Volume 3 Issue 3 November 2017
e-ISSN: 2149-3189

The European Research Journal

The Association of Health Research & Strategy I www.eurj.org

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e-ISSN: 2149-3189

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The European Research Journal

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Review

e-ISSN: 2149-3189

DOI: 10.18621/eurj.310778

Current cancer stem cell biomarkers in laryngeal cancer

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ABSTRACT

Larynx cancer (LCa), being an aggressive malignancy, is the second most commonly diagnosed malignant type of head and neck squamous cell carcinoma worldwide. Although there have been significant improvements in the detection and diagnosis of LCa in the last decades, it is still one of the considerable causes of cancer deaths and an urgent need for identification of novel biomarkers with diagnostic and prognostic significance still remains. The cancer stem cells (CSCs) resemble normal stem cells in terms of biological features and are considered to play critical roles in biological aggressiveness of tumors. Accumulating evidences have proven the existence of CSCs in various tumors including LCa and they are considered as driving force for tumor relapse, metastasis, and chemo-radioresistance. Comprehensive identification and characterization of the CSCs is of paramount importance for their further characterization to develop more effective and targeted therapeutic strategies against cancer. Here, we reviewed and summarized the most current literature to provide an insight into the functions and roles of current CSCs biomarkers in human LCa. We believe that this review will contribute to the knowledge of scientists especially working with LCa CSCs and will help understanding the significance of CSCs biomarkers implicated in LCa pathogenesis.

Eur Res J 2017;3(3):200-206

Keywords: Larynx cancer, cancer stem cell, biomarker

Introduction

Larynx cancer (LCa), being an aggressive malignancy, is the second most commonly diagnosed malignant type of head and neck squamous cell carcinoma worldwide [1] and it is estimated to constitute approximately 1% (13,360) of incident cancer cases in 2017 in the United States alone. It is also predicted to constitute an important fraction of cancer deaths with 3,660 attributed cases in 2017 [2]. LCa incidences have been reported to be increasing each year and the prognosis seems to remain poor along with high mortality rates [2, 3]. Although there have been significant improvements in the detection and diagnosis of LCa in the last decades, it is still one of the considerable causes of cancer deaths and an urgent need for development of novel therapeutic approaches against advanced LCa cases and identification of biomarkers with diagnostic and prognostic significance still remains.

The cancer stem cells (CSCs), constituting a small fraction of tumor cells, resemble normal stem cells in

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Received: May 6, 2017; Accepted: May 31, 2017; Published Online: June 3, 2017

terms of biological features and are considered to play critical roles in the biological aggressiveness of tumors [4]. They have been proposed to have intrinsic and/or acquired capacity to promote initiation, progression, spread, recurrence of the tumor and make it resistant against current clinical treatment strategies [5]. Accumulating evidences have proven the existence of CSCs in a variety of tumors including lung, brain, breast, prostate, colon, ovarian, and head and neck cancers [6, 7] and they are considered as the driving force for tumor relapse, metastasis, and chemo-radioresistance [8-10].

Comprehensive and accurate identification and characterization of the CSCs are of paramount importance for their further characterization to develop more effective and targeted therapeutic strategies against cancer. CSCs are mostly isolated through utilization of certain cell surface markers including CD133, CD44, ALDH1, and ABCG2 [8, 11-14]. Furthermore, CSCs enriched cell populations are known to display elevated expressions of stemness genes like SOX2, OCT4, KLF4, and BMI1 [15] (Table 1 and Figure 1).

Here, we reviewed and summarized the most current literature to provide an insight into the functions and roles of current CSCs biomarkers in human LCa. We believe that this review will contribute to the knowledge of scientists not only working with LCa, but also studying the CSCs in other cancers and diseases, and will help understanding the significance of CSCs biomarkers implicated in LCa pathogenesis.

CD133

Table 1. Laryngeal CSCs biomarkers associated with prognosis of disease.

CSCs Biomarker	Expression Level	Association	Reference
CD133	High	Chemoresistance	[21]
		TNM stage, pathological grade, lymph node metastasis, poor overall survival and disease-free survival	[25]
CD44	High	Poor patient survival	[36]
		Lymph node metastasis	[37]
OCT4, KLF4, and SOX2	High	Poor patient survival	[50]
BMI1	High	Chemoresistance	[55]
ABCG2	High	Clinical stage, lymph node metastasis, and overall survival of patients	[61]
Beta1-integrin	High	Cervical lymph node metastasis, T stage, and histologic differentiation	[68]

CSCs=cancer stem cells

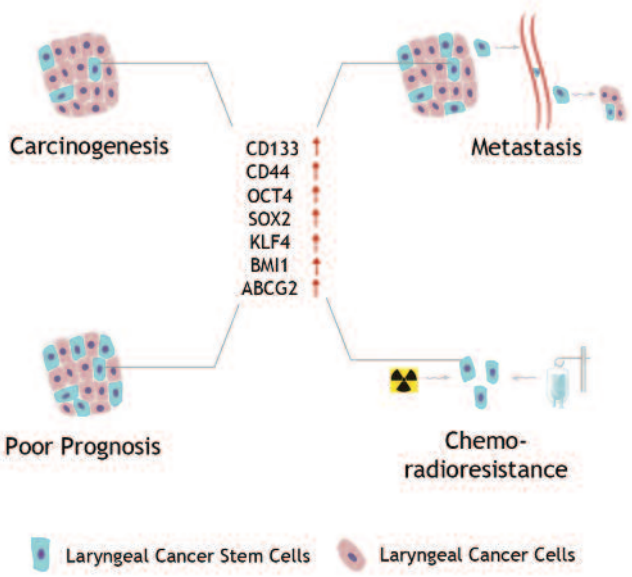

Figure 1. CSCs are characterized by significant overexpression of certain cell surface markers and stemness genes

CD133, a 120 kDa pentaspan transmembrane cell surface glycoprotein [16,17], is a commonly studied potential CSCs marker, which has been demonstrated to be expressed in distinct normal tissue stem cells [18]. It is an apical plasma membrane protein with potential to isolate stem cells from distinct tissues and tumors including LCa [19-21]. CD133 overexpressing cells were shown to possess high self-renewal capacity and multi-lineage differentiating potential both in vitro and *in vivo* [12].

CD133 positive (CD133+) cell fraction in Hep-2 cells, which is a well-studied human LCa cell line, was initially identified in 2007 by Zhou *et al.* [13], and CD133 was proposed as a candidate CSCs marker for LCa. They showed that purified CD133+ cells constituted only a small portion of total Hep-2 cell population and had profoundly increased self-renewal, proliferative, and differentiation capacity *in vitro*. Same group also evaluated the *in vivo* tumorigenic potential of CD133+ cells and found that those cells possessed significantly increased capacity for formation of new tumors *in vivo* when compared to CD133 negative (CD133-) and unsorted Hep-2 cells [14]. Furthermore, CD133+ Hep-2 cells within the side population, which was isolated through Hoechst 33342 dye exclusion, were found to exhibit enhanced cancer stem-like properties compared to corresponding CD133- side population cells both *in vitro* [22] and *in vivo* [23].

CD133, together with CD44, another important stem cell marker, were utilized to isolate cells with stem cell characteristics. When CD44 and CD133 positive cells (CD44+/CD133+) were injected to mice, they produced significantly larger tumors compared to those produced from other cell populations. CD44+/CD133+ cells, with stronger invasive potential, were also found to express several other stem cell markers [24]. In a recent study, Suer et al. [21] investigated the CSC potential of CD133+ cells isolated from freshly resected LCa specimens and found that CD133+ cells strongly express stemness genes such as SOX2, OCT4 and KLF4. Besides, overexpression of CD133 was significantly associated with TNM stage, pathological grade, lymph node metastasis, poor overall survival and disease-free survival in LCa patients [25].

CD133+ CSCs were found to exert stem cell features through upregulation of anti-apoptotic genes and activation of stem cells related signaling pathways like Hedgehog, Wnt and BMI1 [26]. Furthermore, the expression of Glut-1, which is required for transport of glucose (the essential source of energy for both stem cells and cancer cells) through cell membranes, was demonstrated to be significantly upregulated in CD133+ cells than in CD133- cells [27].

Recently, CD133+ cells were also reported to have strong resistance to irradiation and chemotherapy [28-30]. They were shown to have increased expression levels of ABCG2 and CXCR4, which were associated with resistance of tumors to regular chemotherapeutic reagents [21].

Taking these findings into account, CD133 might be considered to serve as an important target for cancer therapy against LCa. In a recent study, CD133+ cells were specifically targeted with mesoporous silica nanoparticles conjugated with chemotherapeutic drugs and siRNA against ABCG2. Interestingly, this CD133 targeted therapeutic approach enhanced the efficacy of chemotherapeutic drug-induced apoptosis through downregulation of ABCG2 in LCa CSCs and effectively inhibited tumor growth *in vivo* in a LCa mouse model, pointing the power CD133 as CSCs specific biomarker to be utilized in targeted therapies against LCa [31].

CD44

CD44 is a conserved single pass transmembrane glycoprotein, which is ubiquitously expressed throughout the body [32]. It interacts with several well-known ligands including hyaluronic acid (HA) and activates distinct signaling pathways involved in tumor progression and acquisition and maintenance of cancer stem cell characteristics [33].

The potential of CD44 in terms of its implication in stem cell characteristics of human laryngeal carcinoma cells was initially evaluated by Yu et al. [34] through isolation of CD44 positive (CD44+) cells from primary cultures of tumor samples obtained from 5 patients. They found that almost half of the cells were CD44+, which showed stronger proliferative capacity compared to CD44 negative (CD44-) cells. These findings pointed that CD44+ cells might be enriched for CSCs in laryngeal carcinoma samples [34].

As an important candidate CSCs biomarker in LCa, a significantly increased CD44 expression along with CD133 was detected in laryngeal tumor tissue samples obtained from LCa patients with lymph node metastasis, implying a potential role for CD44+ CSCs during cancer progression [35]. CD44 has been also proposed as a specific prognostic factor for LCa, where its overexpression in tumor tissues was

significantly associated with reduced 5 year overall survival of patients [36]. Interestingly, the soluble fraction of the CD44 protein in saliva samples of LCa patients served as a valuable prognostic biological marker. Patients with higher concentrations of salivary CD44 v6 tended to have larger primary tumors and metastatic lymph node involvement. Furthermore, patients with advanced tumor (stage III-IV) showed elevated levels of salivary CD44 v6 compared to those with early stage tumors [37]. These findings suggest that increased CD44 expression, which potentially points the enrichments of CSCs within the tumor tissue, would be a critical predictor for aggressiveness of the LCa.

Importantly, the number of CD44+ cells in tumors of most of the LCa patients tested profoundly increased upon fractionated irradiation at a total dose of 10 Gy, indicating their potential for resistance to radiotherapy [38]. Its expression was also demonstrated to significantly associate with response to radiotherapy in early stage LCa patients both at the mRNA and protein levels [39].

Considering the presence of CD44+ cells within the LCa tumor tissues and their potential for predicting the prognosis of patients, CD44 might be considered as an important biomarker for specific targeting of CSCs to eradicate the cancer. In an *in vivo* LCa model, targeting of CD44+ LCa cells via peri-tumoral injection of cisplatin conjugated with HA (a highly specific ligand for the CD44 surface receptors) provided a superior antitumor efficacy and a significant reduction in CD44+ positivity on ex vivo analysis [40]. In another study, a monoclonal antibody recognizing the extracellular domain of a CD44R1 was prepared as a potential immunotherapy tool. When it is injected intraperitoneally one week after the subcutaneous transplantation of HSC-3 human larynx carcinoma cells, it significantly suppressed the tumor growth in mice, which proposed CD44R1 as a possible molecular target for LCa therapy [41].

OCT4, KLF4, and SOX2

OCT4, KLF4, and SOX2 (OKS), as essential stemness factors, are crucial for early development and have been shown to participate in the tumorigenesis of various cancer types [42-44]. They are required for maintenance of self-renewal as well as pluripotency features of embryonic stem cells and CSCs [45-47]. Recent investigations have demonstrated that CSCs obtained from distinct cancer tissues and cell lines express several stem cell markers including OKS factors. They have also been studied in LCa to investigate their involvement in the laryngeal carcinogenesis process and their potential as CSCs markers.

Their expressions have been found to be significantly upregulated in LCa tissue samples [48] and introduction of SOX2 into human laryngeal cancer cell line Hep-2 cells resulted in promoted migratory and invasive capabilities and its overexpression caused induction of epithelial mesenchymal transition [49]. Elevated SOX2 levels was also demonstrated to significantly correlate with poor prognosis of LCa patients [50].

Hypoxia induced CSCs enriched in Hep-2 cells [51] and CD133+ cells isolated from freshly resected laryngeal tumor specimens [21] displayed significantly elevated OKS levels, pointing in their importance as critical CSCs biomarkers for LCa.

However, current literature is limited and further studies are needed to understand the power of OKS factors as CSCs biomarkers for LCa and to enlighten the underlying mechanisms of laryngeal carcinogenesis through unraveling the molecular circuitries of stem cell biology in association with OKS factors.

BMI1

BMI1, with tumorigenic potential in a variety of cancers, has been shown to play critical roles in normal stem cell proliferation [52]. It has been found to induce tumor invasion, metastasis and chemoresistance of solid tumors [53, 54]. As an initial study, Yu et al. [55] demonstrated significant overexpression of oncogenic BMI1 protein,which involves in gene silencing, through chromatin modifications [55], in CD44+/CD133+ cells [34]. It was reported to be upregulated in laryngeal tumor tissue samples and CD133 positive Hep-2 cells, which provided maintenance of cell proliferation and prevented apoptosis [52, 56]. Its knockdown resulted in inhibition of *in vitro* proliferative, clonogenic, invasive capacity, and *in vivo* tumorigenic potential of CD133+ cells through up-regulation of p16(INK4A) and p14(ARF) [57]. More importantly, BMI1 expression was demonstrated to be co-localized with CD133 in LCa specimens [52, 58]. These findings suggested BMI1 as a molecular target to treat patients with LCa.

ABCG2

ABCG2 is known to participate in

chemoresistance and acquisition of stem cell features of tumor cells and its upregulation has been demonstrated in various cancer types [59]. Side population cells with CSCs features isolated from the laryngeal carcinoma cell line as well as primary laryngeal carcinoma cells [60] and CD133+ larynx CSCs isolated from freshly resected laryngeal tumor specimens [21] had significantly higher levels of ABCG2. The presence of ABCG2 in LCa tissue samples was significantly associated with clinical stage, lymph node metastasis, and overall survival of patients [61]. Besides, CD133+ cells, having elevated potential for clonogenicity and invasion, were demonstrated to be more resistant to chemotherapy, which was strongly correlated with higher expression of ABCG2 [62].

Taking these findings into consideration, elevated ABCG2 expression in CSCs population strengthens the idea that these cells are among the strong candidates for chemoresistance and recurrence of cancer.

Other Potential Cancer Stem Cell Biomarkers

Apart from well-characterized surface markers and stemness genes, there are also other factors reported to be as potential CSCs factors including Aldehyde dehydrogenase isoform 1 (ALDH1), h-TERT, and beta1-integrin.

Hep-2 cells overexpressing ALDH1, as a marker of CSCs in head and neck cancers, were reported to exhibit elevated proliferative and tumorigenic potential *in vitro* and *in vivo*, respectively [63]. However, in a very recent study, the relapse rate in patients who underwent curative-intent radiotherapy or chemo-radiotherapy was lower in those with ALDH1 positive tumors [64]. These two studies providing conflicting data about the biomarker potential of ALDH1 for laryngeal CSCs necessitates further investigations to make certain of its potential as a CSCs biomarker.

Another potential CSCs biomarker is human telomerase catalytic subunit (hTERT), which regulates telomerase activity in stem cells. Its expression was found to be increasing from non-cancerous laryngeal samples to grade III LCa specimens [65]. Knockdown of hTERT in Hep-2 cells significantly decreased telomerase activity and cell viability in Hep-2 cells [66]. Besides, its promoter was reported to have lower activity in LCa cells compared to that of radioresistant variant cells [67], pointing its potential as a CSCs biomarker.

CD44+/CD133+ cells obtained from human laryngeal primary carcinoma cells exhibited elevated levels of beta1-integrin (also known as CD29) [24]. Its expression was also significantly associated with cervical lymph node metastasis, T stage, and histologic differentiation [68].

These biomarker candidates need further research to clearly characterize their roles and functions in laryngeal carcinogenesis and their effects on the features of CSCs present in the laryngeal tumor tissue.

Future Perspectives

Experiencing a recurrence after a successful treatment is quite common in various cancer cases including LCa. In addition, current traditional therapies mostly fail to result in a positive outcome in advanced and metastasized tumors, which ultimately causes cancer related death due to disease progression and related organ failure. Recent studies noted that CSCs are crucial contributors of cancer initiation, progression, metastasis, recurrence, and chemo-radioresistance (Figure 2). Although underlying molecular mechanisms of how CSCs participate in cancer pathogenesis is not completely clarified and understood, scientists and clinicians aim to utilize them in therapeutic applications in fight against cancer. Unraveling both genetic and epigenetic circuitries of CSCs is essential to develop effective and successful therapies.

One of the major challenges of CSCs research is the true identification and characterization of CSCs, which is essential for specific targeting of CSCs. Therefore, to develop a therapeutic tool aiming to eradicate CSCs preferentially, the initial goal must be the determination of accurate surface marker(s) and stemness genes in CSCs. By this way, further development of anti-CSCs agents will help overcoming the acquired chemotherapy or radiotherapy resistance of tumors.

Furthermore, early detection and correct diagnosis are especially crucial for determination and application of accurate and effective cancer therapy alternatives in the clinical decision making process. Detailed characterization of circulating tumor cells, especially CSCs, will give opportunity to estimate the prognosis of the disease and decide the therapy strategy for each patient.

Conclusion

As a conclusion, the area of CSCs research is in

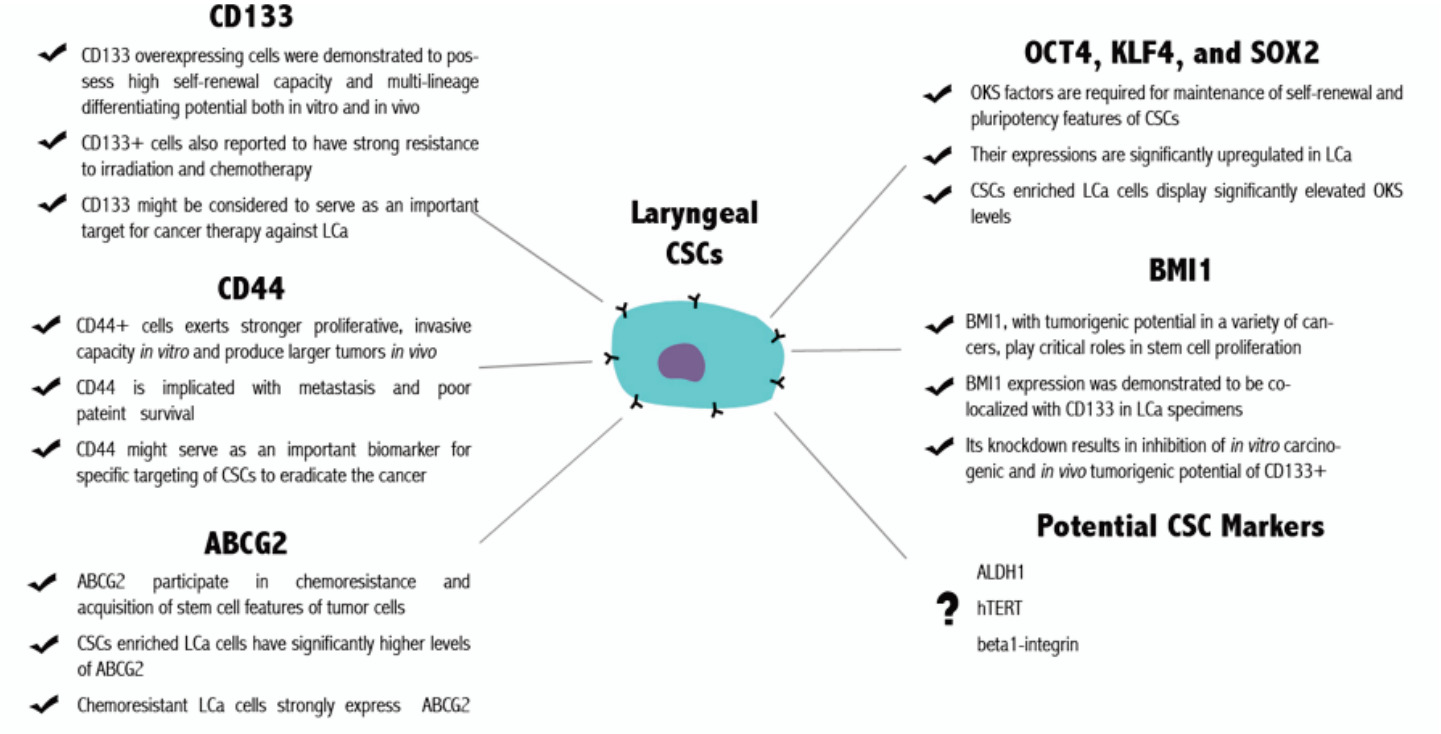

Figure 2. Certain biomarkers are associated with acquisition of stemness features in CSCs, which are crucial contributors of cancer initiation, progression, metastasis, recurrence, and chemo-radioresistance

its infancy and current understanding of the CSCs biomarkers in laryngeal pathogenesis is limited. Therefore, further intense investigations are needed for better understanding of the roles and functions of CSCs biomarkers in LCa biology.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing

The authors disclosed that they did not receive any grant during conduction or writing of this study.

