq27.3 could not be demonstrated in the first cytogenetic studies. Two years later, in repeated cultures of the siblings, 2%- and 5%-fragility was found. The conflicting results of repeated cultures confirmed that the expression variability of fragility might be the reason for false negative cytogenetic results in affected patients with low fragility ratios.

Key words: *Fra (X)(q27.3), Mental Retardation, Martin Bell Phenotype*

ÖZET

FRAJİL X SENDROMU

Ailesel mental retardasyonu nedenleri arasında ilk sırada yer alan Frajil X sendromu, Xq27.3 bölgesindeki frajilite ile birliktelik gösterir. Kalıtım ve klinik bulgularındaki değişkenlik nedeni ile kesin tanısı ancak genetik çalışma ile koyulabilir. Ancak, sitogenetik ekspresyon düzeyleri de değişkenlik göstermektedir ve düşük ekspresyon düzeyi olan hastalara sitogenetik yöntemlerle her zaman tanı koyulamayabilir.

Bu çalışmada, Martin Bell fenotipi gösteren iki olgu sunulmaktadır. Tipik klinik bulgularına ve pozitif aile öyküsüne rağmen ilk sitogenetik çalışmada Xq27.3'te frajilite gözlenememiş, ancak iki yıl sonra tekrarlanan lenfosit kültürlerinde 2% ve 5% oranlarında frajil X saptanmıştır. Bu olguların tekrarlanan kültürlerindeki çelişkili sonuçlar, düşük frajilite oranlarına sahip hastalarda frajilite değişkenliğinin yanlış negatif sonuçlara neden olabileceğini desteklemektedir.

Anahtar Kelimeler: *Fra (X)(q27.3), Mental Retardasyon, Martin Bell Fenotipi*

The fragile X syndrome is the most common cause of familial mental retardation (1). This X-linked mental retardation is associated most typically with conspicuous facial features of high forehead, prominent lower jaw and large ears. Patients with fragile X syndrome may have mild connective tissue abnormalities that lead to fine skin, hyperextensible metacarpophalangeal joints and mitral valve prolapse. Another characteristic finding is macroorchidism, which becomes prominent after puberty (2). Many patients have behavioural difficulties such as hyperactivity,

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Received: Aug 24,2000 Accepted : Feb 08,2001

poor eye contact, short attention span, hand flapping and/or biting, cluttered speech and tactile defensiveness (3).

The syndrome is associated with fragility at Xq27.3a, a rare folate-sensitive fragile site (FSFS). Abnormality is demonstrated under specific conditions. FSFSs are induced by thymidylate stress, which may occur as a result of thymidine/folate deficiency or addition of methotrexate into the medium (4). Only a fraction of cells express this fragility, thus necessitating the screening of a large number of cells. The degree of expression in affected males varies from less than 4% to more than 50%. At least two fragile X-positive cells are necessary for diagnosis (5). The number of cultures and cells required for analysis depends on phenotype and family history.

CASE REPORTS

Case 1

A five-year-old boy was admitted to the hospital because of tonic seizures during sleep. Physical examination revealed macrocephaly, with a head circumference of 53 cm (mean value for this age in Turkey: 49.7±1.2 cm) (6), large ears and large testicles (1.5x2 cm.). Distal phalanxes of index and middle fingers were hypoplastic. The patient was hyperactive and uncooperative, giving an impression of mental retardation. He had mild autistic behaviour characterised by difficult eye contact and poor verbal communication.

The patient was the first child of unrelated healthy parents born after an uneventful pregnancy and delivery. The parents reported that his developmental milestones were late: walking without help, speaking, control of urinary and anal sphincters were gained at two years of age.

On laboratory examination, the patient's blood and urine paper chromatography for aminoacid, blood T3, T4, TSH levels, computerized axial tomography of brain and X-rays of hands and feet were found to be normal. EEG showed irregularity on the background activity and bilateral synchronous paroxysmal discharge of 1-2 Hz sharp and slow waves. Neuropsychological examination with Stanford-Binet test showed an IQ of 62.

Ten months later, the parents reported two more seizures. The patient was put on 30 mg/kg/day valproate treatment, and had no seizures for the following two years. However, his parents stopped giving him valproic acid because he refused to take the medicine. Two months later, his seizures recurred, and he began taking medicine again.

During that two-year period, the patient's appearance became more characteristic of fragile X. In addition, he had a brother with a similar appearance. Because of the patient's typical findings and positive family history, he was re-evaluated. On laboratory examination at that time, EEG showed paroxysmal activity, magnetic resonance of the brain revealed hypomyelinization, and Stanford-Binet test showed an IQ of 70.