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The European Research Journal
http://www.eurj.org
Original Article
e-ISSN: 2149-3189
DOI: 10.18621/eurj.318606

Integrin binding peptide promotes in vitro wound closure in the L929 mouse fibroblasts

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ABSTRACT

Objective. Molecular basis of wound healing process needs to further examined to determine the effective individual biological cues. The objective of this study was to investigate the wound closure, proliferation, and viability of L929 fibroblast when cultured with different concentration of soluble RGD peptid. ***Methods***. RGD peptide was synthesized manually on solid phase. The percentage of healed wound area for control, 0.5 mM, 1 mM, and 2 mM at each time points were analyzed by ImageJ. Cell proliferation and viability were assessed with MTT and live/ dead analysis, respectively. ***Results.*** The results of wound closure area showed that increased RGD peptide concentration in the culture improved cellular migration which enables significantly accelerated wound closure. However, RGD peptide did not dramatically augmented cell proliferation. In addition, cell viability results indicated that dead cell numbers did not critically influence by increasing the RGD peptide concentration in the culture. ***Conclusions.*** The present study showed that soluble integrin binding peptide accelerated the migration and wound closure rate of L929 fibroblasts. Delivery of soluble integrin binding peptides into the wound area may be considered as an alternative wound treatment technique in the near future after proofing the concept study with animal and clinical studies.

Eur Res J 2017;3(3):207-213

Keywords: Integrin binding peptide, *in vitro* scratch assay, cell migration, serum free media

Introduction

Wound healing is a dynamic and complex process involving of synchronized actions including inflammation, regeneration, migration, proliferation, and remodeling [1, 2]. Wound healing process starts right after the skin injury and involves extensive cell-to-cell and cell extracellular matrix (ECM) communications, enhanced cell migration and action of soluble mediator [3-5]. Since wounds remain one of the major clinical problem with high morbidity and mortality rate, many different studies have been carried out to understand the molecular basis of acute and chronic wound healing processes [6, 7]. One of the critical time frame in wound healing is the proliferation and migration of fibroblasts and myofibroblasts in the surrounding tissue,which are stimulated especially for the first 3 days after the

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Received: June 5, 2017; Accepted: August 18, 2017; Published Online: August 24, 2017

injury [8, 9]. The major mediators that accelerate fibroblasts proliferation and migration during wound healing requires to be determined to better understand the molecular mechanism of wound healing. The standard procedure for *in vitro* culture is based on supplementing cell culture media with fetal bovine serum (FBS). FBS is rich in growth factors and ECM such as laminin, fibronectin that enhance cell attachment, proliferation and differentiation. To comprehend individual efficacy of different proteins or bioactive peptides, it would be desirable to culture cells under serum-free media with defined biological cues. It was previously reported that serum free culture media has been developed to prevent the need for presence of bovine serum [10-12]. Kim *et al.* [13] developed serum-free culture conditions by using ECM proteins mimetic peptides to investigate the effect of different biological cues on adhesion and proliferation of chorion derived mesenchymal stem cells (MSCs). In another study, Nyegaard *et al.* [14] was initiated that 5 ng/ml epidermal growth factor (EGF) and 5 µg/ml osteopontin (OPN) in culture media induced migration of human small intestine cells. Current studies also focus on to identify peptide structures which are the small functional units of proteins to design non-immunogenic therapeutic molecules. These peptide structures have key function on cell-to-cell and cell-ECM interaction which are mediated by integrin receptors which are composed of 18α and 8β subunits and placed on cell membrane [15, 16]. The RGD (R: arginine; G: glycine; D: aspartic acid) sequence is the smallest integrin binding unit that presents in most of the ECM proteins including fibronectin, collagen, and laminin [17, 18]. RGD peptide sequence is the primary sequence to effectively modify biomaterials surface to enhance cellular adhesion and migration on synthetic surfaces [15, 16]. It was previously reported that integrin mediated cell spreading activates cell proliferation of surface dependent cells [19, 20]. Immobilized RGD peptide on substrate promotes cellular adhesion, whereas soluble form of RGD peptides inhibits the attachment of the cells and lead apoptosis by covering all the available integrin binding units of the cells [21]. For instance, Patrulea *et al.* [22] reported that RGD peptide functionalization significantly increased fibroblast adhesion and proliferation on chitosan based wound dressing. On the contrary, it has been demonstrated that soluble RGD peptide inhibited the adhesion of non-transformed human lung fibroblasts and trigger apoptosis. It was also previously described that blocking the integrin receptors with soluble RGD peptide may inhibit cell migration. Therefore, soluble RGD peptide was also introduced as possible therapeutics that showed anti-metastatic activity [23, 24]. On the contrary, Reynolds *et al.* [25] demonstrated that soluble RGD peptide can also promote the migration of the tumor cells. Furthermore, Shabbir *et al.* [26] investigated the influence of soluble RGD peptide on cell migration. They seeded HT1080 epithelial cells on RGD peptide immobilized gold substrate and explored the migration of these cells in different RGD peptide containing media. It was reported that cell migration was promoted by the presence of soluble RGD peptides because of the dynamic detachment of the integrin receptors with the substrate [26]. Although couple of studies mentioned the effect of soluble RGD peptide on different cell lines migration capacity, L929 mouse fibroblasts migration and proliferation response in *in vitro* scratch assay when cultured with different concentration of soluble peptide has yet to be studied. Based on these findings, we hypothesized that soluble RGD peptide could enhance the wound closure capacity of mouse L929 fibroblasts which are one of the model cell line of skin tissue by accelerating the migration of the cells. Hence, the objective of this study was to investigate the scratch closure rate of L929 fibroblast when cultured with different concentration of soluble RGD peptide enriched FBS free media. As it was schematically presented in Figure 1, wound closure was observed by analyzing captured micrographs at 24, 48, and 72h. The cell proliferation analysis at 24, 48, and 72 hours were assessed by cell proliferation assay. Live and dead cell analysis was also evaluated to determine cell viability at each time points.

Methods

Peptide Synthesis

All the chemicals used for peptide synthesis were purchased from AAPPTEC (Louisville, KY, USA). GRGDS peptide sequence was synthesized manually on 4-Methylbenzhydrylamine (MBHA) resin (substitution = 0.67 mole/g) according the previously described procedure by Karaman *et al.* [27]. Briefly, 100 mg resin was swelledin 2ml Dimethylformamide (DMF) solution for 20 minutes. Fmoc-protecting group was removed using de-protection solution which is 20% piperidine in DMF for 20 minutes. 2 equivalents (based on resin substitution) of Fmoc-

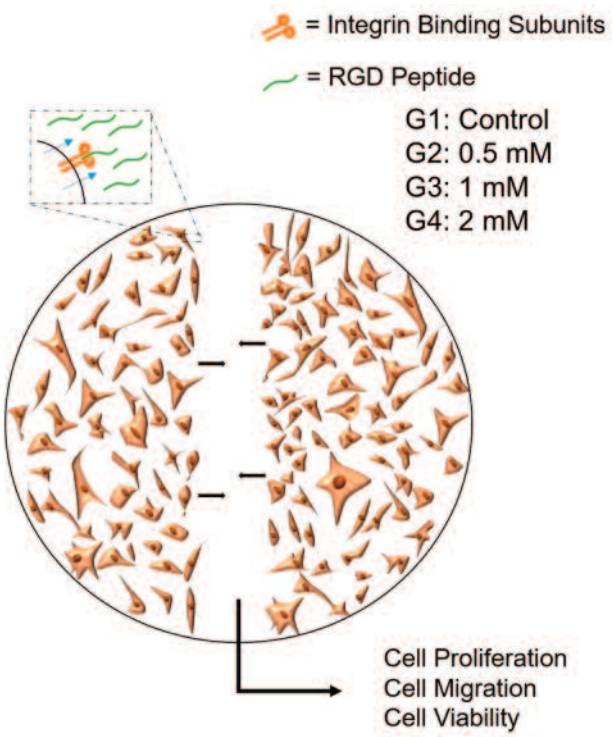

Figure 1. Schematic of the study. Integrin binding (RGD) peptide influence on cell proliferation, migration and viability.

protected amino acid was dissolved in DMF and added to the resin. 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexauorophosphate) (2 equiv, HBTU), hydroxybenzotriazole (2 equiv, HOBt) and N, N-diisopropylethylamine (4 equiv, DIEA) were added to mixture. Tubes were mixed in orbital shaker to proceed coupling reaction for 3 hours. Each coupling and de-protection reaction was confirmedby ninhydrin test. If the result was positive, the resin was washed with DMF and the coupling reaction was repeated until a negative result was obtained. If the test result was negative, the resin was washed with DMF, reacted with 20% piperidine in DMF for 15 min, and again washed with DMF. All amino acids were coupled using the same method. When the last amino acid was coupled, peptide was cleaved from resin by using trifluoroacetic acid (TFA): triisopropylsilane (TIPS) : DI Water solution at ratio of 95 : 2.5 : 2.5. TFA was evaporated with the rotary evaporator. The peptide was precipitated in ice-cold diethyl ether. Next, precipitated peptide was washed by ice-cold diethyl ether for three times. Finally, the resulting pellet was freeze-dried.

Cell Culture

Mouse fibroblasts (L929) were obtained from Ege University Research Group of Animal Cell Culture and Tissue Engineering Laboratory and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, St. Louis, Missouri, USA), supplemented with 10% FBS (Sigma Aldrich, St. Louis, Missouri, USA), 1% L-glutamin (Gibco,Grand Island,USA) and 0.1% penicillin/streptomycin in a humidified incubator containing 5% CO□ and 95% air at 37ºC. Cells were kept in exponential phase and for this study cells at passage three were used.

Wound Scratch Test Assay

5×10^4 cells/cm^2 L929 cells were seeded on 24-well plate and incubated at 37ºC for 24 hours in the 5% of CO2 atmosphere. A linear scratch was created in the confluent monolayer by gently scraping with sterile 200 µl pipette tips. Afterward, the cellular debris was removed by rinsing these cells using sterile PBS. RGD peptide was dissolved in sterilized water, sonicated, sterilized through filtering, and stored at −20ºC. Fresh medium (with no FBS) containing RGD at concentrations of 0.5 mM, 1 mM and 2mM was added to the media. Serum free media with no RGD peptide was used as a control. Promotion of wound closure observed by capturing micrographs with florescence attached phase-contrast microscope (CKX41, Olympus, Hamburg, Germany) at 0h, 24h, 48h and 72h, respectively. The percentage of healed wound area for control, 0.5 mM, 1 mM, and 2mM at each time points were analyzed by using the ImageJ software (NIH, Bethesda, MD, http://www.rsb.info.nih.gov/ij).

Cell Proliferation and Viability Analysis

Cell proliferation analysis was assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Vybrant® MTT Cell Proliferation Assay Kit, Invitrogen, Waltham, MA, USA) assay according to the manufacturer's instructions, after 1, 3, and 5 days of incubation [28]. Briefly, MTT solution (5 mg/mL) was added into culture medium (with 10% concentration) and incubated for 2 h at 37°C. Next, the medium was replaced with 500 µL DMSO (Sigma Aldrich, St. Louis, MO, USA), and the optical density for each well was measured at 540 nm using a Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Measured absorbance values were correlated to the equivalent number of cells by using a calibration curve constructed with reference number of cells. The effect of RGD peptide addition in serum-free media on cell proliferation were determined. After 72 hours of culture, live/ dead double fluorescence staining kit (Dojindo EU,

Munich, Germany) was used to evaluate viability of L929 fibroblasts on well plates by fluorescence microscopy. Briefly, the viable cells (Calcein-AM, green fluorescence) and dead cells (propidium iodide, red fluorescence) were studied using a fluorescence microscope after 15 min of incubation in a culture medium.

Statistical Analysis

Three independent experiments were performed and in each experiment, three cell seeded wells were used for each experimental groups. The images taken from same cell seeded wells for each group at different time points. All data were expressed as mean ± standard error and were statistically analyzed by one-way ANOVA (SPSS 12.0, SPSS GmbH, Germany) and the Student-Newman-Keuls method as a post hoc test. Significant differences between groups were determined at *p* values at least less than 0.05.

Results

Wound Closure Measurement

After L929 fibroblasts reached confluency level, scratch was occurred in order to assess wound healing capacity of cell when cultured with different concentrations of RGD peptide. The micrographs of control, 0.5 mM, 1 mM, and 2 mM RGD at 0, 24, 48, and 72 h were shown in Figure 2. The percentage of healed wound area for control, 0.5 mM, 1 mM, and 2 mM RGD groups at 0, 24, 48, and 72 h was assessed by ImageJ and presented in Figure 3. By 24 h, it is evident that the scratch closure occurred at a faster rate in the presence of different concentration of RGD peptides. For instance, wound closure area significantly increased in 2 mM RGD (29.30 ± 0.9%), 1 mM RGD (28.00 ± 1.1%), and 0.5 mM RGD (22.82 ± 4.03%) compared to control (21.81 ± 0.56%). Similarly, at 48 h, significantly faster wound closure in RGD groups was observed in 2 mM RGD (45.47 ± 1.47%) followed by 1 mM RGD (35.89 ± 1.3%) and 0.5 mM RGD (34.34 ± 0.55 %) compared to control. Additionally, after 72h, 2mM RGD (58,67 ± 1.49%) was the highest in terms of wound closure are among

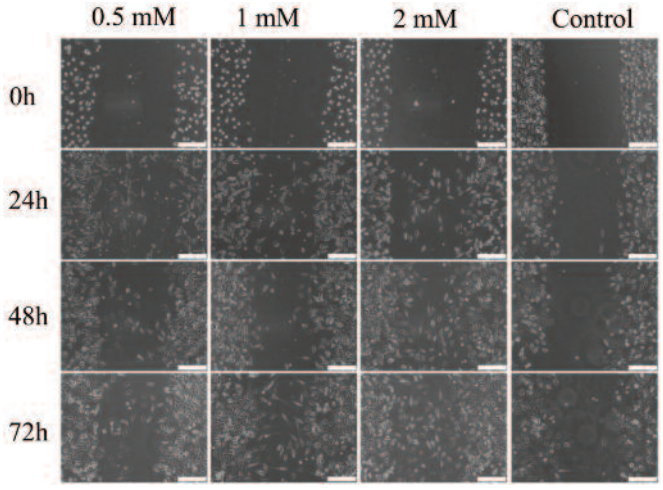

Figure 2. Micrographs of in vitro wound closure assay of control, 0.5 mM, 1 mM, 2 mM RGD peptide groups at 0, 24, 48, and 72 hours. Scale bar is equal to 200 µm.

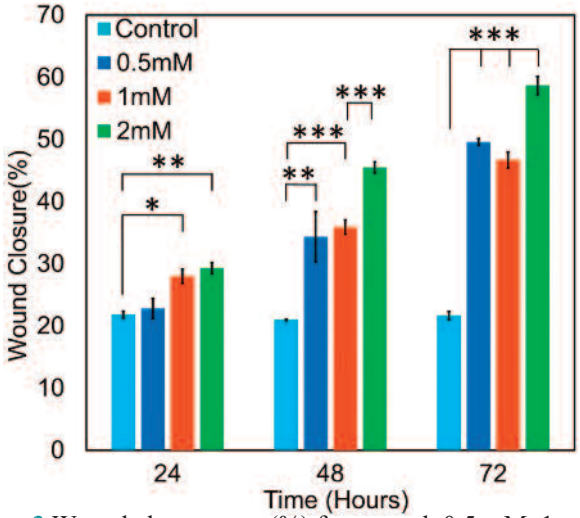

Figure 3. Wound closure area (%) for control, 0.5 mM, 1 mM, and 2 mM RGD peptide groups at 24, 48 and 72 hours by using Image J. Error bars represent mean ± SE (n = 5) (significant differences were determined by one-way ANOVA (Newman-Keuls multiple comparison test, (*p < 0.05, **p < 0.01,***p < 0.001).

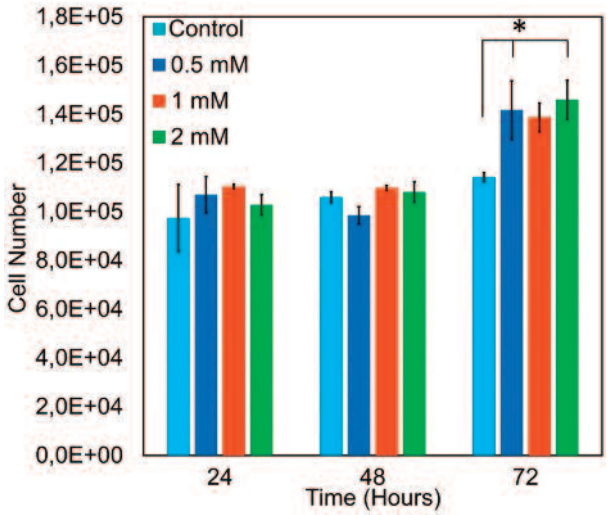

Figure 4. Cell numbers of control, 0.5 mM, 1 mM, and 2 mM RGD peptide groups at 24, 48, and 72 hours. Error bars represent mean ± SE (n = 5) (significant differences were determined by one-way ANOVA (Newman-Keuls multiple comparison test, (*p < 0.05, **p < 0.01,□***p < 0.001).

RGD groups, followed by 0.5 mM RGD (49.57 ± 1.3%) and 1mM RGD (46.69 ± 0.27%). The results of wound closure area showed that RGD treatment improved cellular migration which enables significantly accelerated wound closure.

Cell Proliferation and Viability during Wound Closure

The effect of RGD peptide concentrations of 0.5 mM, 1 mM, and 2 mM on cell proliferation at 24, 48, and 72 h were presented in Figure 4. The results showed that RGD peptide implementation did not affect the cell proliferation at early time points of culture including 24 and 48 h. However, at 72 h, cell number in 2 mM RGD (145,885 ± 8,037), 1 mM (138,663 ± 5,931), and 0.5 mM RGD (141,672.62 ± 12,028) was significantly higher compared to control group (114,114 ± 1,970). The results of MTT assay suggested that increased RGD peptide enriched culture media improved cellular adhesion and proliferation in the late phase of wound healing. Fluorescent microscopy images of live/ dead staining analysis in control, 0.5 mM, 1 mM, and 2 mM at 72 h were shown in Figure 5a, b, c, and d, respectively. The results indicated that dead cell numbers did not critically differ by increasing the RGD peptide concentration in the culture.

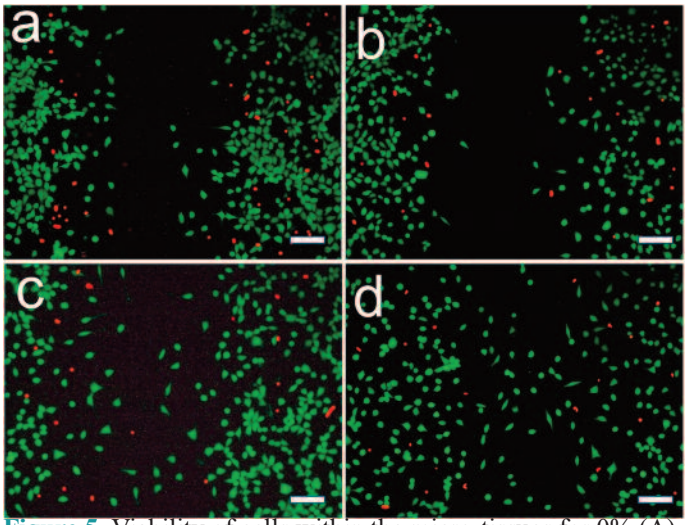

Figure 5. Viability of cells within the micro-tissues for 0% (A), 1% (B), 5% (C), and 10% (D) FBS groups was examined on day 7 (green: LIVE cells, red: DEAD cells) (20x). Scale bar represents 100 µm size.

Discussion

In this study, to precisely observe the effects of three different concentrations of RGD peptides on *in vitro* wound closure, proliferation, and viability of L929 fibroblasts, experiments were conducted with serum-free media. As mentioned earlier, soluble RGD peptides have converse effect on cell behaviors. It was indicated that soluble RGD peptide, when treated with adherent cells prior to cell seeding, prevented the adhesion of cells [21, 23]. The potential reason for this function could be blockage of all integrin binding points of these cells with soluble RGD peptide; therefore, cell adhesion was inhibited. On the other hand, it was also reported that due to the dynamic detachment of the integrin receptor in the presence of soluble RGD peptide, migration of tumor cells was augmented [25].

Our results clearly indicated that increasing the RGD peptide concentration in the culture media significantly accelerated the wound closure of L929 fibroblast (Figure 2 and Figure 3). It could be directly related with the enhanced migration and proliferation capacity of the cells in the presence of RGD peptide. It was also observed that even with higher concentration of soluble RGD peptide did not critically affect cell proliferation specifically at the early time points of *in vitro* wound healing (Figure 4). However, wound closure area was significantly increased by increasing the soluble RGD peptide concentration at each time point. The reason for such rate on cell proliferation might be due to the culture of cells without serum concentration. Although different concentration of RGD peptide was supplemented into the test groups, still there was no serum in the culture therefore no early proliferation rate was observed. From 48 to 72 hours, L929 fibroblasts might start using soluble RGD peptide as a source of amino acids and it might be one of the reason that partially accelerate the proliferation of L929 fibroblasts. Our data showed that although different RGD peptide concentration did not practically affect the proliferation rate, it was clearly seen that higher RGD peptide concentration significantly trigger the migration rate of L929 fibroblasts. Based on these findings, we hypothesize that soluble RGD peptides significantly influence the migration of L929 fibroblasts. The reason of enhanced cell migration caused by soluble RGD peptide may be related with accelerated attachment of the integrin receptors. Similarly, Jones *et al.* [29] reported that addition of RGD sequence including insulin-like growth factor binding protein (IGFBP-1) in the cell culture medium significantly enhanced the migration of CHO cells.

Another possible explanation of such trend on enhanced cell migration by increasing RGD peptide

concentration might the activation ofsignalingpathways such as, focal adhesion,caspase-3,FAK proteolysis [21, 26, 30]. In a previous study, it has been compared the rates of cell migration and viability in the presence of soluble peptide in concentrations. It was reported that the addition of soluble peptide increased the rate of disconnection of focal adhesions at the back of the cell, which may induce higher migration levels and the presence of a soluble peptide helps to block individual communications from reassembling and allows an "unzipping" of the polyvalent complex [26].

Cell viability results revealed that increasing the soluble RGD peptide concentration did not critically affect cell viability. Although it was previously reported that RGD-containing peptides provoked apoptosis, we did not observed similar results [31, 32]. Buckley *et al.* [21] reported that RGD peptides induced apoptosis through direct caspase-3 activation if adherent cells detach from their substrate. In that case, soluble RGD peptides prevented the attachment of the cells by blocking all the available integrin binding units of the cells and lead apoptosis. However, in these study as predicted from live/ dead staining micrographs, soluble RGD peptide did not inhibit cell viability. The potential reason of not leading major cell death might be addition of RGD peptide to already attached fibroblasts culture. It is also recommended that these claim needs to further investigate with apoptosis assay.

Conclusions

In the present study, we investigated the effect of various concentration of soluble RGD peptide on wound closure capacity, proliferation, and viability of L929 fibroblasts *in vitro*. By incorporating soluble RGD with different concentrations including 0.5 mM, 1 mM, and 2 mM to cell culture media, L929 fibroblasts exhibited greater cell proliferation and wound closure capacity compared to control group.Taken together, soluble RGD peptide applications may be an alternative wound treatment technique in the near future after proofing the concept study with animal and clinical studies. Experiments to understand the efficacy of soluble RGD peptide application on an in vivo wound model are underway.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing

The authors disclosed that they did not receive any grant during conduction or writing of this study.

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The European Research Journal
http://www.eurj.org
Original Article
e-ISSN: 2149-3189
DOI: 10.18621/eurj.316421

Importance of preoperative hematological parameters in patients undergoing surgical resection for colorectal cancer

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ABSTRACT

Objective. Colorectal cancer has a high prevalence worldwide, and new predictive and prognostic factors are needed for its early diagnosis, treatment, and follow-up. In this study, we have investigated the relationship between colorectal cancer and neutrophil to lymphocyte ratio (NLR), mean platelet volume (MPV), and platelet to lymphocyte ratio (PLR) values. ***Methods.*** Data from 71 patients admitted to our hospital between May 2013 and May 2015, who underwent surgical resection for colorectal cancer, and met the criteria of the study, was evaluated retrospectively. NLR, MPV and PLR data detected in the preoperative period was noted. Demographic data, the presence of comorbidity, colonic localization of the tumor, colonoscopic findings, and surgical resection type and method were compared with the data of morbidity and mortality, lymphovascular and perineural invasion, lymph node and distant metastasis and stage of the disease. ***Results.*** No statistical significance was detected between preoperative NLR, MPV, and PLR, demographical data, the presence of comorbidity, colonic localization of the tumor, colonoscopic findings, surgical resection type and method and morbidity, lymph node and distant metastasis, lymphovascular invasion, or stage of the disease. However, a statistically significant relationship was detected between mortality development and NLR and PLR values (p=0.030; p=0.043; respectively). There was also a statistically significant relationship between the presence of perineural invasion and PLR (p=0.031). ***Conclusion.*** Hematological parameters (NLR, MPV, and PLR) evaluated in preoperative period in patients who have been applied surgical resection for colorectal cancer may help clinical and pathological staging.

Eur Res J 2017;3(3):214-219

Keywords: Colorectal cancer, surgery, mean platelet volume, neutrophil to lymphocyte ratio, platelet to lymphocyte ratio

Introduction

Colorectal cancer is common worldwide and an important cause of cancer-related deaths [1]. Despite recent advances in treatmentsavailable for patients with colorectal cancer, such as curative resections, chemotherapy, and radiotherapy, 5-year survival rates rangebetween 44% and 93%, decreasing to 12% in patients with advanced stages of the disease [2]. Despite similarclinical phases and pathological

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Received: May 27, 2017; Accepted: July 12, 2017; Published Online: July 31, 2017

features, diversity in the biology of the tumor results in different oncologicaloutcomes for colorectal cancer patients.

Alternative treatments and prognostic evaluation may improve as weknow that angiogenesis and the stromal microenvironment, including inflammation in the body,play an important role in tumor progression and metastasis development [3]. Commonly used biomarkers such as fecal occult blood test(used in diagnosis in the preoperative and postoperative follow-up periods); carcinoembryonic antigen (CEA); and carbohydrate antigen (CA-19.9) have low sensitivity for colorectal cancer and are not specific to the organ [4]. Subsequently, there is a real need formore efficient biomarkers,useful for both early diagnosis and predictingtreatment effectiveness. We have focused on biomarkers that are low-cost, highly efficient, sensitive in the preoperative period, beneficial in clinical usage, and with both prognostic and predictive features. Prognostic biomarkers such as neutrophil to lymphocyte ratio (NLR), mean platelet volume (MPV), and platelet to lymphocyte ratio (PLR) may have strong predictive and prognostic features which can be used in addition to classical pathological staging [5, 6].

In our study, we have retrospectively evaluated the data of colorectal cancer patients who underwenta resection operation; investigating the relationship between systemic inflammatory and hematological parameters detected in the preoperative period (NLR, MPV, and PLR), and patient characteristics and tumor histopathological features.

Methods

Study design and patients

The data of 84 colorectal cancer patients who were admitted to Bursa Yuksek Ihtisas Training and Research Hospital between May 2013 and May 2015 was evaluated retrospectively. Thirteen patients were excluded from the study: these were patients with systemic inflammatory or hematologic disease; patients using anticoagulant drugs; those undergoing emergency operations; those with missing data; and patients operated on for recurrent tumors. Data from the remaining 71 patients who had undergone elective surgical resection and who met the study criteria was evaluated. The following data was taken into consideration: NRL, MPV, and PLR values of the patients detected in the preoperative period; demographic data; presence of comorbidities; colonic localization of tumor; colonoscopic findings; type of surgical resection and method applied; morbidity and mortality; lymph node and distant metastasis; lymphovascular and perineural invasion; and the stage of disease.

A complete blood count was measured on the day previous to the operation, and NLR, MPV, and PLR values were noted. The NLR value was calculated by dividing the neutrophil count by the lymphocyte count. The PLR value was found by dividing the platelet value by the lymphocyte value. The MPV was measured automatically in the complete blood count. All colorectal cancer patients were given oral, enteral supplements and a liquid diet in the preoperative period. Neoadjuvant chemotherapy and radiotherapy were used primarily in patients with a locally advanced stage rectal tumor. No extra preoperative evaluation or postoperative care was employed and mechanical bowel preparations were not used.

Surgical Technique

Low anterior resection (LAR), right or left hemicolectomy, or abdominoperineal resection (APR) were the three types of resection used. In some cases, laparoscopic surgery could not be performed for technical reasons, or in patients with a previous intraabdominal operation history. Laparoscopic surgery, open surgery and a third group of conversion surgery that included patients with a transition from laparoscopic operation to open surgery were defined as our three resection methods. Hybrid attempts were not used in laparoscopic methods. An ostomy was opened in patients with a history of radiotherapy in the rectal area or with anastomosis localized in the lower rectum.

In this study, AJCC (American Joint Committee on Cancer) and TNM (T tumor, N node, and M metastasis) classifications were used for postoperative histopathologic and clinical evaluations for staging.

Statistical Analysis

In our statistical analysis, data was analyzed with SPSS for Mac version SPSS 20.0 (Inc., Chicago, IL) Software Package. Mann-Whitney U test, Kruskal-Wallis H test, and Spearman Correlation Coefficient methods were used to determine the significant difference. The variables of gender, comorbidity, preoperative metastasis, colonoscopic findings, morbidity, mortality, lymph node metastasis, lymphovascular invasion and perineural invasion were analyzed using Mann-Whitney U test as there was not a normal distribution. Kruskal-Wallis H Test was used

Table 1. Patient demographic and clinic characteristics

	n	%	*p* NLR	*p* MPV	*p* PLR
Age (years)					
n, mean(range)	71	63.6 (35-83)	0.545*	0.957*	0.392*
Sex	F :25	35.2%			
	M :46	64.8%	0.380	0.077	0.962**
Tumor localization					
Right colon	16	22.5%			
Left colon	26	36.6%			
Rectum	29	40.8%	0.270	0.388	0.253***
Comorbidity					
(+)	44	62%			
(-)	27	38%	0.281**	0.995**	0.962**
Preoperative metastasis (+)	5	7%	0.608**	0.623**	0.350**
Permeation in colonoscopy					
Narrowing	39	54.9%			
Normal	32	45.1%	0.799**	0.917**	0.908**

F=female, M=male, NLR=neutrophil lymphocyte ratio, MPV=mean platelet volume, PLR=platelet lymphocyte ratio
* Spearman Correlation Coefficient, ** Mann-Whitney U, ***Kruskal-Wallis H methods

for the analysisof variables comprising tumor localization, operation type, and technique, tumor differentiation and stage, which were not normally distributed, and the Spearman Correlation Coefficient test was used forage and tumor diameter variables, also not normally distributed. In the case of non – normal distribution, Mann-Whitney U test replaced the t-test, Kruskal-Wallis H Test replaced Analysis of Variance (ANOVA), and Spearman Correlation Coefficient was used instead of Pearson Correlation Coefficient. *p* values less than 0.05 were considered as statistically significant.

Results

The data of 71 colorectal cancer patients undergoing resection was evaluated. In the study, the NLR median was 2.92 (min: 1.27, max: 54.54); the MPV median was 8.9 (min: 7.30, max: 12.10); and the PLR median came out as 178.57 (min: 6.91, max: 302.27).

Patient characteristics, tumor localization, the presence of comorbidities, preoperative metastasis, and colonoscopic findings were compared with NLR, MPV, and PLR median values;with the statistical results shown in Table 1. Operation type and method, morbidity and mortality values were also compared with NLR, MPV, and PLR median values, and these statistical results are given in Table 2. The most common operation was lower anterior resection (59.1%), and a statistically significant relationship was detected between PLR and this type of operation (*p*=0.040). During the postoperative period, morbidity

Table 2. Operation type and method, morbidity and mortality values; statistical relationship of the data with NLR, MPV, and PLR

	n	%	*p* NLR	*p* MPV	*p* PLR
Operation type					
LAR	42	59.15%			
APR	6	8.45%	0.245**	0.477**	**0.040****
Right Hemicolectomy	16	22.53%			
Left Hemicolectomy	7	9.85%			
Operation technique					
Laparoscopy	32	45.1%			
Open	28	39.4%	0.991**	0.597**	0.667**
Conversion	11	15.5%			
Morbidity (+)	24	33.8%	0.504*	0.733*	0.884*
Mortality (+)	3	4.2%	**0.030***	0.777*	**0.043***

NLR=neutrophillLymphocyte ratio, MPV=mean platelet volume, PLR=platelet lymphocyte ratio, LAR=low anterior resection, APR=abdominoperineal resection *Mann-Whitney U, **Kruskal-Wallis H methods.

Table 3. AJCC (American Joint Committee on Cancer) TNM staging during and following histopathologic evaluation

	n	%	*p*		
			NLR	MPV	PLR
Tumor differentiation					
Good	53	74.6%			
Bad	14	19.7%	0.989***	0.485***	0.400***
High Grade Dysplasia	4	5.6%			
Tumor diameter (mm)					
n, mean (range)	71	52.14 (10-150)	0.136*	0.417*	0.232*
Lymph metastasis (+)	40	56.3%	0.945**	0.245**	0.954**
Lympho-vascular invasion (+)	14	19.7%	0.885**	0.602**	0.840**
Perineural invasion (+)	11	15.5%	0.253**	0.799**	0.031**
Stage					
1	16	22.53%			
2	16	22.53%	0.927***	0.675***	0.981***
3	34	47.88%			
4	5	7.06%			

NLR=neutrophil lymphocyte ratio, MPV=mean platelet volume, PLR=platelet lymphocyte ratio
*Spearman Correlation Coefficient, **Mann-Whitney U, ***Kruskal-Wallis H methods

developed in 24 (33.8%) patients with the most common causes being wound infection in 9 (12.6%)patients; subileus in 4 (5.6%)patients; and anastomosis leakage in one (1.4%) patient. Mortality was seen in three (4.2%) patients, with statistically significant relationships between mortality and NLR, PLR (p=0.03; p=0.043 respectively). Mortality developed in one (1.4%) patient due to fulminant liver metastasis, one (1.4%) due to necrotizing fasciitis and one (1.4%) due to myocardial infarction during the postoperative period. While the NLR median was found to be 2.90 (min: 1.27, max: 54.5) and the PLR median to be 175.44 (min: 6.91, max: 302.7) in patients without mortality, the NLR median climbed to 7.29 (min: 4, max: 7.35) and the PLR median to 332.43 (min: 214, max: 361.53) in patients with mortality.

Histopathologic evaluation with NLR, MPV and PLR is statistical relationship given in Table 3. Perineural invasion was detected in 11 (15.5%) patients, and a statistically significant relationship was found between this invasion and the PLR value (p=0.031).

Discussion

The main factors determining the progression of the disease and survival in cancer patients are the tumor's characteristics and the host's immune response [7]. Chronic inflammation increases the risk of colon cancer development by affecting carcinogenesis at different stages [8]. It has been shown that host or immune response can be evaluated as an important prognostic indicator in addition to TNM staging [9].

Lymphocytes and neutrophils play important roles in systemic inflammatory response. As the NLR is found by dividing the number of neutrophils by lymphocyte values, an increase in the NLR may be linked to an increase in the number of neutrophils or a decrease in the lymphocyte number. High NLR levels have been shown to be not only an indicator of inflammation in different cancer types but also an independent prognostic factor for short survival, with various threshold values reported [7, 10]. In colorectal cancer, it is reported that NLR>5 [10]. Another study describes NLR>3 as a negative independent prognostic factor for disease-free survival in colorectal cancer patients [11]. A significant relationship between high NLR values, and advanced age and high T-stage has also been mentioned. It has been reported that a NLR>2.5 value in colorectal cancer patients aggravates cancer-specific survival; NLR>4 negatively affects disease-free survival as an independent factor, and it may be used to distinguish patients for adjuvant chemotherapy in stage IIA [12]. In a study involving 243 patients, it was shown that preoperative NLR and PLR values statistically and significantly decrease general survival [13]. In colorectal cancer patients, although NLR has a prognostic value, a standard cut-off value has clearly not yet been determined [14]. In our study, the NLR value was found to be statistically related only to mortality (p=0.030).

MPV is measured automatically with either

electrical impedance or optic fluorescence methods [15]. MPV activated platelet index is used in evaluating disease activity and the effectiveness of anti-inflammatory treatment [16]. Moreover, it is indicated as a diagnostic marker in hepatocellular carcinoma and pancreas cancers [17, 18]. The role of MPV in the diagnosis and treatment of colorectal cancer could not be completely revealed. In our study, the median MPV value was 8.90 (min: 7.30, max: 12.10). Any relationship with patient and tumor characteristics in the preoperative period, postoperative surgical period or histopathologic examination could not be determined.

Platelets have important duties in hemostasis, inflammation and tissue repair [19]. Activated platelets play an important role in cancer metastasis with the cytokines they secrete, chemokinesis and adhesion receptors [20]. Moreover, activated platelets cause thrombosis development and then mortality as they cause coagulation [21]. Also, the usage of antiplatelet drugs has been seen to inhibit the invasion of tumor cells [22]. PLR values vary between 150 and 300 in colorectal cancer patients [23]. In our study, we found the median PLR value to be 178.57. This high PLR is shown as an independent predictor factor regarding T stage (tumor invasion depth) in colorectal cancer patients [24]. It is reported that high PLR values are an independent factor in decreasing general survival in colorectal cancers, and can shorten the recurrence time [25, 26]. In a study involving 200 patients, preoperative high PLR values were an independent factor for decreased general survival, and had a significant relationship with metastatic positive lymph nodes [26]. In our study, a statistical relationship was detected regarding our PLR values and both mortality and perineural invasion positivity (p=0.043, p=0.031, respectively).

The Limitations of the Study

There are certain limitations to our study. Evaluation of the data of a small group of 71 patients, absence of randomization, retrospective assessment of the data and non-evaluation of survival may be listed as examples of these limitations.

Conclusions

NLR, MPV, and PLR values are parameters that can be easily detected during routine preoperative evaluation. With more prospective randomized studies with extensive patient involvement, these biomarkers can be added to the classical staging system and contribute as prognostic and predictive factors in colorectal cancer patients.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing

The authors disclosed that they did not receive any grant during conduction or writing of this study.

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The European Research Journal

http://www.eurj.org

Original Article

e-ISSN: 2149-3189

DOI: 10.18621/eurj.278899

The effect of levosimendan on Tp-e interval, Tp-e/QT ratio, Tp-e/QTc ratio and QRS interval in heart failure patients

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ABSTRACT

Objectives. Increased Tp-e interval (from the peak-to-the-end of the electrocardiographic T wave) and Tp-e/QT ratio are associated with malignant ventricular arrhythmias. We aimed to evaluate the acute effects of levosimendan on Tp-e interval, Tp-e/QT ratio, Tp-e/QTc ratio and QRS interval in patients with severe heart failure. ***Methods.*** The study included 85 patients with decompansed heart failure, who were treated with levosimendan in our cardiology department . We evaluated the patients retrospectively. QT and Tp-e interval were assessed in the precordial leads. QRS duration was determined in the single lead which had the longest QRS. Electrocardiographic measurements was performed the basal (just before the levosimendan) and 24th hour after the levosimendan infusion (just after the levosimendan infusion). ***Results.*** No significant differences were found between before and after treatment of levosimendan with respect to Tp-e and QTc interval, QRS duration, Tp-e/QT and Tp-e/QTc ratio (pretreatment versus 24th hour values; p>0.05). Subgroup analysis was performed in the patients with inotropic therapy including dopamine and/or dobutamin (34 patients) and without inotropic therapy (49 patients) during the levosimendan infusion. The analysis showed that pretreatment and 24th hour values of Tp-e interval and Tp-e/QT ratio were significantly higher in the inotropic therapy group; (Pretreatment; Tp-e: 100.12±22.96 milliseconds [ms] versus 89.59±17.67 ms; p=0.03, Tp-e/QT: 0.26±0.05 versus 0.23±0.04; p=0.007, 24th hour: Tp-e: 101.41±27.09 ms versus 88.77±15.89 ms; p=0.009, Tp-e/QT: 0.26±0.07 versus 0.23±0.05; p=0.03). However intra-group changes of these parameters, before and after levosimendan treatment , were not significant (p>0.05). ***Conclusion.*** Our results suggested that, therapeutic doses of levosimendan infusion don't have a significant effect on Tp-e and Tp-e/QT parameters. However inotropic therapy significantly increases these parameters.

Eur Res J 2017;3(3):220-226

Keywords: Levosimendan, arrhythmia, Tp-e interval, QT interval, heart failure

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Received: December 18, 2016; Accepted: March 27, 2017; Published Online: May 24, 2017

Introduction

Ventricular repolarization is commonly assessed using QT interval and T wave measurements. Recent studies indicated that the Tp-e interval, which is the interval from the peak to the end of the electrocardiographic (ECG) T wave, can be used as an index of the total (transmural, apico-basal, global) dispersion of repolarization. Increased Tp-e interval and Tp-e/QT ratio may be associated with malignant ventricular arrhythmias [1, 2].