Case 2

The second child of this family, an 18-month-old boy, came to our attention because of his brother. After an eventful pregnancy, this child was born by Caesarean section, due to delayed effective uterine contractions. Like his older brother, his development was also delayed, with the following milestones: his first word came at 12, he walked with help at 16 months, he was still not able to walk without help at admission.

On physical examination, he was found to have large ears, despite his normal head circumference of 48 cm (N: 47±1.3 cm) (6). He appeared to be hyperactive, and a Denver Developmental Screening test showed his development as abnormal.

On laboratory investigation, blood and urine aminoacid levels were normal. Magnetic resonance imaging (MRI) findings were compatible with hypomyelinization.

Because of his brother's typical findings, the decision was made to analyze the chromosomes of both children for fragile X.

Cytogenetic Analysis and Results

Cytogenetic analyses were done on peripher-

al blood lymphocytes. Whole blood was cultured 72 hours at 37C in 5 ml folate-depressed medium prepared according to the following protocol: 100ml M199 w/o folic acid, 5 ml fetal calf serum, 5 ml phytohemagglutinin, 100 IU/ml penicillin, 100 g/ml streptomycin. 0.04 g/ml colchicine was added at the 71st hour (7). Two hundred giemsa (5%) stained metaphases were analysed for each patient. Slides were decolored after screening, and GTG banding was performed on metaphase chromosomes to define the fragile chromosomes.

Fragility at Xq27.3 was not found at first time. However, when cultures were repeated two years later, 2% and 5% fragility was observed in Case 1 and Case 2, respectively (Fig. 1). The mother's chromosomes were normal in 200 metaphases obtained from peripheral lymphocytes under folate-depressed culture conditions.

DISCUSSION

After the discovery of the association of Martin-Bell phenotype with fragility at Xq27.3 (8), early diagnosis of this most frequent heritable cause of mental retardation has become more important. A better knowledge of the clinical characteristics and variations are necessary to recognize this condition. This may lead to early diagnosis, giving families the advantages of better help, more information and genetic counselling, including prenatal diagnosis.

Fragile X syndrome can only be diagnosed through genetic studies. However, significant variation has been reported for cytogenetic expression of fragility (9, 10, 11, 12, 13). Fragile-site expression is strongly correlated with the repeat number of unstable trinuclotide (CCG) sequences at Xq27.3, which vary in length (14). Variability is a result of amplification, which may be due to unequal cross-over during female meiosis (15, 16). However, recombination can also occur during mitosis of somatic cells (16, 17). Slippage of Okazagi fragments at DNA synthesis may be responsible for the variation of repeat numbers among the different cells of an individual (18). Repeat number variation may cause somatic mosaicism, which may result in heterogenous expression of fragility (18). It has also been noted that sampling from different tissues may be responsible for variations of expression in an individual (19). Steinbach et al reported that expression is generally higher in peripher-