Levosimendan is an inotropic agent that improves cardiac contractility without increasing myocardial oxygen consumption. Levosimendan binds to the N-terminal domain of troponin C and stabilizes the troponin molecule with subsequent prolongation of its effect on contractile proteins [3]. Levosimendan is a calcium sensitizer that increases the contractile force of the myocardium by enhancing the sensitivity of myofilaments to calcium without increasing intracellular calcium concentration unlike other inotropic agents [4, 5].

Ambulatory electrocardiographic and electrophysiological evaluation did not detect any pro-arrhythmic effect of intravenous levosimendan [6]. But, according to the REVIVE study, the rate of ventricular tachycardia, atrial fibrillation, and ventricular extra-systoles in the levosimendan group were increased compared to placebo [7]. At this present study, we aimed to evaluate the acute effects of levosimendan on Tp-e interval, Tp-e/QT ratio, Tp-e/QTc ratio and QRS interval in patients with severe heart failure.

Methods

Study population

A total of 85 consecutive patients with decompansed heart failure, who were treated with levosimendan in our cardiology department were enrolled to our study. Patients with presented New York Heart Association Class IV symptoms and left ventricular systolic dysfunction of ischemic or dilated origin were enrolled to the study. The study was approved by the local ethics committee of our Institute.

Exclusion criteria were: acute or chronic infectious or inflammatory diseases; recent myocardial infarction (<8 weeks) or active myocardial ischemia; hypersensitivity to levosimendan or any of its metabolites, severe renal failure (creatinine >2.5 mg/dl), hepatic failure, 2nd or 3rd-degree atrio-ventricular blocks, overt bundle branch blocks, history of ventricular tachycardia or ventricular fibrillation, heart failure due to restrictive or hypertrophic cardiomyopathy or to uncorrected stenotic valvular disease.

Levosimendan was administered as a continuous 24 hour infusion under continuous haemodynamic monitoring. An initial loading dose of levosimendan of 12 µg/kg was infused over 10 min, followed by a continuous infusion of 0.1µg/kg/min for 24 hours. Thirty-four patients with hypotensive were also treated with concomitant inotropic therapy (dopamine and/or dobutamin) during the levosimendan infusion.

Electrocardiography

Resting 12-lead surface ECG was recorded from all the patients before and 24 hours after the start of drugs infusion. The ECG recordings were obtained at a paper speed of 25 mm/sec. and signal size of 1 mV/cm (ECG machine; Cardiofax M, 1350K, Nihon Kohden, Tokyo, Japan).

We evaluated the patients' ECG retrospectively. The 12 lead ECG was scanned at 600 dpi and ECG parameters were measured on a high resolution computer screen by 2 independent observers blinded to all other patient's data and an average of two measurements was accepted as a final result. The QT interval was measured in as many of the 12 leads as possible. Tp-e interval was assessed in the precordial leads. The Tp-e interval was defined as the interval from the peak to the end of T wave. The end of the T wave was defined as the intersection of the tangent to the downslope of the T wave and isoelectric line. The QT interval was measured from the beginning of the QRS complex to the end of the T wave, and corrected for heart rate by using the Bazett's formula, where QTc = QT/√(RR in seconds). QRS duration was determined in the single lead which had the longest QRS. The ECG measurements performed the baseline (just before the levosimendan) and 24th hour after the levosimendan infusion (just after the levosimendan infusion).

Statistical Analysis

Statistical evaluation was performed using the SPSS program (Statistical Package for the Social Sciences version 10.0, SPSS Inc, Chicago, Illinois, USA). Numerical variants were defined as mean ± standart deviation and categorical variables were

defined as percentage. Continuous variables were compared by Student t-test or Mann-Whitney U test, while categorical variables were compared by chi-square or Fisher's exact test when appropriate. Wilcoxon signed ranks test was used to compare continuous variables before and after drug therapy. Differences were considered significant at $p< 0.05$.

Results

Two patients were excluded because their ECG records were not sufficient. Therefore; we analysed 83 patients. Baseline demographic, echocardiographic and biochemical characteristics of the study patients are shown in Table 1. No significant differences were found before and after treatment of levosimendan with respect to Tp-e interval, QTc interval, QRS duration, Tp-e/QT and Tp-e/QTc ratio (pretreatment versus 24th hour values; Tp-e: 93.85±20.53 milliseconds [ms] versus 93.97±21.97 ms, QTc: 465.49±53.38 ms versus 460.82±53.78 ms, QRS duration: 107.10±31.21 ms versus 107.71±30.69 ms, Tp-e/QT: 0.24±0.05 versus 0.24±0.06, Tp-e/QTc: 0.20±0.05 versus 0.20±0.04) (p>0.05) (Table 2).

Subgroup analysis was performed in the patients with inotropic therapy including dopamine and/or dobutamin (34 patients) and without inotropic therapy (49 patients) during the levosimendan infusion. The comparison of both groups for demographic,

Table 1. Baseline demographic, echocardiographic and biochemical characteristics of the study patients

Parameters	Value
AGE (year)	66.5±10.6
GENDER (male/female)	63 (75.9%)/20 (24.1%)
BMI (kg/m2)	26.2±4.4
SBP [mm Hg]	
Before*	108.7±18.3
After **	104.1±21.5
DBP [mm Hg]	
Before	63.8±10.8
After	62.3±12.1
Heart rate [bpm]	
Before	85±16.7
After	87±17.2
GFR (mL/min)	67.4±29.1
BNP (pg/ml)	1731.2±1138.6
LVEF [%]	23.09±7.05
LVEDD (mm)	59.4±8.1
LVESD (mm)	47±10.1
IVS (mm)	10±2.09
PW (mm)	9.8±1.6
LA (mm)	47.3±6.1
WBC (10^3 /µL)	10489±3791
HgB (g/dL)	11.2±1.7
PLT (10^3 /µL)	234215±88280
GLUCOSE (mg/dL)	141.9±53.5
UREA (mg/dl)	44.6±22.7
CREATININE (mg/dl)	1.26±0.4
AST (IU/L)	44.4±36.7
ALT (IU/L)	54.8±100
Na (mEq/L)	134.1±14.4
K (mEq/L)	4.2±0.5

*before = pretreatment, ** after = 24th hour of treatment, BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, GFR = glomerular filtration rate, BNP = B-type natriuretic peptide, LVEF = left ventricular ejection fraction, LVEDD = left ventricular end diastolic diameter, LVESD = left ventricular end systolic diameter, IVS = Interventricular septum, PW = posterior wall, LA = left atrium; WBC = white blood cell count, HgB = hemoglobin, PLT = platelet count, AST = aspartate aminotransferase, ALT = alanine aminotransferase, Na = sodium, K = potassium

Table 2. QRS duration, QTc interval, Tp-e interval, Tp-e/QT and Tp-e/QTc ratio parameters before (pretreatment) and after treatment (24th hour of treatment) of levosimendan.

	Levosimendan (n=83)		*p* Value
	Before*	After**	
QRS duration (ms)	107.10±31.21	107.71±30.69	0.66
QTc interval (ms)	465.49±53.38	460.82±53.78	0.29
Tp-e (mean) (ms)	93.85±20.53	93.97±21.97	0.95
Tp-e/QT ratio	0.24±0.05	0.24±0.06	0.34
Tp-e/QTc ratio	0.20±0.05	0.20±0.04	0.43

*before = pretreatment; ** after= 24th hour of treatment

echocardiographic and biochemical characteristics are shown in Table 3. The analysis showed that pretreatment and 24th hour values of Tp-e interval and Tp-e/QT ratio were significantly higher in the inotropic therapy group; (pretreatment; Tp-e: 100.12±22.96 ms versus 89.59±17.67 ms; *p*=0.03, Tp-e/QT: 0.26±0.05 versus 0.23±0.04; *p*=0.007, 24th hour: Tp-e:101.41±27.09 ms versus 88.77±15.89 ms; *p*=0.009, Tp-e/QT: 0.26±0.07 versus 0.23±0.05; *p*=0.03) (Table 4). However; the QT and Tp-e parameters did not significantly change with levosimendan infusion in both subgroups (inotropic therapy group and without inotropic therapy group) (p>0.05) (Table 4).

Discussion

The results of this present study revealed that the therapeutic doses of levosimendan infusion did not have a significant effect on QRS duration, QTc interval, Tp-e, Tp-e/QT and Tp-e/QTc ratio parameters. Nevertheless levosimendan and concomitant inotropic therapy significantly increase these parameters that the increasing is related with inotropic (dopamine and dobutamine) therapy.

Arrhythmia is one of the disadvantages and a restriction of usage of positive inotropic drugs. Therefore, it is important to determine the proarhythmic potential of any new inotropic agent intended for treatment of the heart failure. Levosimendan is a novel calcium sensitizer which has inotropic effect by increasing sensitivity of Ca2+ in the contraction site. Levosimendan increases the contractile force of the myocardium by enhancing the sensitivity of myofilaments to calcium without increasing intracellular calcium concentration or intracellular cyclic Adenosine Monophosphate (cAMP) [4, 5].

Tp-e interval was evaluated in different patients' population about its arrhythmias prediction [1, 2, 8]. Recent studies showed that the Tp-e interval can be used as an index of the total (transmural, apico-basal, global) dispersion of repolarization and also, increased Tp-e interval and Tp-e/QT ratio may be associated with malignant ventricular arrhythmias [1, 2]. So, at this present study, we aimed to evaluate the acute effects of levosimendan on Tp-e interval, Tp-e/QT ratio, Tp-e/QTc ratio, and QRS interval in patients with severe heart failure as a proarhythmic potential effect.

Arrhythmic potential of the levosimendan has been previously studied in the several major trials and small-sized studies [9, 10]. The Levosimendan Infusion versus Dobutamine (LIDO) study and the Randomized Study on Safety and Effectiveness of Levosimendan in Patients with Left Ventricular Failure after an Acute Myocardial Infarct (RUSSLAN) are the major trials evaluating levosimendan. The RUSSLAN study was a double-blind, placebo-controlled trial conducted in 504 patients who had recently experienced an acute myocardial infarction and the patients were allocated to placebo or one of four dose regimens of levosimendan. At the end of the study, the frequency of atrial and ventricular arrythmias were similar to placebo in all of the four levosimendan regimens and also, the risk of arrhythmic events showed no dose-relation [9]. In LIDO study, 203 patients with severe heart failure randomly received a 24 h infusion with either levosimendan or dobutamine. At dobutamine group, there was a higher rate rhythm disorders [11]. Flevari *et al.* [12] examined effects of short-term levosimendan infusion on ventricular arrhythmias. Forty-five patients with heart failure were randomized to levosimendan or placebo group. 24-hour Holter

Table 3. The comparison of both groups for demographic, echocardiographic and biochemical characteristics

	Levosimendan and Inotropic Therapy (n=34)	Levosimendan (n=49)	*p Value*
Age (year)	67.09±10.8	66.2±10.6	0.72
BMI (kg/m2)	25.4±4.2	26.9±4.5	0.37
SBP [mm Hg]			
Before*	105.6±16.5	110.8±19.3	0.21
After**	106.8±13.5	102.3±25.5	0.37
DBP [mm Hg]			
Before	63.7±11.5	63.9±10.5	0.93
After	63.4±9.4	61.6±13.7	0.52
Heart rate [bpm]			
Before	91.2±16	81±16	0.007
After	95.2±18	82.3±14	0.001
Causes of HF (ischemic /nonischemic)	29/5	39/10	0.50
GFR (mL/min)	62.9±28.8	70.7±29.6	0.45
BNP (pg/ml)	1802.5±590.1	1695.6±1372.4	0.88
LVEF [%]	22.5±5.7	23.5±7.9	0.51
LVEDD (mm)	59.4±7.5	59.3±8.5	0.98
LVESD (mm)	47±9.9	46.9±10.3	0.96
IVS (mm)	10.1±2.2	9.9±1.9	0.64
PW (mm)	9.8±1.5	9.8±1.7	0.94
LA (mm)	47.1±5.9	47.4±6.3	0.81
WBC (10^3 /μL)	10497±3884	10483±3765	0.98
HgB (g/dL)	11±1.3	11.3±1.9	0.56
PLT (10^3 /μL)	232382±84068	235487±91927	0.87
Glucose (mg/dL)	151.5±61.5	133.8±44.9	0.16
Urea (mg/dl)	48.6±23	41.9±22.3	0.19
Creatinine (mg/dl)	1.34±0.46	1.21±0.44	0.18
AST (IU/L)	50.8±50.7	39.9±21.7	0.18
ALT (IU/L)	72.9±148.7	41.9±36.4	0.16
Na (mEq/L)	137.1±6.2	132.1±17.9	0.12
K (mEq/L)	4.1±0.4	4.2±0.6	0.40
Ca (mEq/L)	8.6±0.9	8.9±0.7	0.23
Mg (mEq/L)	2±0.4	2.2±0.2	0.15

*before = pretreatment, ** after = 24th hour of treatment, BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, GFR = glomerular filtration rate, BNP = B-type natriuretic peptide, LVEF = left ventricular ejection fraction, LVEDD = left ventricular end diastolic diameter, LVESD = left ventricular end systolic diameter, IVS = interventricular septum, PW = posterior wall, LA = left atrium; WBC = white blood cell count, HgB = hemoglobin, PLT = platelet count, AST = aspartate aminotransferase, ALT = alanine aminotransferase, Na = sodium, K = potassium, Ca = calcium, Mg = magnesium

recording was performed to assess changes in ventricular arrhythmogenesis, 24-hour heart rate variability indexes, QTc, QT variability, and QT/RR slope. Episodes of non-sustained ventricular tachycardia were found increased with levosimendan group (21.9±9.6 versus 3.0±1.2, $p<0.05$) [12]. Lilleberg *et al.* [13] assessed levosimendan to generate cardiac arrhythmias by analysing ECG recordings from clinical studies on intravenously administered levosimendan in heart failure patients. Their database consisted of continuous 1□day recordings, of which 366 were during levosimendan and 142 during placebo comparison. At the end, there was no difference appeared between levosimendan and control groups in the occurrence of atrial fibrillation (12% versus 13%), SVT (28% versus 30%), or VT

Table 4. Subgroup analysis and comparative effects of levosimendan according to inotropic therapy on QT, QTc, Tp-e,Tp-e/QT and Tp-e/QTc ratio parameters.

	Levosimendan and Inotropic Therapy (n=34)	**Levosimendan (n=49)**	***p* Value**
QT interval (ms)			
Before*	385.29±54.72	389.38±57.35	0.74
After **	373.82±51.22	380.20±55.65	0.59
p	0.08	0.14	
QTc interval (ms)			
Before	474.44±59.58	459.82±48.35	0.22
After	476.44±61.48	449.98±45.27	0.02
p	0.60	0.33	
Tp-e (mean) (ms)			
Before	100±22.96	89.59±17.67	0.02
After	101.47±27.09	88.77±15.89	0.009
p	0.98	0.91	
Tp-e/QT ratio			
Before	0.26±0.05	0.23±0.04	0.007
After	0.26±0.06	0.23±0.04	0.03
p	0.44	0.51	
Tp-e/QTc ratio			
Before	0.21±0.04	0.19±0.04	0.06
After	0.21±0.04	0.19±0.03	0.11
p	0.95	0.28	

*before = pretreatment, ** after = 24th hour of treatment

(41% versus 44% of all recordings; all p>0.05) and also the frequency of VT was similar (0.55±3.89 vs 0.20±1.08 episodes/h; *p*>0.05) [13].

In the light of these aforementioned studies, we assessed the short-term effects of levosimendan therapy on QRS duration QTc interval, Tp-e, Tp-e/QT and Tp-e/QTc ratio parameters in surface ECG in 83 patients who had severely depressed left ventricle functions. The analysis showed that levosimendan did not have a significant effect on QRS duration, QTc interval, Tp-e, Tp-e/QT and Tp-e/QTc ratio parameters. So, our analysis supports the previous studies' results that short□term levosimendan therapy of heart failure had no tendency to increase cardiac arrhythmias [14, 15]. We performed subgroup analysis to the patients with inotropic therapy (dopamine and/or dobutamin) during the levosimendan infusion (34 patients) and without inotropic therapy (49 patients). We found that pretreatment and 24th hour values of Tp-e interval and Tp-e/QT ratio were significantly higher in the inotropic therapy group. On the other hand, levosimendan and concomitant inotropic therapy may significantly affect these parameters but actually these alterations were related with the inotropic therapy. Because; the QT and Tp-e parameters did not significantly change with levosimendan infusion in the inotropic and without inotropic treatment group. Paksoy *et al.* [16] investigated the effect of intravenous levosimendan and dobutamine on QT parameters and found that levosimendan and dobutamine did not have a significant effect on QT parameters (16). In our study group we used dopamine/dobutamine and levosimendan concomitant at inotropic therapy. However, Paksoy *et al.* [16] used dobutamine and levosimendan in two different groups separately. QT

prolongation is usually associated with cardiac ischemia. QT is not affected by inotropic therapy unless patients have cardiac ischemia. There was no ischemic event in our study patients. This point could explain our results.

The Limitations of the Study

This study has a number of limitations. Small number of patients, non randomized, retrospective analysis are the major limitations. However our analysis is the first study which evaluate the Tp-e and Tp-e/QT parameters in patients with levosimendan treatment. When viewed from this aspect this study could flash on novel investigations.

Conclusions

In conclusion, our study supported that short□term therapeutic doses of levosimendan infusion for heart failure had no tendency to increase cardiac arrhythmias according to Tp-e and Tp-e/QT parameters. However levosimendan and concomitant inotropic therapy may have arrhythmia potential, that is related with inotropic therapy.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing

The authors disclosed that they did not receive any grant during conduction or writing of this study.

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The European Research Journal

http://www.eurj.org

Original Article

e-ISSN: 2149-3189

DOI: 10.18621/eurj.345667

Cardiac myxomas: an analysis of 39 patients

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ABSTRACT

Objectives. Myxomas are the most common benign primary heart tumors. They have serious complications including intracardiac blood flow obstruction and embolic events. The aim of the study was to assess our experiences related to patients undergoing surgical resection for cardiac myxomas. ***Methods.*** The medical records of 39 patients, aged 16 to 76 years (mean, 47.5 years), who were operated on for primary cardiac myxomas between January 1994 and December 2016 at our clinic were retrospectively evaluated. Demographic, clinical, operative and postoperative data were obtained from these hospital medical records. Cardiac myxomas were diagnosed by transthoracic echocardiography. Preoperative coronary angiography was performed in patients over 40 years of age and those with symptoms of coronary disease. In routine follow-up after discharge the patients were checked by echocardiography. Long-term cumulative survival was analyzed using the Kaplan-Meier method. ***Results.*** There was no in-hospital mortality. The majority (61.5%) of patients were female. The most common encountered localization of myxoma was the left atrium (76.9%), and the classic posterior approach from interatrial groove was preferred in 32 (82.1%) patients. Mean follow-up was 6.05 ± 3.75 years (range, 1-10 years). Five (12.8%) patients were lost on long-term follow-up. Kaplan-Meier curves, cumulative proportion surviving of patients at 1-, 2-, 5-, and 10-year were 97.4%, 91.7%, 84.7%, and 84.7%, respectively. No hospital mortality was observed in any of the patients. There was no recurrence in our series. ***Conclusions.*** Myxoma is the disease that can lead to complications such as embolic events and intracardiac blood flow obstruction. It can be excised with a low rate of morbidity and mortality. Surgical resection should be performed promptly after diagnosis in order to prevent potential complications.

Eur Res J 2017;3(3):227-233

Keywords: Cardiac tumors, myxomas, cardiac surgery, echocardiography

Introduction

Myxomas are the most common seen primary benign heart tumors. Although they can be found in all chambers of the heart, 75% of cases is localized in the left atrium [1, 2]. Despite their benign nature, they have serious complications including intracardiac blood flow obstruction and embolic events [3]. If left untreated, they are unappeasable progressive and result in fatal outcomes.

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Received: October 22, 2017; Accepted: October 26, 2017; Published Online: October 27, 2017

The most effective treatment of myxomas is surgical removal. Many surgeons agree that the surgical excision of the tumor should be performed promptly after diagnosis in order to avoid potential serious complications. Surgical resection of the myxoma has good results. However, patients should be followed-up closely after surgery because recurrent myxomas may occur, requiring reoperation [4].

The aim of the study was to evaluate our experiences related to patients undergoing surgical resection for cardiac myxomas and to review the literature.

Methods

Patients

Thirty-nine patients undergoing surgical resection for primary cardiac myxomas between January 1994 and December 2016 at our institution were retrospectively evaluated. Demographic, clinical, operative and postoperative data were obtained from hospital records. All medical records including echocardiography (transthoracic and/or transesophageal), electrocardiography, exercise tests, coronary angiography, surgical, pathological and postoperative reports were analysed. Twenty-four (61.5%) of the patients were female, 15 (38.5%) were Male. The mean age was 47.5 ± 13.12 years and ranged in age from 16 to 76 years. Myxoma was diagnosed primarily by transthoracic two-dimensional echocardiography, in only one patient diagnosis made by echocardiography performed after abnormal coronary artery vascularization detected during coronary angiography. Cardiac myxoma diagnosed by transthoracic echocardiography was confirmed by transesophageal echocardiography. Preoperative coronary angiography was performed in patients over 40 years of age or in cases of anginal symptoms. This study was approved by the Local Institutional Ethics Committee and and a waiver of informed consent was obtained.

Surgical Technique

Median sternotomy was performed in all cases. Routine cardiopulmonary bypass was instituted with aortic and bicaval cannulation and cardiac arrest was provided using antegrade cardioplegia. Myocardial protection was achieved firstly by antegrade cold crystalloid 'St Thomas' cardioplegia, subsequently, intermittent blood cardioplegia. and surgical procedures were performed under aortic cross-clamp. Surgical approach was performed according to the localization of the myxoma and radical excision with the attachment base of mass was performed. Where necessary, septal defects caused by myxoma excision were repaired with a patch of autologous pericardium and after the operation all excised tumor materials were sent for routine histopathological examination (Figure 1A and 1B). Cardiac chambers were irrigated with saline to prevent possible microembolisms after excision. If applicable, additional concomitant surgical procedures were performed for cardiac pathologies accompanied by myxomas. In routine follow-up after

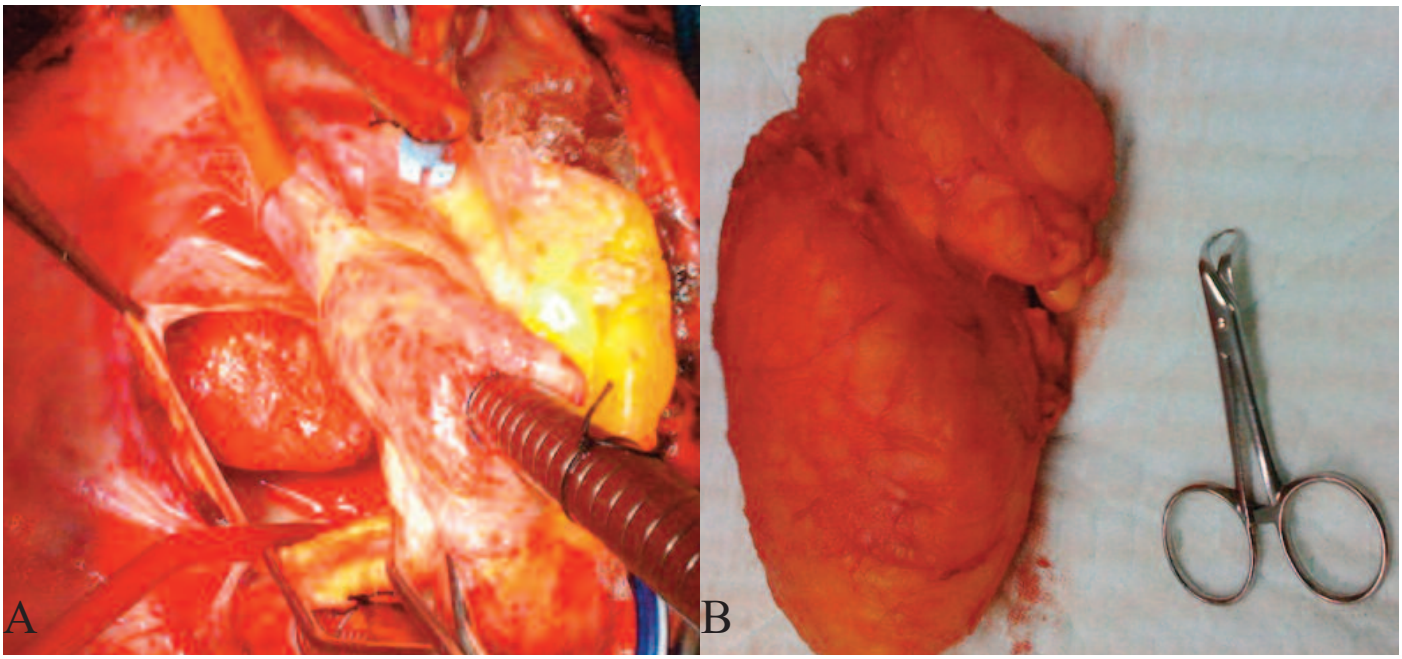

Figure 1. Intraoperative appearance of the myxoma (A) and excised myxoma (B).

discharge, patients were checked by echocardiography.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD) for all continuous variables or as minimum and maximum values if there is not normal distribution or as numbers with percentages for complication and recurrence rates, including information about preoperative status where appropriate. Long-term cumulative survival was analyzed using the Kaplan-Meier method. Statistical analysis data were analyzed with the Statistical Package for the Social Sciences (IBM SPSS Statistic Inc. Version 21.0, Chicago, IL, USA).

Results

The preoperative clinical status and age distribution of the patients are shown in Table 1. The majority (61.5%) of patients were female and patients were found most frequently in the age range of 40-60 years. Thirty (76.9%) of the detected myxomas were originated from the left atrium and in half of them left atrial trombus was found. Peripheral arterial thromboembolism was the cause of admission to the hospital in 6 (15.4%) of the patients. Preoperative coronary angiography performed in 20 (51.3%) patients and 9 (23.1%) of these patients underwent coronary artery bypass grafting with tumor excision. Surgical procedures applied to the patients are shown in Table 2. Histopatologic study of the mass confirmed the diagnosis of myxoma.

In 32 (76.9%) patients with myxoma originated from the left atrium, posterior mitral valve myxoma and biatrial and biventricular recurrent myxoma, posterior approach was applied from interatrial grove. The septum was repaired with a pericardial patch after excision in 6 of the myxomas arising from the left atrial septum. Mitral valve replacement was performed in 3 (7.7%) patients with concomitant mitral stenosis and in one (2.6%) patient with myxoma arised from posterior mitral leaflet. In addition to tumor resection, mitral valve annuloplasty was performed in the 5 (12.8%) patients with mitral regurgitation. Right

Table 1. Demographic and clinical characteristics of the patients

	n (%)
Age (years)	47.15 ± 13.2 (16-76)
Gender, female	24 (61.5)
Localization	
Left atrial septum	21 (53.8)
Left atrial wall	9 (23.1)
Mitral valve posterior leaflet	1 (2.6)
Right atrial septum	7 (17.9)
Biatrial + biventricular (multiple)*	1 (2.6)
Symptoms	
Dyspne	26 (66.7)
Palpitation	19 (48.7)
Angina	3 (7.7)
Syncope	2 (5.1)
Heart failure	3 (7.7)
Acute pulmonary edema	1 (2.6)
Peripheral thromboembolism	6 (15.4)
Co-morbidity	
Coronary artery disease	9 (23.1)
Dilate cardiomyopathy	1 (2.6)
Left atrial thrombus	17 (43.6)
Tricuspid valve regurtation	4 (10.3)
Mitral valve stenosis	6 (15.4)
Mitral valve regurtation	4 (10.3)
Atrial fibrillation	8 (20.5)

Data are shown as mean ± standard deviation (range) or number (percent). *Patient who had underwent myxoma surgery at another center 8 years ago

Table 2. Surgical procedure

Procedure	n (%)
Resection	17 (43.6)
Resection+CABG	9 (23.1)
Resection+MVR	4 (10.2)
Resection+MR	4 (10.2)
Resection+TR	4 (10.2)
Resection+MR+TR	1 (2.6)

Data are shown as number (percent). CABG = coronary artery bypass grafting, MR = mitral repair, MVR = mitral valve replacement, TR = tricuspid repair

atriotomy was performed in 8 (20.5%) patients with right atrial myxoma, and biatrial and biventricular recurrent myxoma. Due to the large resection of the interatrial septum in 2 (5.1%) patients, the septum was repaired with pericardial patch while primary repair was sufficient in 6 (15.4%) patients. In 5 (12.8%) patients with concomitant moderate or severe tricuspid regurgitation, De Vega tricuspid annuloplasty was performed.

As a remarkable event, in one patient, a mass found incidentally in the left atrium was excised during mitral valve surgery. After pathological examination the diagnosis of the mass was confirmed as myxoma.

In echocardiographic examination of another case with acute cardiac insufficiency findings who had episodes of syncope and pulmonary edema, a pedicled myxoma was found in the left atrium. It was observed that the mass was prolapsed to the left ventricle during each diastole and returned to the left atrium during systole. The patient had arrhythmia and low cardiac output due to intermittent obliteration of the mitral valve orifice by the mass. The case underwent to emergency operation.

Another patient with dilated cardiomyopathy, whose ejection fraction was measured as 18%, was operated electively for hemodynamic instability and syncope episodes because of occlusion at the mitral valve orifice. The myxoma was successfully removed under a very short cross-clamp time.

Biatrial and biventricular recurrent myxomas were detected in echocardiography performed due to the shortness of breath in one (2.6%) patient who underwent resection for left atrial and right ventricular myxoma at another center 8 years ago [4]. Atrial and ventricular myxomas were successfully excised by performing biatrial incision. At the same time, mitral and tricuspid valve repair was performed.

In a patient admitted to our hospital with symptoms of stable angina pectoris, coronary angiography was performed after a positive evaluation of the effort test. While coronary arteries were found normal on coronary angiogram, a large mass revealed which has very advanced vascularity and feeding from the circumflex and the right coronary artery. Echocardiography revealed a myxoma in the left atrium and anginal symptoms disappeared after removal of the myxoma.

No hospital mortality was observed in any of the patients. The postoperative course was uneventful and follow-up examination showed normal doppler echocardiographic parameters and clinical recovery. Mean follow-up was 6.05 ± 3.75 years (range, 1-10 years). Five (12.8%) patients were lost on long-term follow-up. There were three deaths due to traffic accident in one case and cardiac reasons related to coronary artery disease in 2 cases. Two patients could not be reached because of the remote rural area. Kaplan-Meier curves, cumulative proportion surviving of patients at 1-, 2-, 5-, and 10-year were 97.4%, 91.7%, 84.7%, and 84.7%, respectively (Figure 2).

Discussion

The primary cardiac tumors are benign at the rate of 75% and myxomas are the most common encountered tumors. Fibroelastoma, hemangioma and fibroma are the other types of cardiac benign tumors [3]. Most common malign primary cardiac tumor is angiosarcoma and metastatic tumors frequently originate from lung and breast carcinomas. Myxomas are thought to develop from primitive endothelial or subendocardial cells, or from multipotential mesenchymal cells [5].

Myxomas can occur in all chambers of heart and 75% of myxomas located in the left atrium [1]. The majority of the myxomas detected in our study were of left atrial origin. Clinical symptoms are usually seen in adults, and most often between the third and sixth

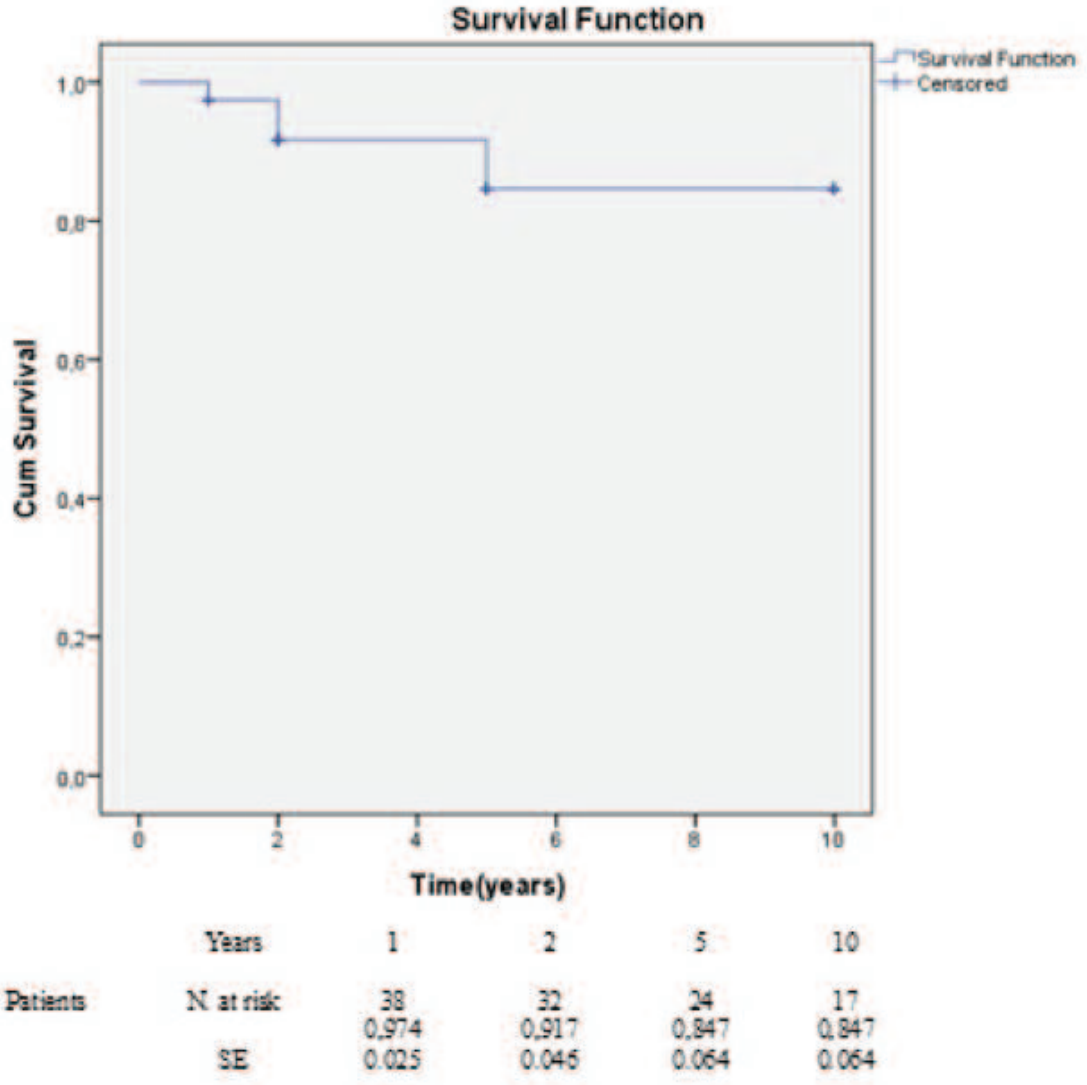

Figure 2. Long-term cumulative survival curves (Kaplan-Meier) after surgery. The proportion of patients following 1 year was estimated as 0.97 ± 0.025, 2 years as 0.91 ± 0.046, 5 years and 10 years as 0.84 ± 0.064. SE = standart error.).

decades and women are more frequently affected. Our patient's average age was 47.15 and female-male ratio was 1.6.

Most of myxomas are idiopathic. However 5% of cases have familial history. Familial form generally is known as Carney's syndrome. This syndrome has an autosomal dominant transition and frequently seen in younger males. Mucosal myxoma, skin hyperpigmentation and endocrine organ hyperactivity accompanies to this syndrome [3].

The clinical presentation of cardiac myxomas are classified in three main categories: tumor-related obstruction of intracardiac blood flow, tumor-related embolic events, and systemic symptoms [3]. Clinical features are related with size, location and mobility of the myxoma. Meşe *et al.* [6] reported a case with a giant left atrial myxoma that completely obstructed the left atrioventricular flow leading to acute pulmonary edema. We had a case that underwent operation urgently with similar symptoms in our series. As stated in the literature, the most common symptoms in our series with cardiac myxomas were also congestive heart failure, dyspnea and palpitation [3, 5, 7, 8]. In particular, tumors originating from the septum exhibit symptoms of congestive heart failure, whereas those that do not originate from the septum are more associated with embolic events [9].

Embolic events may be the first sign of cardiac myxomas. Many embolic events such as cerebral embolism, lower extremity embolisms, visceral organs and coronary embolism may be resulted from an intracardiac myxoma [3]. Papillary and fragmented myxomas with irregular surface are more prone to embolism than solid ones. Coating by thrombus of

irregular surface of papillary tumor contributes to embolism process. In two studies comparing papillary and solid myxomas, embolism predisposition was found more to favor papillary myxoma [10, 11]. Taş et al. [8] detected the embolic events of 25% in their study and reported that 75% of them were cerebral. Kaplan *et al.* [7] reported that they identify cerebral embolism of 2% versus peripheral embolism of 11%. In our study we detected embolism of 15.4% and all of them were peripheral events. It should also be kept in mind that the right-side myxomas may lead to pulmonary embolism but uncommon, while the left side ones can rarely cause pulmonary embolism in the presence of atrial septal defect and ventricular septal defect [12]. In a retrospective analysis, He *et al.* [13] reported that location and size of the tumor, macroscopic appearance, mean platelet volume, and high platelet count are closely associated with embolic events in patients with cardiac myxomas.

Echocardiography is the most important diagnostic method for diagnosis of the cardiac myxomas. It can give an accurate information about the size, localization and origin of the tumor. In our clinic, myxomas was diagnosed by two-dimensional transthoracic echocardiography and transesophageal echocardiography have been used to confirm the diagnosis of myxoma. Likewise, postoperative echocardiography shall be sufficient for control. Other imaging modalities that are equally useful for diagnosis but are not easily accessible, and are generally more costly and time-consuming, are thoracic CT and MRI, on which myxomas appear as spherical or ovoid intracavitary masses of heterogeneous composition [14].

Treatment of cardiac myxomas is surgery. Surgical treatment should be performed as soon as the diagnosis is made. Surgical approaches vary according to myxomas origin. Different techniques such as posterior approach from interatrial groove, transseptal approach, biatrial approach, T-shaped biatrial approach, and superficial septal approach are available for myxoma excision [6]. No matter which surgical technique, it is necessary to avoid unnecessary manipulations until the tumor is excised to prevent the intraoperative embolism. The surgical approaches should provide adequate exposure for a complete resection, and allow for the inspection of all four heart chambers in order to minimize the risk of recurrence. In addition, right atrium myxomas can be injured during cannulation. In surgical manipulation this issue should not be overlooked.

The classic posterior approach from interatrial groove may be suitable in most cases especially for thin pedicled myxomas and the defect can be closed primarily. However, in order to reduce the recurrence rate, a large resection of the septum and a patch for its closure may be required. Taş *et al.* [8] reported that about half of the cases were operated with classical left atrial approach in their study. In our series classical left atrial incision were used for myxomas which originated from the left atrium and only in three cases pericardial patch was used for repair of the defect due to wide septum resection. The transseptal approach is performed through right atriotomy. Kaplan *et al.* [7] described that the transseptal approach is well suited for clear exploration, comfortable resection and keeping under control of all cardiac chamber. The transseptal approach gives good access to the myxoma with minimum handling and allows inspection of all cardiac chambers [15]. The biatrial approach of left atrial myxomas is considered more appropriate in the literature [1]. The biatrial approach includes both a vertical incision made in the posterior of the interatrial groove and a transverse incision on the right atrium. In our study we could be able to resect easily the myxomas by biatrial approach in a case which we found recurrence in four cardiac chamber. Meşe et al. [6] reported that they excised a giant left atrial myxoma by performing superior septal approach. The right atrium and the right ventricule myxomas are approached through the right atrium.

Recurrence may occur after surgical resection of myxomas. Inadequate resection, being familial type, intraoperative implantation and embolization of tumor fragment are among the causes of recurrence [3, 4]. Garatti *et al.* [10] reported a recurrence rate of 1% in the series of large resection and patch use, whereas McCarty *et al.* [16] reported that this rate was as high as 10-30% in familial cases. There was no recurrence in our series, however we determined a case with multiple localized recurrent myxoma 8 years after surgery for left atrial and right ventricular myxoma in another center [4]. It should be noted that appropriate approach according to localization, wide resection and checking the other cardiac chambers may decrease reccurence rate. Normally echocardiographic follow-up should be performed each year to detect early myxoma relapses.

Cardiac myxomas may exist with concomitant cardiac disease. Injury of mitral subvalvular apparatus or leaflets can cause mitral regurgitation [5, 17]. Gucu *et al.* [18] presented a case of myxoma that was

located in the mitral valve with mitral insufficiency and accompanying intracranial tumor. In our series, mitral valve repair or replacement was applied at rate of 25%.

In the literature, the incidence of coronary artery disease is reported to be between 20% and 36% in patients with myxoma due to age and atherosclerosis. Furthermore, the likelihood of causing coronary artery embolism of myxoma is reported as 0.06% [19, 20]. Consistent with the literature, we performed coronary artery bypass grafting in addition to myxoma resection in 23.1% of our patients. Because of this high association, preoperative coronary angiography for patients with symptoms and over 40 years is necessary.

Conclusion

As a result, despite being benign, cardiac myxomas are pathologies that can lead to complications such as embolic events and intracardiac blood flow obstruction. It can be excised with a low rate of morbidity and mortality. Surgical resection should be performed promptly after diagnosis in order to prevent potential complications and even possible fatal outcomes.

Authorship declaration

All authors listed meet the authorship criteria according to the latest guidelines of the International Committee of Medical Journal Editors, and all authors are in agreement with the manuscript.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing

The authors disclosed that they did not receive any grant during conduction or writing of this study.