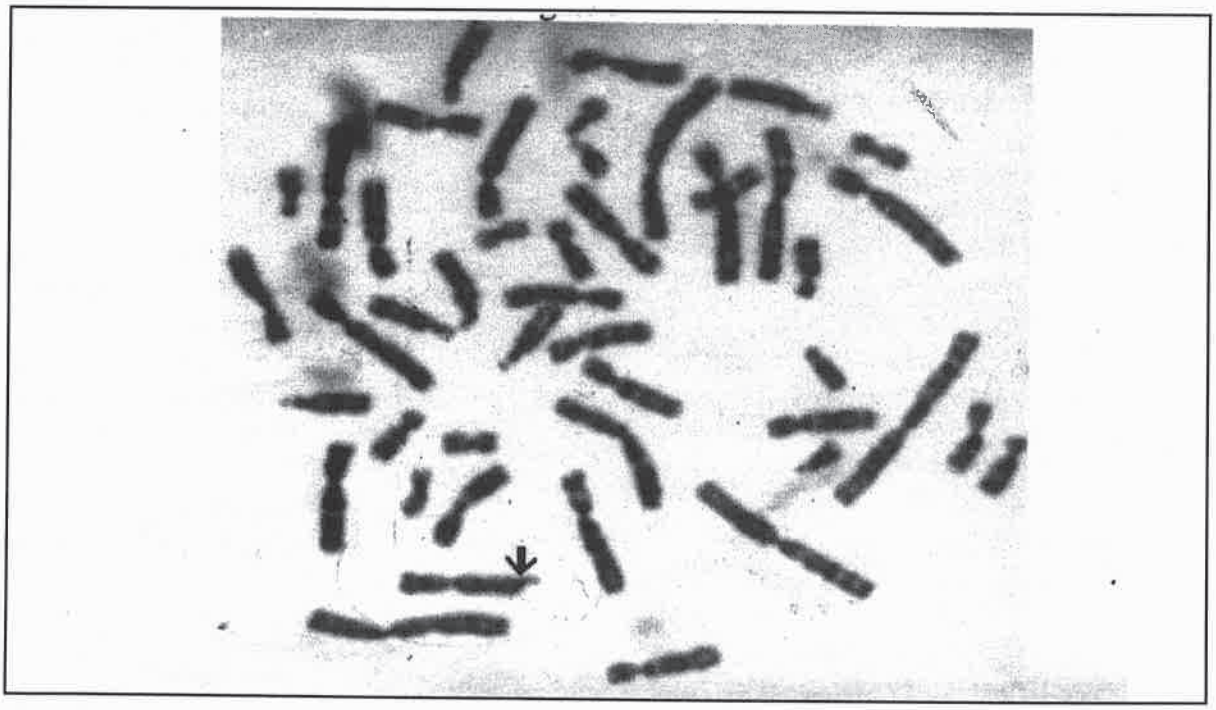

Figure 1: Fragile X chromosome in one metaphase from Case 2.

al blood lymphocytes (20). Nevertheless, fragile X has been found in different cells, indicating that it is not a cell-specific phenomenon (21). Cantu and Jacobs suggested that the fragile site could not be expressed in all cell lineages of an individual. It has also been noticed that the occurrence of fragile X in a cell did not indicate its presence in previous divisions (10). As a matter of fact, a very small difference in expression from time to time in the same individual's lymphocytes has been reported (22). Although Silverman et al (23) reported that expression of fragile X in more than four percent of cells was precisely associated with Martin Bell phenotype, it is not possible to define the lowest expression limit for Martin Bell phenotype. Tommerup et al reported that some affected males with a lower degree of fragility could not be identified using cytogenetic techniques (24).

Our conflicting results from repeated cultures confirmed that the expression variability of fragility might be the reason for false negative cytogenetic results in affected patients with low fragility ratios.

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A CASE OF OPHTHALMIC ANENRYSM CAUSED BY GUNSHOT INJURY

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SUMMARY

This article presents a case of ophthalmic artery aneurysm and briefly reviews the literature on the subject. A patient was admitted to our hospital with loss of vision due to a gunshot injury. A plain radiography revealed an arrow-shaped bullet in the skull. Cerebral angiography confirmed a diagnosis of ophthalmic artery aneurysm, and surgery revealed a false traumatic ophthalmic aneurysm.

Key Words: *Aneurysm, Ophthalmic Artery, Trauma*

ÖZET

ATEŞLİ SİLAH YARALANMASININ NEDEN OLDUĞU OFTALMİK ARTER ANEVRİZMA VAKASı

Bu yazıda travma kaynaklı bir oftalmik anevrizma vakası sunulmuştur.Hasta kliniğimize ateşli silah yaralanmaya bağlı görme kaybı ile başvurmuştur.Direk kafa grafisinde sivri uçlu kurşun parçası görülmüştür.Yapılan serebral anjiografi ile oftalmik arter anevrizması tanısı konulmuştur.Operasyon sırasında oftalmik arterde yalancı anevrizma ile karşılaşılmıştır.Bu konudaki literatüre bilgileri kısaca tartışılmıştır.

Anahtar Kelimeler: *Anevrizma, Oftalmik Arter, Travma*

Traumatic aneurysms are rare, representing only 0.04%-0.09% of all cerebral aneurysms. They usually involve either the peripheral cerebral arteries or the basal portion of the internal carotid artery. Several theories have been proposed to explain the pathogenesis of traumatic aneurysms.(1, 2) Digital subtraction angiography provides the standard for diagnosing traumatic aneurysms, and the primary treatment is surgical. The purpose of this study is to add to the literature a case of traumatic ophthalmic aneurysm and to briefly discuss the pathogenesis, possibilities of early diagnosis and treatment modalities available for traumatic ophthalmic aneurysms.

Case Report

A previously healthy 19-year-old male presented with loss of vision in the left eye. One week before admission, he had received a gunshot injury to the left eye inflicted by a toy rifle at a shooting gallery, after which he developed a progressive loss of left-eye vision. Physical examination revealed exophthalmus and chemosis of the left eye. A neurological examination revealed

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Received: June 02,2000 Accepted: Jan 08,2001

visual acuity at the level of perception and projection only. Plain skull radiographs revealed an arrow-shaped bullet in the skull. Because of the possibility of aneurysm, a digital subtraction angiography was performed, after which an aneurysm in the orbital part of the ophthalmic artery was diagnosed. (Fig1) Following diagnosis, the patient was prepared for surgery, and a left frontal craniotomy and orbital unroofing was performed. The optic canal was drilled, and the optic nerve and ophthalmic artery were identified in the orbita. The ophthalmic artery was followed distally in the orbita, and an aneurysm and laceration of the full arterial wall were discovered, both of which were occluded by a hematoma. The distal part of the proximal ophthalmic artery was clipped. The patient's neurological status did not change after the operation.