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The European Research Journal

http://www.eurj.org

Original Article

e-ISSN: 2149-3189

DOI: 10.18621/eurj.278518

Causes of elevated parathyroid hormone levels in postmenopausal women

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ABSTRACT

Objectives. In this study we aimed to investigate causes of hyperparathyroidism and related factors in postmenopausal women. ***Methods.*** The study was conducted on 156 postmenopausal women, 43 with normal serum parathyroid hormone (PTH) levels and 113 with elevated serum PTH levels. Serum levels of 25-OH vitamin D, calcium and phosphorus, 24-hour urine calcium, phosphorus and calcium/creatinine ratio were compared between study groups. Also, bone mineral density, age of menopause, educational level, occupation, clothing style, daylight exposure time and daily dietary calcium consumption of subjects and relationships of these parameters with parathyroid hormone levels were investigated. ***Results.*** Causes of elevated serum PTH level were vitamin D deficiency in 92.9% and primary hyperparathyroidism in 4.4% of study group. Serum PTH levels were significantly higher in housewives (p<0.001), women with less than a high school graduates (p=0.008), and the veiled women (p=0.025). Serum 25-OH vitamin D levels were significantly lower in the veiled covered (p=0.002) and participants with less than a high school graduate (p=0.041). Significant negative correlation was detected between serum 25-OH vitamin D and the logarithmic value of serum PTH levels (r= -0.188; p=0.019). ***Conclusions.*** Vitamin D deficiency was common in all postmenopausal women but especially in those with lower education level and the veiled. Postmenopausal women should be screened for vitamin D deficiency and encouraged to benefit more from sunlight. Also, enriching foods in the markets with vitamin D may be helpful for decreasing hyperparathyroidism in this population.

Eur Res J 2017;3(3):234-242

Keywords: Primary hyperparathyroidism, secondary hyperparathyroidism, vitamin D deficiency, postmenopausal women, parathyroid hormome

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Received: December 17, 2016; Accepted: March 3, 2017; Published Online: May 30, 2017

Introduction

Primary hyperparathyroidism is a calcium metabolism disorder characterized by excessive production of parathyroid hormone (PTH) from the parathyroid glands without any stimulus known or described. Primary hyperparathyroidism affects 0.3% of the general population and 2.1% of the postmenopausal women [1]. Secondary hyperparathyroidism is described as increased secretion of PTH as a result of the stimulation of the calcium sensitive receptors secondary to a decrease in serum calcium level. Secondary hyperparathyroidism affects 1% of the general population and is usually secondary to low oral intake of calcium and/or vitamin D deficiency [2]. Secondary hyperparathyroidism secondary to vitamin D deficiency leads to mineralization disorders of the bones, low bone mineral density, osteoporosis and ultimately an increased risk of bone fracture in adults [3]. Calcium and vitamin D replacement leads to reduction in fracture incidence in elderly people but treatment with only vitamin D does not provide significant benefits [4].

In this study, we screened the etiologies of elevated PTH levels in postmenopausal women without concomitant diseases or use of drugs that may interfere with PTH levels. Also, we aimed to determine the incidence of clinically asymptomatic primary hyperparathyroidism, the causes of secondary hyperparathyroidism, social risk factors of hyperparathyroidism and the measures that can be taken to avoid hyperparathyroidism and its complications in postmenopausal women.

Methods

One hunded and fifty-six postmenopausal women, 113 with elevated PTH levels and 43 with normal PTH levels, admitted to the department of Internal Medicine outpatient clinics of Uludag University Hospital (from October 2010 to May 2011), aged ≥50 years, had no menstruation for at least two years and no disease to affect calcium metabolism were included. Premenopausal women aged <50 years, postmenopausal women aged ≥75 years, women with a previous diagnosis of parathyroid gland disease, chronic renal failure, malabsorption, biphosphonate, thiazid or corticosteroid drug use and acute infection were excluded. Serum PTH levels higher than 68.3 pg/ml was considered as hyperparathyroidism according to Uludag University Laboratory parameters. The study protocol was approved by Medical Research Ethics Committee of Uludag University (Date: 28.09.2010; Nr: 2010-9/15) and written informed consent were taken from all patients included to the study.

Education, occupation, average duration of exposure to sunlight per day during summer and winter, consumption of calcium-rich foods were recorded. Participants were divided into four groups according to their educational levels: had no formal education, primary school, high school and college graduates; two groups according to their clothings: veiled and non-veiled; three groups according to their occupational status as housewives, workers and retired people; two groups according to sunlight exposure insufficient (less than 20 minutes per day) and sufficient (more than 20 minutes per day); two groups according to daily calcium consumption adequate and inadequate. Daily calcium intake of subjects were calculated by using a questionnaire form which prepared by Turkish Dietitians' Association. The questionnare form was designed to assessment calcium content of dietary records.

Venous blood samples were collected after 10-hours of overnight fast and centrifuged. Levels of calcium, phosphorus, creatinine, alkaline phosphates, albumin and follicle stimulating hormone (FSH) were determined using commercially available assay kits with an Abbott Architect C16000 auto-analyzer. PTH levels were measured using Abbott G200 kits with an Abbott Architect i2000sr analyzer. 25-OH vitamin D levels were measured using THERMO HPLC analyzer. 24-hour urinary calcium, phosphorus and creatinine excretion were measured. Patients with high serum creatinine levels and FSH levels lower than 20 IU/ml were excluded from the study.

Bone mineral density measurements in all participants were detected by dual-energy x-ray absorptiometry (DEXA) method using with Hologic QDR-4500A S/N 45130 analyzer (Hologic Inc. Bedford, MA, USA). The measurements of the lumbar spine and the left femur were recorded. The mineral density (g/cm²) of the second lumbar (L) vertebra and the average mineral density of the L 1-4 vertebrae were compared statistically. Also the mineral density (g/cm²) of the left femoral neck and the average mineral density of the femoral neck, trochanteric and intertrochanteric regions were compared.

Statistical Analysis

Continuous variables were expressed as mean ± standard error, median, minimum and maximum values. Categorical variables were expressed as number and percentage values. The suitability of the normal distribution of continuous variables was analyzed by Shapiro Wilk test. According to the test results, in cases where there is compliance with the normal distribution, independent-samples t test was used for between groups comparisons. Correlation analysis was used for determination the relationship between continuous variables and Pearson correlation coefficient was calculated.

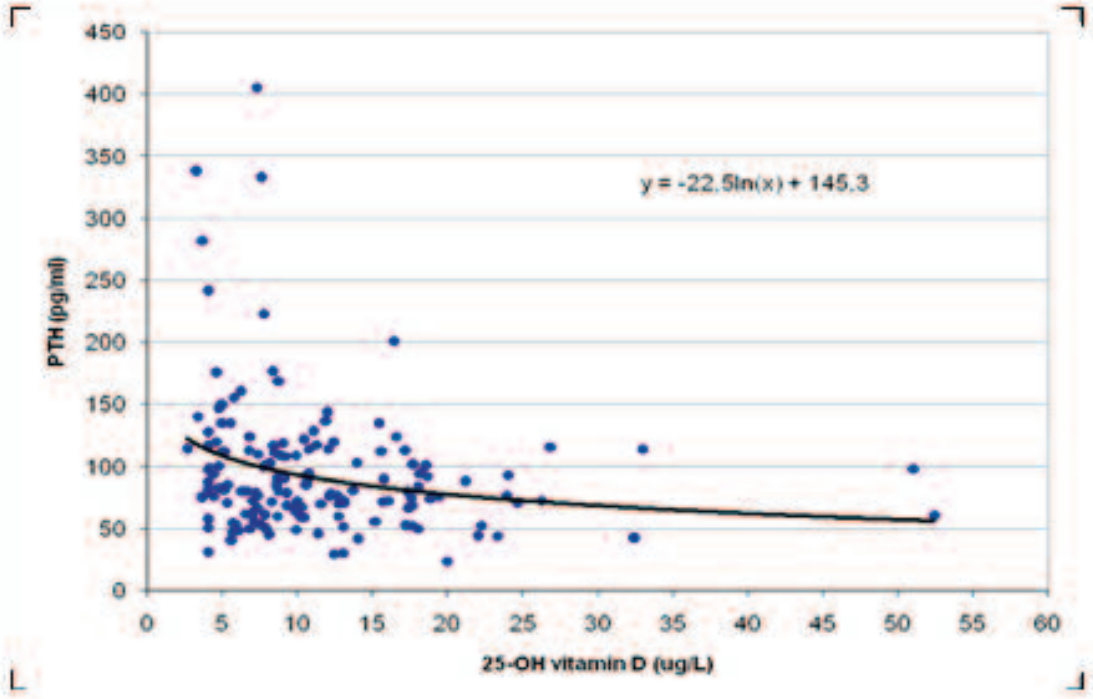

Figure 1. The relationship between 25-OH vitamin D levels and logarithmic values of serum PTH levels

Table 1. Comparison of some clinical and laboratory characteristics of the study group and the control group

	Study Group (n=113)	Control Group (n=43)	*p*
Age (year)	56.9±0.5	57.9±0.7	0.087
Menopausal age (year)	10.2±0.6	9.7±0.9	0.834
BMI (kg/m²)	30.2±0.5	29.4±0.5	0.753
Creatinine (mg/dl)	0.7±0.0	0.6±0.1	0.070
Chlorine (mmol/l)	104.2±0.2	104.2±0.3	0.865
ALP (IU/l)	83.6±2.3	81.3±3.1	0.362
Calcium (mg/dl)	9.5±0.04	9.5±0.05	0.588
Adjusted calcium (mg/dl)	9.6±0.04	9.6±0.05	0.355
Phosphorus (mg/dl)	3.3±0.04	3.6±0.7	**<0.001**
Creatinine clearance (ml/min)	110.6±2.2	108±3.5	0.530
24hUCa (mg/24s)	132.3 ± 7.3	168.5±11.8	**0.006**
24hUp (mg /24s)	637.6±23.4	653.4±42.7	0.956
TRp (%)	86.0±0.4	86.7±0.6	0.478
25- OH vitamin D (µg/l)	10.7±0.6	12.1±1.3	0.439
Uca /Ucr	0.009±0.004	0.012±0.004	**0.001**

Data are given as mean±SE (standart error). ALP=alkaline phosphates, BMI=body mass index, These parameters are statitistically insignificantly different, 24hUCa=24-hour urinary calcium excretion, 24hUp=24-hour urinary phosphorus excretion, TRp=tubular phosphorus reabsorption, Uca /Ucr=urinary calcium-creatinine ratio

Table 2. Comparison of the study group and the control group in terms of bone mineral density

	Study Group (n=113)	Control Group (n=43)	*p*
L2 (g/cm²)	0.90±0.12	0.89±0.12	0.840
L1-L4 total (g/cm²)	0.90±0.12	0.92±0.02	0.360
Femoral neck (g/cm²)	0.75 ± 0.11	0.75±0.01	0.680
Femur total (g/cm²)	0.95±0.01	0.95±0.01	0.970

Table 3. Social factors in the study and control groups

		STUDY	CONTROL	*p*
Social life	Living alone	(n=13; 11.5%)	(n=8; 18.6%)	0.294
	Living with partner/children	(n=100; 88.5%)	(n=35; 81.4%)	
Educational level	Less than a high school graduates	(n=93; 68.2%)	(n=25; 58.0%)	**0.003**
	More than a high school graduates	(n=20; 17.8%)	(n=18; 41.0%)	
Occupation	Housewives	(n=73; 64.6%)	(n=22; 51.1%)	0.144
	Workers / Retired	(n=40; 35.4%)	(n=21; 49.9%)	
Smoking	Yes	(n=16; 14.1%)	(n=9; 20.9%)	0.332
	No	(n=97; 85.9%)	(n=34; 79.1%)	
Be covered	Yes	(n=79; 69.9%)	(n=21; 48.8%)	**0.016**
	No	(n=34; 30.1%)	(n=22; 51.2%)	
Sunlight exposure	Sufficient	(n=40; 35.3%)	(n=23; 53.4%)	**0.046**
	Insufficient	(n=73; 64.7%)	(n=40; 46.6%)	

PTH=parathyroid hormone

Results

The mean age was 56.9 (range; 50-74) years in patients (n=113) with hyperparathyroidism and 57.9 (range; 50-72) years in the control group (n=43). There were no significant differences between the groups in terms of menopausal age, body mass index, serum calcium levels and serum 25-OH vitamin D levels but serum phosphorus levels were significantly lower in the study group (Table1). 24-hour urinary calcium excretion (24hUca) was reduced in the study group and 24-hour urinary phosphorus (24hUp) excretion was similar in both groups (Table1). Creatinine clearance was 110.6±2.2 ml/min in control study group and 108±3.5 ml/min in control group which was not statistically significant (p=0.530). 24hUca was 132.3±7.3 mg/24h in study group and 168.5±11.8 mg/24h in control group which was statistically significant (p=0.006). Tubular phosphorus reabsorption (TRp) was 86.0±0.4% in study group and 86.7±0.6% in control group, which was not statistically significant (p=0.478). Urinary calcium-

Table 4. Serum PTH levels of all participants

		PTH (pg/ml)	*p*
Social life	Living alone (n=21)	84.8±8.8	0.478
	Living with partner/children (n=135)	95.5±4.8	
Educational level	Less than a high school graduates (n=118)	101.4±5.4	**0.008**
	More than a high school graduates (n=38)	75.1±4.5	
Occupation	Housewives (n=95)	99.4±5.7	**<0.001**
	Workers / Retired (n=61)	88.0±6.5	
Smoking	Yes (n=25)	88.0±7.9	0.544
	No (n=131)	96.3±4.9	
Be covered	Yes (n=100)	103.2±6.1	**0.025**
	No (n=56)	80.2±4.1	
Calcium consumption	Sufficient (n=49)	94.7±6.4	0.680
	Insufficient (n=107)	95.1±4.3	

PTH=parathyroid hormone

Table 5. Serum 25-OH vitamin D levels of all participants

		25-OH vitamin D (µg/l)	*p*
Social life	Living alone (n=21)	12.4±1.6	0.242
	Living with partner/children (n=135)	10.9±0.6	
Educational level	Less than a high school graduates (n=118)	10.4±0.6	**0.041**
	More than a high school graduates (n=38)	13.2±1.4	
Occupation	Housewives (n=95)	10.3±0.7	0.439
	Workers / Retired (n=61)	12.2±1.0	
Smoking	Yes (n=25)	10.5±0.7	0.828
	No (n=131)	11.2±0.6	
Be covered	Yes (n=100)	9.9 ± 0.7	**0.002**
	No (n=56)	13.2±1.0	
Calcium consumption	Sufficient (n=49)	12.0±1.2	0.337
	Insufficient (n=107)	10.7±0.6	

Table 6. Causes of elevated serum PTH levels in patients with hyperparathyroidism

	n=113*
25-OH vitamin D deficiency	92.9%
Primary hyperparathyroidism	4.4%
Normocalcemic with hyperparathyroidism	3.5%
Calcium renal leak	1.8%
Hypocalciuric hypercalcemia	1.8%

*some patients have both of diseases, PTH=parathyroid hormone

creatinine ratio (Uca/Ucr) was 0.009±0.004 in study group and 0.012±0.004 in control group which was statistically significant (p=0.001) (Table 1).

Significant negative correlation were detected between logarithmic values of serum PTH levels and serum 25-OH vitamin D levels of both groups (r= -0.188; p=0.019) (Figure 1) but correlation coefficient was low showing a weak correlation.

There was no statistically significant difference between groups in terms of bone mineral density (Table 2). However, the mean mineral density of the L 1-4 vertebrae were in negative corerelation with the increase in serum PTH levels which was statistically significant (r= -0.175; p=0.029) despite this significance correlation coefficient was low showing a weak correlation, similarly the mean mineral density of the femoral neck, wards triangle and trochanteric region did not show any correlation.

Based on our data, serum PTH levels were significantly higher in housewives (p<0.001), women with less than a high school graduates (p=0.008), and the veiled women (p=0.025). Living alone and smoking were found to have no effect on serum PTH levels. PTH levels were not significantly different between groups with adequate calcium consumption and inadequate calcium consumption and also PTH levels were significantly different between groups with insufficient sunlight exposure and sufficient sunlight exposure (Tables 3 and 4).

Serum 25-OH vitamin D levels were significantly lower in the veiled covered (p=0.002); in participants with less than a high school graduate (p=0.041). Living alone, being a housewife or retired, smoking, sunlight exposure, calcium consumption found to have no effect on serum vitamin D levels (Table 5).

When the causes of elevated serum PTH levels were examined, 25-OH vitamin D deficiency was detected in 105 of cases and primary hyperparathyroidism was detected in five cases with 25-OH vitamin D deficiency (Table 6).

Discussion

Causes of elevated serum PTH levels are adenoma, hyperplasia and carcinoma of the parathyroid gland. Elevated PTH levels may be a physiological response which is called secondary hyperparathyroidism especially seen in cases with low serum calcium levels. Decreased oral intake or intestinal absorption of calcium, vitamin D deficiency,

increased renal calcium losses, impaired renal function results in elevated serum PTH levels. In our study, serum calcium levels were not statistically different between the study group and the control group but 24-hour urine calcium excretion was significantly lower in the study group. It was understood that vitamin D deficiency was the most important reason for this situation Reduction of renal phosphorus reabsorption is expected in vitamin D deficiency depending on the decreased intestinal absorption of phosphorus and the increased PTH activity leading to a decrease in serum phosphorus level. The levels of serum phosphorus were significantly lower in the study group than the control group. These results are expected in vitamin D deficiency. Although statistically significant reduction of the calculated rate of tubular reabsorption was not detected.

Serum 25-OH vitamin D levels were low in both control and study groups. A negative correlation was detected between serum PTH levels and 25-OH vitamin D levels. However, PTH levels were not increased in the control group with low 25-OH vitamin D levels Albertazzi *et al.* [5] divided the subjects in their study according to the levels of serum PTH and observed that serum 25-OH vitamin D levels were significantly lower in the group with high serum PTH levels. Sahota *et al.* [6] reported that vitamin D deficiency was common in active elderly people living at home but they also reported that secondary hyperparathyroidism was not so common among these elderly. In other studies, secondary hyperparathyroidism was observed in approximately only 30-50% of elderly people with low serum 25-OH vitamin D levels [7]. There may be several reasons for this situation. PTH levels may not be increased in people with vitamin D deficiency that consumes calcium-rich foods [8]. There may be personal differences in terms of intestinal calcium absorption, even if there is not a consumption of calcium-rich foods [9]. Also, the measured serum vitamin D is 25-OH vitamin D which is a reliable method in terms of showing the levels of stored vitamin D. However, the effect of serum PTH levels on [1.25(OH)2D], which is active vitamin D, is not clear [10].

One of the main problems encountered with increased levels of serum PTH is decrease in bone mineral density in long term. Decrease in bone mineral density is seen not only in hypercalcemic patients with primary hyperparathyroidism and also seen in normocalcemic patients with primary hyperparathyroidism [1]. Renjmark *et al*'s [11] study showed that bone mineral density was lower especially in the femoral neck and the risk of osteoporotic bone fracture was 59% in normocalcemic patients with hyperparathyroidism. Decrease in bone mineral density was seen not only in patients with primary hyperparathyroidism and also seen in patients with secondary hyperparathyroidism due to vitamin D deficiency. Nakamura *et al.* [12] showed a significant decrease in bone mineral density in patients with hyperparathyroidism but they also showed a decrease in bone mineral density in patients with vitamin D deficiency independently from PTH levels. There are no adequate data about what levels of increase in serum PTH may adversely affect bone mineral density [13]. Albettazzi *et al.* [5] compared the cases with elevated serum PTH levels and with normal serum PTH levels in their study but they could not detect a significant relationship between serum PTH levels and the bone mineral density of the lumbar spine and the femoral neck. Similarly, Kaya *et al.* [14] found no association between bone mineral density and elevated serum PTH levels in their study. But Silverberg *et al.* [15] demonstrated slightly decrease in bone mineral density with elevated PTH levels in their study. In our study, there is no significant difference between the study and control group in terms of bone mineral density and it may be related to the duration of hyperparathyroidism. Our study was a cross-sectional study and we did not know how long the patients had hyperparathyroidism. Probably, due to short-term exposure of elevated PTH levels and the small number of the study group, a significant difference could not be identified between the two groups in terms of bone mineral density. In addition, the levels of vitamin D deficiency in both groups were not very different. Therefore, it is considered that the levels of secondary hyperparathyroidism, the main factor that causes bone resorption, were not at the levels of negative effect on bone mineral density.

The reduction of bone mineral density is usually detected in all of the measurement regions in patients with primary hyperparathyroidism [16]. However, increase in bone resorption due to hyperparathyroidism is expected in cortical bones [15, 17]. Proximal radius is the region with highest cortical bone. In our study, due to the fact that the bone mineral densities of the lumbar vertebra, which is mainly trabecular, and femur, which is mainly cortical, were measured and the bone mineral density of proximal radius was not measured, a significant difference could not be identified in patients with hyperparathyroidism.