Discussion

Cerebral aneurysms are usually classified as congenital, arteriosclerotic, mycotic or traumatic. Traumatic intracranial aneurysms most frequently occur after blunt head injury, with only 10% resulting from projectile injury and 4% resulting from non-projectile penetrating head injury.(3, 4, 5)Traumatic intracranial aneurysms are usually false aneurysms. Although rare, true traumatic intracranial aneurysms have also been noted after head injury.(6) In sharp contrast to congenital aneurysms, over 50% of traumatic aneurysms occur in patients under 30 years of age. Traumatic intracranial aneurysms frequently involve the distal cerebral vascularite.(7, 8, 3, 5, 10) Iatrogenic trauma to cerebral arteries during intracranial surgery is also a cause that is well-recognized. Aneurysms occurring after a penetrating gunshot wound injury to the orbita are rare.(1, 2, 11)

Figure 1: Digital subtracted left carotid angiogram, lateral projection, showing left ophthalmic artery, arrow-shaped bullet and aneurysm caused by gunshot injury.

Traumatic aneurysms may also be differentiated on a histological basis into true, false, mixed and dissecting aneurysms. True aneurysms arise when the arterial wall is only partially disrupted; the internal elastic lamina and media are damaged, but the adventitia remains intact, forming the outer wall of the aneurysm. A false traumatic aneurysm is the most common and results from laceration of the full arterial wall, which is occluded by a hematoma; subsequent fibrous organization and hemodynamic excavation of the hematoma result in aneurysm development.(2, 4) Traumatic cerebral injuries have an unpredictable course and may undergo spontaneous thrombosis, enlargement, or catastrophic rupture.(2, 11, 12) Digital subtraction angiography is standard for diagnosis, but other methods such as plain radiographies, computerized tomography and magnetic resonance imaging should be employed. Traumatic aneurysms are distinguished on cerebral angiography by a broad base, irregular appearance, slow filling and delayed emptying.(4, 11)

Our report presents a case of traumatic ophthalmic artery aneurysm after a penetrating projectile injury. The first case of a traumatic superior cerebellar artery and ophthalmic artery aneurysm was reported by Ferry and Kempe in 1972. Ligation of the ophthalmic artery and superior cerebellar artery was performed with a good outcome, leaving the patient with a blind left eye.(12) The second case of traumatic artery

aneurysm was reported in a 15-year-old following a blunt head injury, which was treated by clipping the ophthalmic artery just proximal to the aneurysm. (9) Although spontaneous healing of post-surgical aneurysms and post-traumatic aneurysms has been reported, we recommend that a patient with a traumatic aneurysm undergo definitive surgical treatment whenever possible because of the unpredictable course of this entity and the high mortality rates associated with hemorrhage resulting from the progressive increase in the size of the sac.

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MALIGNANT OPTIC GLIOMA OF CHILDHOOD

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SUMMARY

Optic glioma is known as a tumor of childhood. However, malignant glial tumors of the anterior visual pathway are rarely encountered in children, but in the elderly. The tumor presents with visual deterioration. Malignancy may occur primarily, or a preexisting low-grade glioma may become malignant following factors such as radiation therapy.

A 6-year-old girl presented with visual deterioration for about two months. Radiological evaluation revealed an intra, para and suprasellar lesion. The patient was operated on through a right pterional craniotomy and a subtotal tumor resection performed. The histopathologic diagnosis was malignant glioma of the optic chiasma.

Although considered to be a pathology of adults, malign optic glioma may occur in childhood. Presentation may occur with visual deterioration or endocrinological abnormality. Therapeutic intervention is similar to that of the management combination for a malignant glial tumor of the cerebral cortex and optic glioma.

Key Words: *Malignant Optic Glioma, Optic Glioblastoma Multiforme*

ÖZET

MALİNG OPTİK GLİOMA

Optik glioma genellikle çocukluk çağı tümörü olmasina rağmen anterior vizual yolun malign glial tümörleri az rastlanan bir patolojidir ve genellikle ileri yaşlarda ortaya çikarlar. Görmede bozulmayla kendini gösterir. Malignensi primer olarak ortaya çıkabilir veya düşük grade'li gliomada radyoterapi gibi bazi faktörleri takiben malign tümöre dönüsebilir.

6 yaşında kız çocuğu 2 aylık görme bozukluğu şikayetiyle başvurdu. Hastanın radyolojik değerlendirmesinde sellar, parasellar ve suprasellar lezyon tespit edildi. Hasta sağ pterional kraniotomiyle opere edildi. Subtotal tümör rezeksiyonu yapıldı. Histopatolojik tanı optik kiazmanın malign gliomu olarak geldi. Bu patoloji ileri yaşlarda seyrekde olsa görülebilir, fakat çocukluk çağında oldukça nadirdir. Hastalık görme bozukluğu veya endokrinolojik anormalliklerle kendini gösterir.

Anahtar Kelimeler: *Malign Optik Glioma, Optik Glioblastome Multiforme*

A malign optic glioma is a rather rare tumor that usually develops in adults. The tumor is located predominantly in the optic chiasm and presents with progressive loss of vision. In a verbal pre-school child, typical presentation is visual impairment with optic canal enlargement and optic atrophy. The clinical course is extremely fatal. In a review article Taphorn et al. reported 30 cases of malign optic glioma (1). Diagnosis depends on neuroradiological and opthalmological findings (2). We present a child with histologically confirmed malign optic glioma.

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Received: June 30,2000 Accepted: Dec 04,2000

Case Report

A 6-year-old girl complained of progressive deterioration of vision in both eyes for two months. On examination, she was cooperative and alert. Opthalmological examination revealed ambylopia of the right eye. Visual acuity was decreased in both eyes to 5/20, and a visual field examination showed bitemporal hemianopsia, indicating the involvement of the chiasm. Optic discs were normal. On T1-weighted Magnetic Resonance Imaging (T1W-MRI), enhanced with gadolinium, a lesion was observed in the chiasmatic region, extending both upward and downward and heterogenously. In the center of the lesion there was a hypointense region, indicating necrosis (Figures 1a, 1b). A malignant glioma of the optic tract was suspected, and a right pterional craniotomy was performed with the partial resection of the tumor. As expected, the tumor arose from the chiasma and infiltrated both temporal lobes and the hypothalamus. The part infiltrating the hypothalamus and temporal lobes was dissected and resected. There were necrotic and hemorrhagic areas in the center of the tumor. Histological evaluation revealed a glioblastome multiforme with anaplastic hyperchromatic cells, atypical mitoses, vascular proliferation and areas of necrosis. In the postoperative course, the patient developed abundant metabolic disorders, including resistant hypernatremia (up to 175mg/dl) and diabetes insipidus, and her general condition worsened. She died on the postoperative sixth day.

Discussion

Gliomas of the anterior visual pathway are rare orbital lesions occurring principally among children in the first decade of life. They appear to be true neoplasms that characteristically show early growth, followed by stability in many patients. Visual prognosis is fair, and the outlook for life depends on tumor location. When initially confined to the optic nerve alone, overall mortality is about 5%. Once the hypothalamus becomes involved, mortality rises sharply to 50%. With involvement of the chiasm or hypothalamus, no form of therapy significantly alters the final outcome. Because of their indolent course, gliomas may be conservatively followed when confined to the optic nerve. In these cases, surgery is indicated only when blindness and pain or severe proptosis intervene. However, all such cases should be followed radiologically for evidence of posterior extension. When the chiasm is threatened, surgical excision is warranted to prevent subsequent hypothalamic or third ventricle involvement.

Malignant optic glioma is a distinct disease primarily affecting middle-aged adults. The chiasm is always involved, and rapid progression to bilateral blindness is usual. The disease is uniformly fatal (3,4). When occurring in a verbal pre-school child, the typical presentation is visual impairment with optic canal enlargement and optic atrophy. An intraorbital location leads to axial irreducible, nonpulsatile proptosis, whereas an intracranial location may disturb hypothalamic and pituitary function and produce hydrocephalus. Ocular findings may include limited motility, a pupillary-related afferent defect, nystagmus and nonspecific field defects. Roentgenographic studies may show concentric unilateral enlargement of the optic canal with preservation of a well-corticated margin, a fossa under the anterior clinoid process in continuity with the optic canal (J-shaped sella) and increased intracranial pressure (3,5).

Malignant gliomas of the optic nerve and chiasm are extremely rare, but may develop after radiotherapy for a preexisting malignancy (6). Although malignant optic glioma patients receive radiotherapy and chemotherapy, these measures do not significantly improve survival rates. The infiltrative nature of the tumor into the normal brain and the presence of tumor foci in regions far from the main tumor burden make cure using current therapies virtually impossible. Management therefore consists of tumor control and maintaining the patient's quality of life. Craniotomy decreases the overall tumor burden and provides room for normal brain, edema and recurrent tumors (3,6,7,8,9).