Vitamin D deficiency/insufficiency is one of the most discussed topics. What should be the normal range of serum 25-OH vitamin D levels are not exactly certain for the definition of vitamin D deficiency. The level that stimulates parathyroid gland for PTH secretion is generally accepted as cut off for vitamin D deficiency [18]. In 1985, Peacock *et al.* [19] defined vitamin D insufficiency in addition to vitamin D deficiency. They proposed to define serum vitamin D level less than 10 ng/ml as deficiency and serum vitamin D level less than 20 ng/ml as insufficiency. The minimum level of serum 25-OH vitamin D necessary to normalize the increased bone turnover due to elevated serum PTH was 20 ng/ml (80 mmol/l) [20, 21]. Generally, serum vitamin D level less than 20 ng/ml is defined as deficiency and serum vitamin D level less than 30 ng/ml is defined as insufficiency [15, 22-24]. In a study performed in our country, sufficient serum 25-OH vitamin D levels were observed in only 18% of postmenopausal women and elderly men. In addition, there was no relationship between the levels of serum 25-OH vitamin D and some parameters such as serum PTH levels, age, body mass index and dietary calcium content [25]. 25-OH vitamin D level less than 20 ng/ml was defined as vitamin D deficiency in our study. We detected sufficient serum 25-OH vitamin D levels in only eight patients (7.4%) with hyperparathyroidism and in only six patients (13.9%) without hyperparathyroidism.

The frequency of vitamin D deficiency was detected as 50-97% in several studies in different populations [26, 27]. This increased incidence was not only reported in countries with insufficient exposure to sunlight Vitamin D deficiency is detected up to 90% in countries such as Greece, Italy and Spain during winter [28]. The different results detected in the studies were thought to be associated with the habits of vitamin D-rich food consumption and the use of drugs containing vitamin D. In many studies synthesis of vitamin D has been shown to be decreased with increasing age [29, 30]. Therefore, the daily requirement of vitamin D is known to be increased in advanced age. In patients requiring vitamin D supplementation, daily 400 units of vitamin D supplementation is considered insufficient and it is accepted that this dosage should be increased to 800 units/day. Apart from all these, the reason for the detection of extremely low serum 25-OH vitamin D levels may be related to the adequacy of the study method. It was shown in studies that there may be significant differences between the assays used in the measurements of serum 25-OH vitamin D levels and also significant deviations were found between the measurements performed in different laboratories, although the same study method [31, 32]. HPLC (Recipe ®) method was used for the measurement of vitamin D in our study. It is a very reliable method with 2.7% intraassay deviation and 3.7% interassay deviation. In some studies, HPLC method has been reported to be more sensitive than chemiluminescent immunoassay or radio immunoassay methods [33].

In our study, participants were divided into groups based on various social features. Participants were mainly composed of people with hyperparathyroidism. The control group were only 27.5% of all participants. In our society, the most important factors leading to vitamin D deficiency were being veiled and having low levels of education. Since, living alone, being housewife or retired, smoking, sunlight exposure, calcium consumption found to have no effect on serum vitamin D levels Our study proved once more that vitamin D deficiency is the most important cause of hyperparathyroidism.

The incidence of primary hyperparathyroidism was calculated as 4.4% in our study group. It was a very high prevalence although the average age of the group was not very advanced. In comprehensive studies, the prevalence of primary hyperparathyroidism ranged from 3/1000 to 21/1000. However, in our study, patients with elevated levels of serum PTH were selected and there were sufficient numbers of patients as a control group. Therefore, the frequency of detected primary hyperparathyroidism reflected primary hyperparathyroidism frequency of patients with elevated PTH levels rather than primary hyperparathyroidism frequency of general population. Primary hyperparathyroidism was detected in 5 cases. The remaining 108 patients had elevated serum PTH levels but normal serum calcium levels. After vitamin D deficiency, calcium renal leak, and hypocalciuric hypercalcemia excluded; we evaluated 4 patients (3.5%) as normocalcemic with hyperparathyroidism. In studies on long-term follow-up these patients, it has been shown that the possibility of hypercalcemic hyperparathyroidism development was very low [1]. Some studies reported that there may be adverse effects on bone mineral density in these cases [34].

Conclusions

Consequently, the results of this study showed that the most important reason for the increase of PTH secretion in postmenopausal women was vitamin D deficiency. Additionally, vitamin D deficiency was common in patients with or without hyperparathyroidism. Especially, all postmenopausal women should be evaluated for vitamin D deficiency to be informed about vitamin D deficiency. The normal range of serum 25-OH vitamin D level is not exactly certain, vitamin D deficiency incidence shows seasonal changes, there are problems with the measurement accuracy and the measurements of vitamin D levels are quite expensive. Because of all these reasons vitamin D deficiency screening and treating are not easy. Vitamin D supplementation for all society is a more rational approach to avoid vitamin D deficiency. Enrichment of milk and milk products with vitamin D seems to be the most appropriate solution for the elimination of vitamin D deficiency.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing

The authors disclosed that they did not receive any grant during conduction or writing of this study.

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The European Research Journal
http://www.eurj.org
Original Article
e-ISSN: 2149-3189
DOI: 10.18621/eurj.271084

Physician versus the smoking habit of the patient

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ABSTRACT

Objectives. This study has aimed to investigate the bases, conditions and the characteristics of the counselling approach of physicians serving in diverse branches of medicine in our hospital. ***Methods.*** Volunteering physicians working in our hospital were given a questionnaire designed to investigate their approach in counselling against cigarette smoking habits of patients arriving at the emergency services, the polyclinics and the specialized clinics. The relationships between the answers given to the questions asked and the professional branches or the characteristics of the approach to counselling was analysed statistically. ***Results.*** The study enrolled 64 volunteering physicians. There were statistically significant differences in the approaches of the physicians working at the emergency services, polyclinics and the clinics ($p<0.001$). The strongest counselling approach was estimated in the specialized clinics and the weakest in the emergecy services. Whereas there were no statistically significant differences in counselling with respect to the branches of medicine at the clinics ($p=0.271$) and the emergency services ($p=0.542$); the awareness on the subject was found to be higher among the physicians at the pulmonology, thoracic surgery and ear-nose-throat (ENT) polyclinics as determined statistically ($p=0.013$). ***Conclusions.*** To counsel consulting patients against this habit is therefore a fundamental duty of the physician. The study has shown that not all physicians are equally aware of and informed on the necessity of this counselling duty.

Eur Res J 2017;3(3):243-249

Keywords: Physician, smoking, cigarette, duty

Introduction

Encouragement of the patient against the cigarette smoking habit by the physician, even during very short clinical interventions, has been found to be effective [1-3]. In the United States of America, over 70% of the smokers consult a physicians at least once per year [4]. Such consultations can be exploited for counselling against the smoking habit by the physician who has a strong potential for this purpose. However, this potential is not being used appropriately [5, 6]. The World Health Organization (WHO) has drawn attention to the potential of all healthcare professionals in fighting against the cigarette smoking habit [7]. Serving at any branch or any platform of medicine, the healthcare professional has to query the smoking habit

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Received: December 02, 2016; Accepted: April 21, 2017; Published Online: May 09, 2017

and to advise against it with an aim to promote public health.

The aim of this study has been to investigate the differences in the counselling approach of the physicians serving in the same hospital, when meeting patients on different consultation platforms with differing conditions.

Methods

Out of a total 98 physicians contacted in our hospital, in Bursa, Turkey, 64 (65.3%) accepted to participate in our study. The volunteering group of 64 physicians were asked to complete a specially designed questionnaire consisting of 3 parts:

1) Demographic information

2) Questions related to the approaches made to find out the smoking habit of the consulting patient and the counsellinggiven (Table 1). The questions were designed after presenting the preliminary form to the pulmonology and thoracic surgery specialists at our hospital. The final format was organized with the help of the psychology department. Each question was given alternative choices with scores ranging from 1 to 5. The scores of the physicians were evaluated with statistical analyses.

3) The Maslach burnout inventory

Physicians involved in preclinical branches and in the healthcare of pediatric patients were excluded on grounds of the inapplicability of the questionnaire. Two pulmonologists who held certificates of a course for stopping smoking were also excluded from the study as their response would not represent the general attitude of the physicians to the smoking habit. Otherwise, it was attempted to reach all physicians working in the hospital.

Statistical Analysis

Existence of any relationship between the answers given by the physicians and their respective branches of medicine and also the characteristics of their counselling approach were statistically evaluated by means of the Statistical Package for Social Sciences (SPSS) (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.). Numerical values were expressed by the mean and the standard deviation; and their distribution was tested with the Kolmogorov-Smirnov test. Categorical values were evaluated with percentages. The mean scores on the answers given to the questions in the questionnaire form and the median (minimum-maximum) of the patients seen at the emergency services, the polyclinics and the specialized clinics were compared using the Kruskal-Wallis test. The mean scores of the internal medical and the surgical branches were compared by using the independent samples t test. The relationship between the mean scores on the questionnaire in the emergency services, the polyclinics and the specialist clinics and the mean number of the monthly seen patients together with the scores on the burnout inventory were analyzed by using correlation analysis and Pearson correlation coefficient was computed. The relationship between the demographic details of the physicians and the scores on the questionnaire form were assessed by the Chi-Square test. A p value of <0.05 was accepted as statistically significant.

Table 1. Contents of the Questionnaire completed by the physicians at the emergency services, the outpatient clinics and the specialist clinics.

A- Do you follow up inpatients at the specialist clinics? (If your answer is Yes, please answer the questions on this page. If your answer is No, you may continue on the following pages)
1) What is the mean number of patients you see in the outpatient clinics during one month?
2) I query the patients on whether they smoke or not.
A) Never B) Rarely C) Generally D) Frequently E) Always
3) I give inform the polyclinics patients on the harmful effects of smoking.
A) Never B) Rarely C) Generally D) Frequently E) Always
4) I advise the patients that they should quit smoking.
A) Never B) Rarely C) Generally D) Frequently E) Always
5) I refer the smoking patients to the smoking cessation outpatient clinics when their treatment is completed.
A=1 point, B=2 point, C=3 point, D=4 point, E=5 point

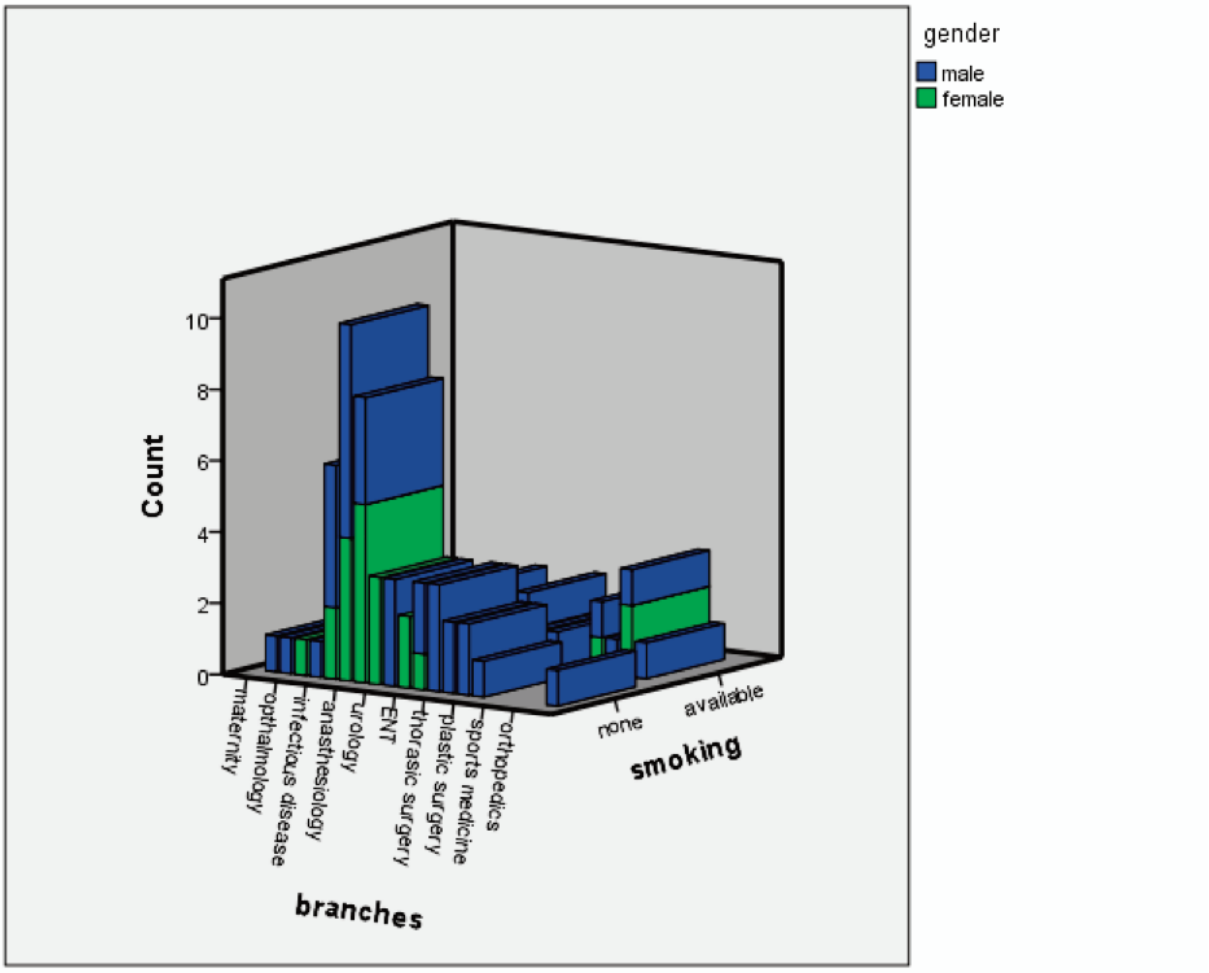

Figure1. According to branches, the number of male and female doctors who smoker and do not smoker.

Results

The study enrolled 64 physicians with a mean professional experience of 15.13 (min=1, max=33) years and a mean age of 39.73 (min=20, max=57) years. The group consisted of 23 (35.9%) females and 41 (64.1%) males; and in the total group, 16 (25%) were smokers while 48 (75%) were non-smokers (Figure 1). The mean number of the total patients seen monthly was found to be 697.27±443.47. The mean number of patients seen per month at the emergency services, the polyclinics and the specialist clinics were 158.44±349.28, 569.03±446.24 and 44.15±45.6; respectively.

The percentage values for the answers given by the physicians to the questionnaire are summarized in Table 2. The mean percentage of physicians in the total group generally or frequently questioning the consulting patient on "if they smoked cigarettes" was 56%; the distribution in the emergency services, the polyclinics and the clinics being 34.9%, 58.1% and 75%; respectively. The values for the physicians who frequently or generally gave "information on the adverse effects of smoking on health" in the above named hospital departments were 21%, 43.6% and 62.5%; respectively, the value being 42.36% in the total group of 64 physicians; the corresponding values for generally or frequently advising the patient "to give up smoking" being, 32.6%, 62.9% and 75%; and 56.83%; respectively, for the total group of 64 physicians. The values for the physicians at the emergency services, the polyclinics and the specialist clinics who generally or frequently referred the patients with a smoking habit to the smoking cessation polyclinics (SCP) were 23.3%, 38.8% and 27.1%; respectively, the mean value for the whole group of 64 physicians being 29.3%. The approaches of the physicians working at the emergency services, the polyclinics and the specialist clinics to the cigarette smoking habit of the patients differed significantly (p<0,001). The strongest care on the subject was seen in the specialist clinics and the lowest in the

Table 2. The percentage expression of the answers given to the questionnaire

Questionnaire items	Platform	Always (%)	Frequently (%)	Generally (%)	Rarely (%)	Never (%)
Querying	Outpatient clinics	19.4	21	17.7	33.9	8.1
	Specialized clinics	37.5	16.7	20.8	18.8	6.3
	Emergency services	9.3	2.3	23.3	44.2	20.9
Informing	Outpatient clinics	9.7	12.9	21	46.8	9.7
	Specialized clinics	22.9	14.6	25	31.3	6.3
	Emergency services	4.7	4.7	11.6	53.5	25.6
Advise to quit	Outpatient clinics	19.4	25.8	17.7	29	8.1
	Specialized clinics	27.1	20.8	27.1	18.8	6.3
	Emergency services	7	4.7	20.9	46.5	20.9
Referral to SCP	Outpatient clinics	8.1	11.3	19.4	37.1	24.2
	Specialized clinics	10.4	6.3	10.4	52.1	20.8
	Emergency services	7	4.7	11.6	39.5	37.2

(SCP=smoking cessation polyclinic)

emergency services (Figure 2).

Although there were not significant differences in the counselling against smoking with respect to the branches of medicine at the emergency services and the clinics; the awareness on the duty to advise against smoking was significantly higher at the at the pulmonology, thoracic surgery, and the ear-nose-throat (ENT) polyclinics (p=0.013) in contrast to the observations in the emergency services (p=0.54) and the clinics (p=0.27) (Figure 3).

When the internal diseases and the surgical branches , excluding the pulmonogy , thoracic surgery and the ENT branches, were compared as two groups, statistically significant differences were not observed in the groups working at the polyclinics (p=0.44) and the specialist clinics (p=0.37). However, the mean score of the internal branches at the emergency service was significantly elevated (p=0.029). Correlation were not observed between the scores on the burnout inventory and the questioning of the smoking habits of patients at the emergency services (p=0.28, correlation coefficient=-0.172), the polyclinics (p=0.48, correlation coefficient=0.092) and the specialist clinics (p=0.37, correlation coefficient=0.132).

The approach to counselling against smoking did not differ between the different gender groups at the emergency services (p=0.4); the polyclinics (p=0.92) or at the clinics (p=0.79). Also, there were no significant differences between the smoking and the non-smoking physicians at the emergency services (p=0.1), the polyclinics (p=0.1) and the clinics (p=0.42). Although there were no statistically significant differences in the marital status of the physicians working at the polyclinics (p=0.76) and clinics (p=0.47), those working at the emergency services were found to be of younger age (p=0.01).

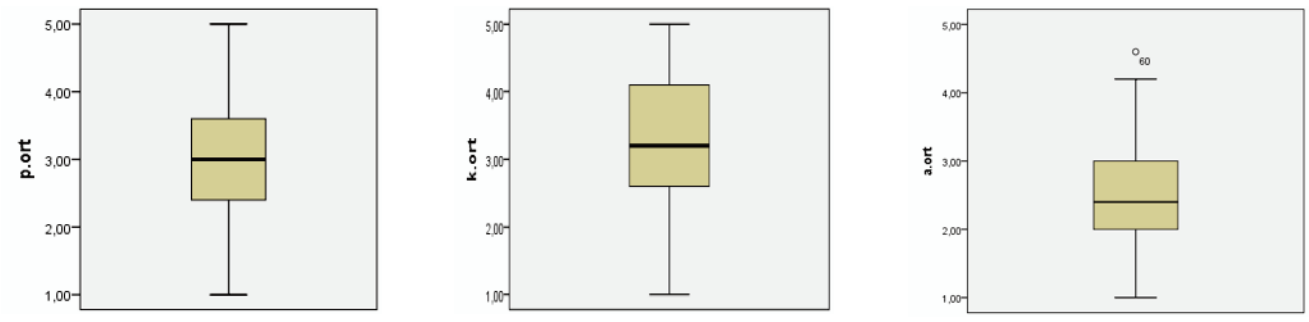

Figure 2. The mean scores (counts) on the questionnaire completed by the physicians in the Outpatient clinics, the Clinics and the Emergency Services.