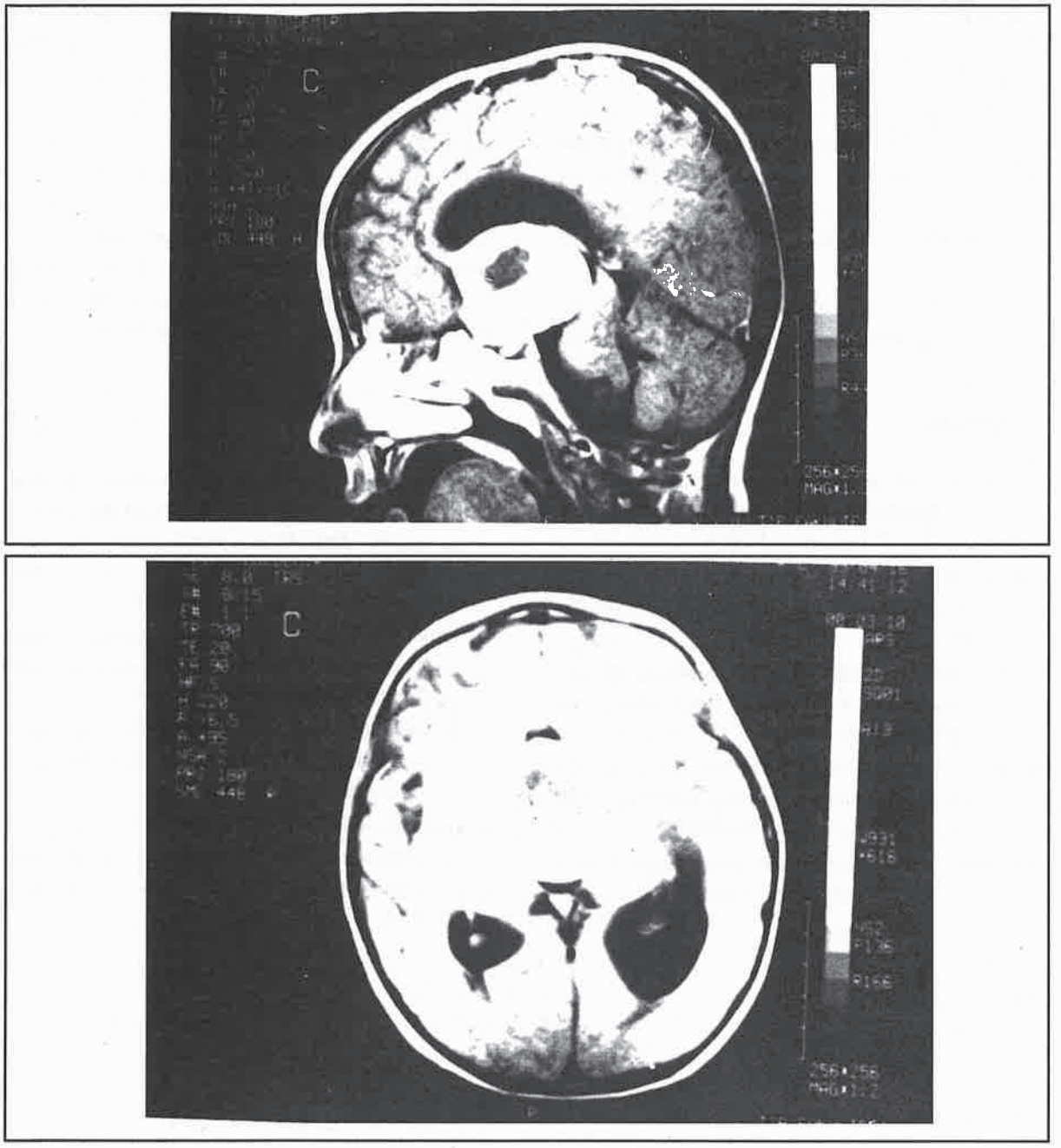

Figure 1: TIW MRI (a: sagital, b: coronal) sections of the tumor enhanced after GDTPA administration; the central hypointensity, indicating necrosis, should be noted.

Our case is unique in that the malignant form of the tumor is extremely rare in childhood. Presentation and radiological evaluation is no different than in adult cases, but the postoperative course is worse in children than in adults. Progression is similar to that of a craniopharyngioma infiltrating the hypothalamic region; therefore, a surgeon who opts for a radical resection of the lesion must keep in mind the presence of postoperative metabolic changes and may require the assistance of neuroendocrinological expertise.

Careful pre- and postoperative neuroendocrinological and opthalmological evaluation is necessary. Since our patient did not survive the early postoperative course, we cannot evaluate how radiotherapy and chemotherapy might have affected the outcome. However, the literature on adult cases of malign optic glioma indicate such measures have little effect on increasing survival rates.

In conclusion, childhood malign optic gliomas are rather rare and present with a variety of visual problems. Once the lesion infiltrates the hypothalamus, the course is usually fatal. The postoperative course may be complicated by endocrinological abnormalities that increase morbidity and mortality. Postoperative radiochemotherapy has not played a significant role in affecting the general outcome. However, surgical planning may affect the postoperative course. Attempts at total resection may increase morbidity and mortality by decreasing vision and causing postoperative endocrinological problems.

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MORGAGNI HERNIA: AN UNCOMMON CAUSE OF INTESTINAL OBSTRUCTION IN ADULT

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SUMMARY

We present a patient with intestinal obstruction due to Morgagni hernia. The case was preoperatively diagnosed as a diaphragmatic hernia by direct chest and abdominal x-rays. An incarcerated Morgagni hernia was found in laparotomy and repaired with primary sutures.

Key Words: *Morgagni Hernia, Diaphragmatic Hernia, İntestinal Obstruction.*

ÖZET

Bu yazıda, Morgagni hernisine bağlı bir barsak tıkanıklığı olgusu sunulmuştur. Vaka, preoperatif olarak, PA akciğer grafisi ve direk karın grafisiyle diafragmatik herni tanısı aldı. Laparotomide inkarsere Morgagni hernisi saptandı ve primer onarım yapıldı.

Anahtar Kelimeler: *Morgagni Hernisi, Diafragma Hernisi, Barsak Tıkanıklığı.*

The Morgagni hernia is the rarest type of all diaphragmatic hernias (1). Also known as anterior diaphragmatic, retrosternal, parasternal, and Larrey's hernia, it has been referred to primarily as a Morgagni hernia since it was first described by Morgagni in 1761. (2-4). The present report illustrates a case of intestinal obstruction due to incarcerated Morgagni hernia and includes clinical presentation, diagnostic procedures and surgical approach.

Case Report: A 70-year-old male patient with a history of intestinal obstruction was admitted to the emergency room with a three-day history of vomiting and abdominal distension. His blood pressure was 100/70 mmHg, pulse rate 110/min, temperature 38°C, respiratory rate 15/min, white blood count 11000/mm3 and hemoglobin 13g/dl. The patient was moderately dehydrated and had a distended, tender abdomen with hyperactive bowel sounds. The lungs were normal to auscultation. Posteroanterior and lateral chest x-rays showed dilated bowel loops above the diaphragm in the anterior mediastinum and in the abdomen (Figure 1). The patient had no past history of trauma.

A diagnosis was made of mechanical intestinal obstruction due to diaphragmatic hernia. Following preoperative resuscitation, the patient was taken to the operating room. An exploratory laparotomy was performed through an upper midline abdominal incision, and a Morgagni hernia involving the right diaphragm was found. The transverse colon and the greater omentum occupied the hernia and were incarcerated. The hernia content was reduced, and the previously incarcerated bowel segment was detected in order to observe its viability. The diaphragmatic defect was sutured using interrupted polypropy-

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Received: Nov 17,1999 Accepted: Oct 26,2000

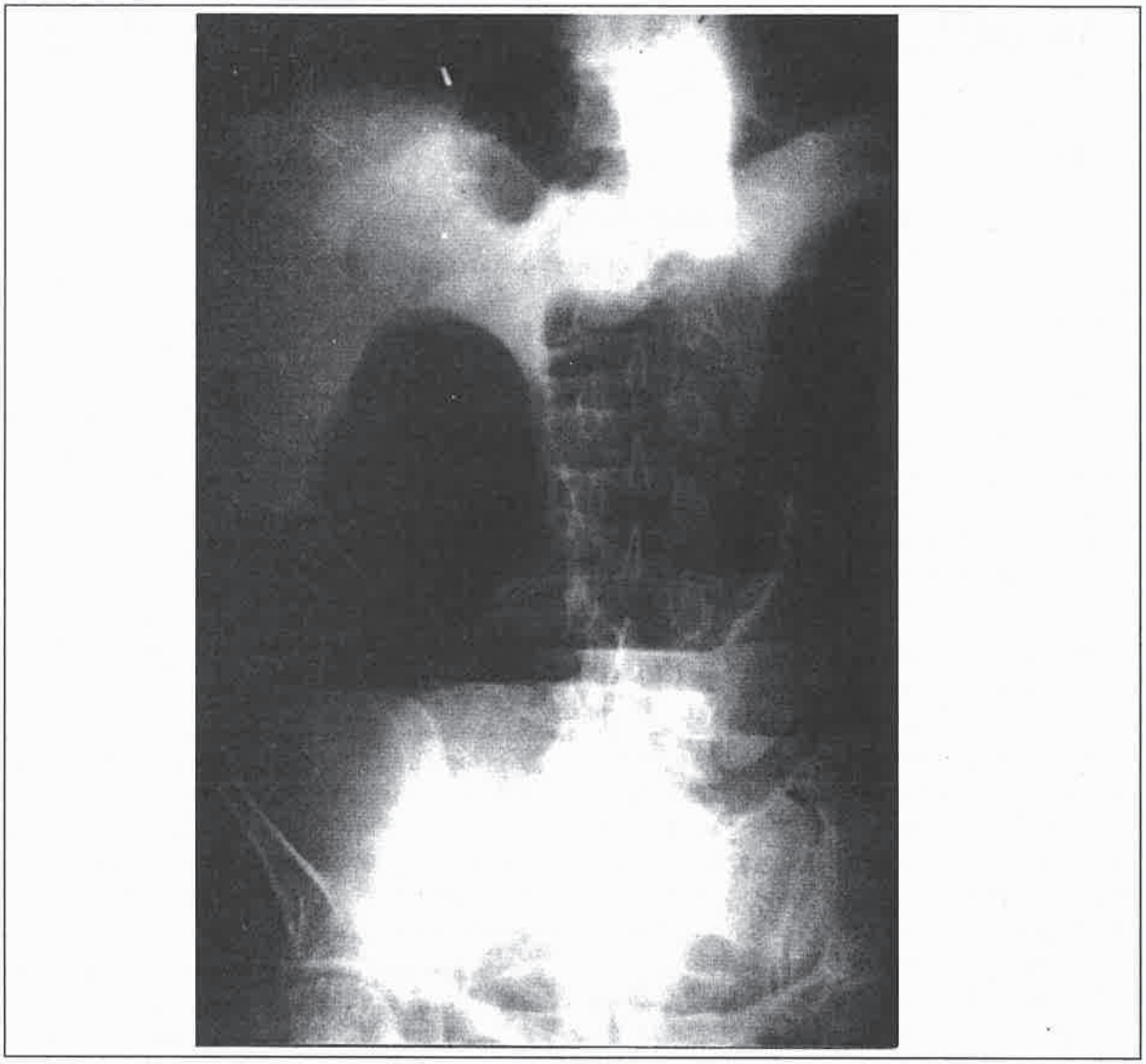

Figure 1: An abdominal x-ray shows a distended bowel loop in the paraesophageal area, air-fluid levels and distended bowel loops in the abdomen.

lene sutures. The postoperative period was uneventful, and the patient was discharged on the tenth postoperative day. The patient is doing well at the end of the first postoperative year.

Discussion: Approximately 20% of all surgical operations for acute abdominal conditions are due to intestinal obstruction (5). Incarcerated Morgagni hernia is a rare cause of intestinal obstruction. The incidence of Morgagni hernia is 3% among diaphragmatic hernias (3,4), with 90% bilateral, 8% right-sided and 2% left-sided (2-4,6). The rarity of an isolated left-sided Morgagni hernia defect is most likely due to the reinforcing effect of the heart and pericardium. Although Morgagni hernias are commonly congenital in origin, some of them are caused by blunt trauma. Blunt injuries that damage only the left leaflet of the diaphragm are probably a result of the protective role of the liver on the right side. If herniation occurs, it may not always become symptomatic immediately after injury (1). Morgagni hernia may be missed, misdiagnosed,

or result in complications. The differential diagnosis should include pericardial cyst, a large pericardial fat pad and a solid tumor (4). The majority of patients who present with symptoms of Morgagni hernia are over 40 years of age. A large proportion are asymptomatic, with hernia diagnosed incidentally on routine chest x-rays or barium contrast studies (6,7). In patients who are symptomatic, symptoms are commonly related to complications due to delayed diagnosis (6). Complications of delayed diagnosis include herniation, obstruction and strangulation of the stomach, colon or liver. Bowel incarceration as a complication of Morgagni hernia is rare, with only a few reports appearing in the literature (6,8,9).

Repair of a Morgagni hernia is easily accomplished through an abdominal approach, which also allows repair of an unexpected bilateral hernia. In a patient with a large defect, a plastic marlex mesh prosthesis may be used to secure closure of the defect in the diaphragm. Recently, laparoscopic repair of Morgagni hernia have been performed, but only after preoperative diagnosis of Morgagni hernia and in cases where emergency abdominal surgery is not required. (10)

The present case shows an unusual cause of intestinal obstruction in an aged patient. Such rare cases may be revealed following a systemic physical examination, which we therefore recommend be performed on every patient admitted for intestinal obstruction.

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