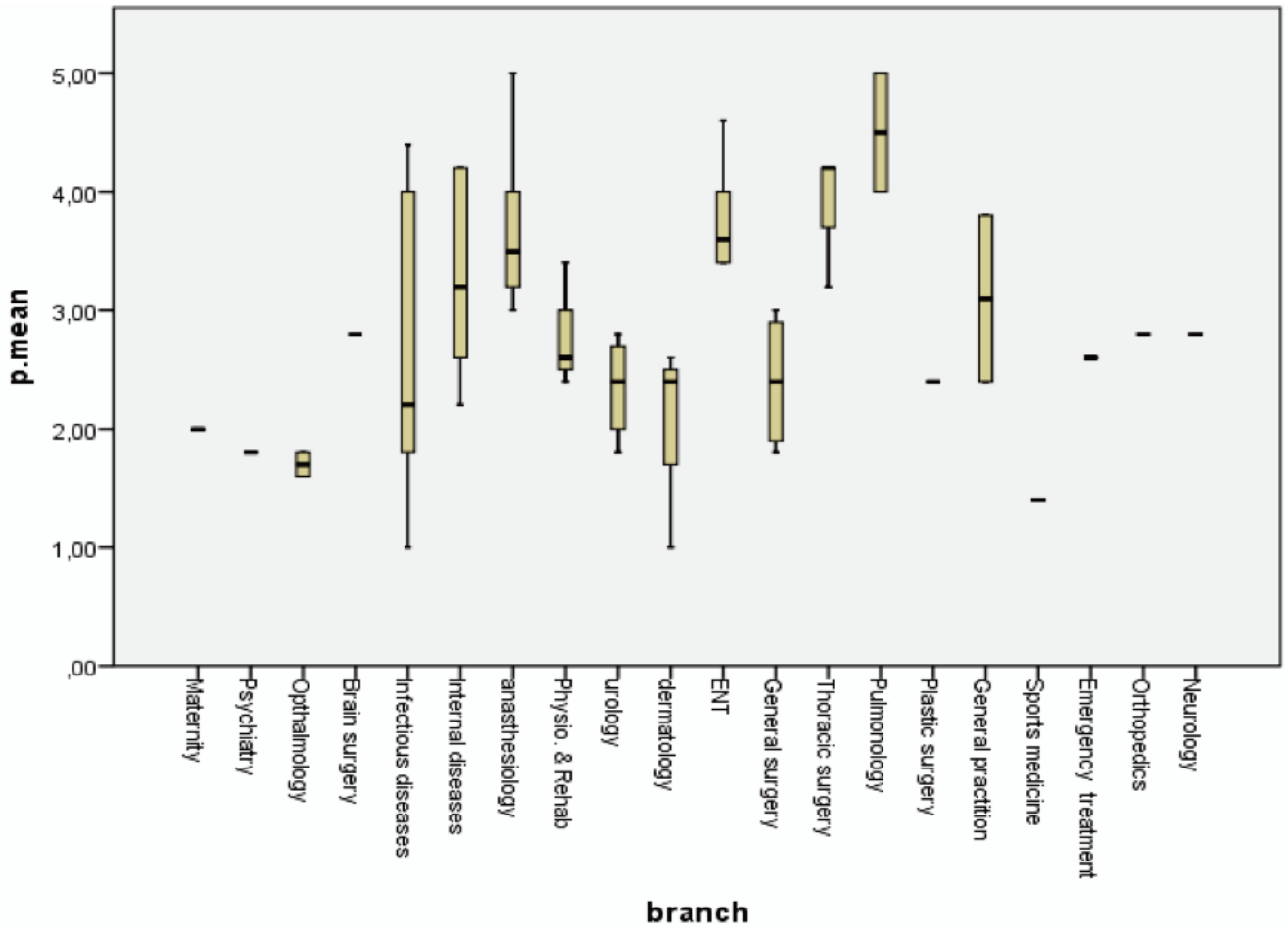

Figure 3. The mean values in all branches of medical services given in the outpatient clinics.

Discussion

Cigarette smoking habit is globally ranked as the first cause of mortality among the high income nations, and the second cause of mortality in the entire world [8]. It must, therefore, be emphasized that the notable result of this study is the observation of a significantly lower approach to the smoking habit of the patient among the physicians specialized in branches not directly related to the pulmonary system and employed in departments other than the pulmonology, thoracic surgery and the ENT polyclinics. In a study by King *et al.* [9], on the venture of healthcare professionals to give up smoking, not having evaluated the physicians separately with respect to branches of medicine has been given as a limitation to the study. We have not found in the literature scanned another study discerning the approach on counselling against smoking in different branches of medicine.

Evaluation of the physicians in counselling patients on smoking in the emergency services, the polyclinics and the specialized clinics has given the highest results in the clinics and the lowest at the emergency services. This can be explained by the clinicians having more time to give to the fewer inpatients under treatment, while the time spent with the patient is much more limited at the emergency services and the polyclinics. However, we believe that relating the causes of the complaints of patients consulting the emergency services to the smoking habit, which would not take further than 1-2 minutes, would be of significant contribution to the cessation of this habit. It has been reported that a few minutes given by the physician to counselling against smoking has been effective to the extent of 5-15% increase in giving up the habit [5]. King *et al.* [9] have estimated that 87.9% of the physicians do inquire if the patient smokes or not. The corresponding value determined by Demir *et al.* [10] for physicians who generally or frequently inquired the smoking habit of the patient was 56.7%. Our result was 56% for the physicians working at the polyclinics, indicating that a significant improvement has not taken place over the passing 3

years.

Advice given to the patient on giving up smoking has been rated as 65.8%; 71.5% and 48.6% by King *et al.* [9], Demir *et al.* [10] and Lindorff *et al.* [11], respectively, while our result was 56.83%. In comparing these results, as there cannot be incidences of advising to give up smoking that exceed those of inquiring about it, we believe that these values in hand represent the percentage of those physicians who advise against smoking. In our study 42.36% of the physicians have reported informing the patient on the harm done by smoking. We have not met a similar evaluation in the studies carried out previously by others.

While the individual efforts result in 3-5% success in giving up smoking, receiving help from the smoking cessation polyclinics increases the success to nearer the 40% [12]. Despite the presence of a 7-day serving SCP at our hospital, the mean percentage of physicians referring patients to this service was found to be 29.7%. Demir *et al.* [10] have reported that about one third of the physicians were unaware of the existence of the SCP service while only 57.3% (making up 38.2% of the physicians joining the investigation) who knew about it made the necessary referrals.

Although some studies have reported a higher percentage of nonsmoking physicians get concerned with the smoking habit of patients as compared to the physicians who are smokers [10, 13, 14], our results have not determined a similar difference. This observation may be due to the increased awareness of the cigarette smoking physicians despite continuing with their personal habit. However, it should be kept in mind when making this assumption that the number of the participants in our study has been low.

There was not any difference on the approaches of the physicians on counselling against smoking on the basis of gender difference; but, unmarried physicians working at the emergency services were observed to be more sensitive to the smoking habit of the arriving patients. The relatively fewer physicians working at the emergency services may have given this statistical result.

The Limitations of the Study

The primary limitation to the study reported here may be having worked with a small number of physicians at the same hospital, the dependence of our results on the answers given by them to the purpose made questionnaire, and not having had the opportunity to observe the physicians at the emergency services, the polyclinics and the specialist clinics.

Conclusions

Habitual cigarette smoking is one of the most important threats to human health in the current century. A very serious duty falls on the physician to combat this habit. It is quite obvious that not all physicians working in any branch of medicine are aware of the obligation to query the smoking habit, to give information about it and to advice against it. We believe that not only the physicians treating disorders related to pulmonary functions, but all physicians in diverse branches ofmedicine should be trained comprehensively on the subject.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing

The authors disclosed that they did not receive any grant during conduction or writing of this study.

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The European Research Journal
http://www.eurj.org
Original Article
e-ISSN: 2149-3189
DOI: 10.18621/eurj.304447

Developmental screening of healthy children between 6-24 months

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ABSTRACT

Objectives. Developmental screening of healthy children from early childhood provides to identify high-risk children, early diagnose of developmental delay, better prognosis, correspond direction of family and treatment efficacy. The aim of this study was to evaluate the developmental stages for healthy children referring to the pediatric clinic for early childhood check-ups. Methods. The development of 328 children was evaluated. It is found that; awareness skills of infants with siblings were better than those without siblings. The infants attended to by caregivers had limited word usage compared with those cared for by mothers and relatives. The developmental stages were evaluated by using Social-Communication Area Screening Test for Infants (SCASI). Results. The developmental stages of infants whose mothers were graduated from university were better than the other infants in terms of awareness skills. In addition, social content skills in terms of communication levels and total points were better in infants cared for by mothers and relatives than the others, and it appears that those cared for by relatives were better compared to those only looked after by mothers. Our study indicated that according to SCASI scores, 6.1% of infants were in the risk group. Conclusions. During the first years, regular check-ups and recording of the development are very important in terms of ensuring that there is early intervention in the case of any delay in development stages. Screening tests that can be used easily, regularly repeatable, including observation of parents and having short evaluation process should be extended.

Eur Res J 2017;3(3):250-258

Keywords: Infant development, developmental screening, developmental delay

Introduction

The developmental process and, more specifically, the issue of developmental regression in the infant and early childhood period is an area of intense interest for researchers [1] The first years of life after leaving the mother's womb are very important in terms of mental and physical development, and brain development in this period is known to be rapid [2, 3]. Currently, it is thought that the sources of many neurological and psychiatric problems are in some way related to this particular period [4, 5].

Developmental regression observed at rates of 5-30% in the infant and early childhood period may cause delays in one or more areas of growth [6-8]. Though parents may become aware of developmental

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Received: April 7, 2016; Accepted: May 5, 2017; Published Online: June 2, 2017

problems in this period, both initial reporting and diagnosis take time [9, 10, 11]. Early diagnosis and the beginning of rehabilitation for developmental regression is very important for prognosis and treatment efficacy, as well as for offsetting increasing costs [12-14].

Families commonly share some of the problematic experiences in the early period with pediatric clinicians who they meet regularly. Because of that, pediatric clinicians should be competent in child development and aware of developmental problems that can arise. Currently, it is recommended that screening scales must be used during routine health checks for research into developmental delays [15-17]. Especially screening scales that are in accord with the norms of society that the children grow up in and that can be easily administered by pediatric clinicians are needed [18-20].

Screening tests generally consist of a short evaluation process used to identify high-risk children who are in need of more detailed examination [21, 22]. The screening test to be administered by clinicians working with children should be practical and capable of being implemented within a short period of time; it should also be appropriate for that population, and should be regularly repeatable.

This study evaluated a current screening test assessing the developmental levels for healthy babies referring to the pediatric clinic for early childhood check-ups with the objective of identifying high-risk children in terms of developmental delay.

Methods

Three hundred and twenty-eight healthy children brought into the pediatric outpatient clinic between August 2016 and October 2016 for routine vaccination were included after the written and verbal informed consent of their parents was obtained. Local ethics committee approval was obtained for the study. Age, gender, number of siblings, education levels of the mothers and information about the caregivers were all noted. The Social-Communication Area Screening Test for Infants (SCASI) was conducted with the information given by the parents. The relationship between the SCASI subscores and total scores and gender, number of siblings, caregiver information and education levels of mothers were analysed.

SCASI is a test applied to the children at 6th, 9th, 12th, 15th, 18th and 24th months after birth. For this reason only the children at these months were included. In order to compare the results of children at different age groups, we used SCASI score percentages instead of SCASI score points.

A detailed medical history of the children was obtained. Physical and neurological examinations were conducted. Infants with the existence of risk factors such as prematurity, asphyxia, new-born convulsions, hyperbilirubinemia, low birth weight, congenital hypothyroidism and epilepsy were excluded.

The parents were informed about the developmental state of their infants. The children at-risk were first identified and then referred to playgroups or kindergartens by the researchers. Increasing the levels of interaction with the children was recommended to their parents.

Social-Communication Area Screening Test for Infants (SCASI)

SCASI is a developmental screening test focused on the social communication area for infants between 6 and 24 months of age. It is created to assess the development of healthy children at 6th, 9th, 12th, 15th, 18th and 24th months after birth and to determine the children at-risk.It is parent-reported, and may be filled out by either the mother or the father. It can also be completed by the clinician with responses in line with the answers of parents. Taking about 10 minutes to complete and score, it was first developed by Sertgil and colleagues [23]. They studied norm determination and the validity of the criteria [23].

The item "Not yet" was scored as one point, "Sometimes" was scored as two points, and "Often" was scored as three points. The other four questions had options from 1 to 5, and the first option scored as one point and the fifth as five points for each question. SCASI is a test with 43 items and a two-factor structure. The first factor consists of communication-oriented social skills, and the second consists of skills including awareness. Five different scores are derived, including the following subparts: communication-oriented social skills (F1), preverbal skills (F1a), vocabulary (F1b) and skills including awareness (F2). The test has strong internal consistency: α is 0.962, and 0.961 and 0.811 for factors I and II, respectively. The children are identified to be normal or at-risk (at the 20–30% percentile) according to the cut-off score determined for each part.

Socio-Demographic Data Form

Table 1. Distribution of sociodemographic features of children

Age Group (n=328)	**n (%)**
6 month	201 (61.3)
9 month	42 (12.8)
12 month	36 (11.0)
15 month	16 (4.9)
18 month	17 (5.2)
24 month	16 (4.9)
Gender	
Boy	182 (55.5)
Girl	146 (44.5)
Existing of a Sibling	
No	240 (73.2)
Yes	88 (26.8)
Child Care Providers	
Mother	262 (79.9)
Relatives	36 (11.0)
Caregiver	27 (8.2)
Kindergarten	3 (0.9)
Education Levels of Mothers	
Primary School	8 (2.4)
High School	56 (17.1)
University	264 (80.5)

Data are given as number (percent).

This data form was prepared according to the purpose of the study to collect specific information such as gender, age, childcare and education levels of mothers. The physician completed the form based on the parents' statements during the interviews.

Statistical Analysis

The analysis of the data was conducted using SPSS for Windows 22.0 (SPSS Inc., Chicago, IL). The identifying analysis was initiated as means ± standard deviations, frequency distributions, and percentages. Pearson's chi-squared test and Fisher's exact test were used to analyse the categorical variables. The fitness with normal distribution of the variables was assessed by using the Kolmogorov-Smirnov/Shapiro-Wilk test, and visually through a histogram and graphics. In the intergroup comparisons, participants were evaluated using the Mann-Whitney U test when the number of independent groups was two, and with the Kruskal-Wallis test when there were more than two. The Bonferroni correction was used to find the source of the difference when a level of significance was found in three independent samples. The significance level was statistically determined as $p<0.05$.

Results

The development of 328 children was evaluated in this study. Sociodemographic features (distribution by age group, gender, number of siblings, child care providers, and education levels of mothers) are shown in Table 1.

Table 2. SCASI score percentages of children

SCASI (%) (n=328)	**Data**
F1a	67.21±23.50
F1b	73.29±15.45
F1	66.23±23.79
F2	62.71±28.25
Total	67.27±22.97

Data are given as mean ± standard deviation. SCASI=Social-Communication Area Screening Test for Infants, F1a=preverbal skills, F1b=vocabulary, F1=communication-oriented social skills, F2=skills including awareness

The SCASI total and subpart scores of participants were analysed. As shown in Table 2, the mean of the F1a subpart (preverbal skills) scores was 67.21±23.50,

for F1b (vocabulary) scores was 73.29±15.45, for F1 (communication-oriented social skills) scores was 66.23±23.79, for F2 (skills including awareness) scores was 62.71±28.25 and for total scores was 67.27±22.97 in terms of percentages.

Table 3. The Distribution of risk statement of children according to SCASI scores

SCASI (%) (n=328)		n (%)
F1a	Normal	296 (90.2)
	Risky	32 (9.8)
F1b	Normal	323 (98.5)
	Risky	5 (1.5)
F1	Normal	296 (90.2)
	Risky	32 (9.8)
F2	Normal	315 (96.0)
	Risky	13 (4.0)
Total	Normal	308 (93.9)
	Risky	20 (6.1)

Data are given as number (percent). SCASI=Social-Communication Area Screening Test for Infants, F1a=preverbal skills, F1b=vocabulary, F1=communication-oriented social skills, F2=skills including awareness

According to the SCASI score percentages, the number of children-at-risk was determined (Table 3). Thirty-two (9.8%) of the 328 children with respect to F1a, five (1.5%) children with respect to F1b, 13 (4.0%) with respect to F2 and 20 (6.1%) with respect to the SCASI total scores were determined to be in the at-risk group.

The correlation analyses of the SCASI score percentages and gender, the existence of a sibling, child care providers and education levels of the mothers was also evaluated (Table 4). There was no significant difference found between the SCASI total and subpart scores and gender (p>0.05). The SCASI F1a, F1b, F1 and total score percentages of the children with or without siblings were similar (p>0.05). However, the SCASI F2 score percentages were significantly higher among children with siblings (p<0.05). Evaluating child care providers, we divided the sample into three groups according to children looked after by their mothers, those looked after by relatives, and those with other caregivers. It is shown that the SCASI F1a, F1b, F2 and total score percentages were not significantly different between the groups (p>0.05). On the other hand, the SCASI F1 score percentage was different between the groups. The post-hoc tests showed that the significance was derived from the two groups looked after by mothers and relatives (p<0.05). The SCASI F1 scores were

Tablo 4. Distribution of SCASI score percentages according to gender, existing of a sibling, child care providers and education levels of mothers

(n=328)	n	SCASI (%) F1a	F1b	F1	F2	Total
Gender						
Boy	182	66,35±23,86	72,66±16,12	65,06±24,42	62,16±28,60	67,00±23,27
Girl	146	67,96±23,09	74,08±14,59	67,69±22,99	63,40±27,89	67,62±22,67
	p	0.555	0.545	0.386	0.672	0.873
Existing of Sibling(s)						
No	240	66.04±24.26	72.48±16.14	65.22±24.46	60.05±28.15	66.47±23.64
Yes	88	69.86±21.17	75.51±13.22	68.98±21.76	69.97±27.38	69.37±21.11
	p	0.289	0.156	0.343	**0.003**	0.458
Child Care Providers*						
Mother	262	65.93±23.51	73.72±13.46	64.50±23.82	62.58±27.96	65.97±22.93
Relatives	36	72.22±22.66	73.06±20.68	73.40±22.54	63.06±30.43	73.45±20.75
Caregiver	27	70.74±24.64	68.70±23.92	72.78±23.63	63.24±29.82	72.62±25.91
	p	0.158	0.596	**0.017**[a]	0.930	0.063[a]
Education Levels of Mothers						
Primary or high school	64	63.16±26.24	74.14±15.57	63.71±25.01	54.23±30.30	61.04±24.66
University	264	68.01±22.74	73.09±15.44	66.84±23.50	64.76±27.40	68.76±22.35
	p	0.265	0.528	0.385	**0.013**[b]	**0.035**[b]

Data are given as mean ± standard deviation. SCASI=Social-Communication Area Screening Test for Infants, F1a=preverbal skills, F1b=vocabulary, F1=communication-oriented social skills, F2=skills including awareness, a=According to post-hoc tests, the difference is found to be between the children looked after by mothers and by relatives, b=According to post-hoc tests, the difference is found to be between the mothers graduated from university and the mothers graduated from high school

Table 5. Distribution of SCASI risk Statement according to gender, child care providers and education levels of mothers

(n=328)	SCASI Risk Statement									
	F1a		F1b		F1		F2		Total	
	Normal n (%*)	Risky n (%*)	Normal n (%*)	Risky n (%*)	Normal n (%*)	Risky n (%*)	Normal n (%*)	Risky n (%*)	Normal n (%*)	Risky n (%*)
Gender										
Boy	163 (89.6)	19 (10.4)	179 (98.4)	3 (1.6)	161 (88.5)	21 (11.5)	173 (95.1)	9 (4.9)	168 (92.3)	14 (7.7)
Girl	133 (91.1)	13 (8.9)	144 (98.6)	2 (1.4)	135 (92.5)	11 (7.5)	142 (97.3)	4 (2.7)	140 (95.9)	6 (4.1)
p	0.641		1.000		0.225		0.309		0.178	
Existing of Sibling(s)										
No	216 (90.0)	24 (10.0)	236 (98.3)	4 (1.7)	217 (90.4)	23 (9.6)	228 (95.0)	12 (5.0)	223 (92.9)	17 (7.1)
Yes	80 (90.9)	8 (9.1)	87 (98.9)	1 (1.1)	79 (89.8)	9 (10.2)	87 (98.9)	1 (1.1)	85 (96.6)	3 (15.8)
p	0.806		1.000		0.862		0.198		0.218	
Child Care Provider**										
Mother	233 (88.9)	29 (11.1)	260 (99.2)	2 (0.8)	234 (89.3)	28 (10.7)	254 (96.9)	8 (3.1)	246 (93.9)	16 (6.1)
Relatives	35 (97.2)	1 (2.8)	35 (97.2)	1 (2.8)	34 (94.4)	2 (5.6)	32 (88.9)	4 (11.1)	34 (94.4)	2 (5.6)
Caregiver	25 (92.6)	2 (7.4)	25 (92.6)	2 (7.4)	25 (92.6)	2 (7.4)	26 (96.3)	1 (3.7)	25 (92.6)	2 (7.4)
p	0.265		**0.023**		0.567		0.069		0.953	
Education Levels of Mothers#										
Primary or high school	54 (84.4)	10 (15.6)	63 (98.4)	1 (1.6)	56 (87.5)	8 (12.5)	58 (90.6)	6 (9.4)	58 (90.6)	6 (9.4)
University	242 (91.7)	22 (8.3)	260 (98.5)	4 (1.5)	240 (90.9)	24 (9.1)	257 (97.3)	6 (2.7)	250 (94.7)	14 (5.3)
p	0.078		1.000		0.410		**0.024**		0.244	

Data are given as number (percent). SCASI=Social-Communication Area Screening Test for Infants, F1a=preverbal skills, F1b=vocabulary, F1=communication-oriented social skills, F2=skills including awareness, *=Line percentage; **=The 3 children attending kindergarten were excluded, #=The mothers graduated from primary or high schools were combined in a group

higher in the group looked after by relatives in comparison to the scores for the group looked after by mothers. As the mothers who had graduated from primary or high school only were few in number, we divided the mothers into two groups: those graduating from university, and those graduating from primary or high school. Evaluating the education of mothers in these two groups, we found a significant relationship between education levels and the SCASI F2 and total score percentages ($p<0.05$). The SCASI F2 and total score percentages of children whose mothers graduated from university were higher than those of the others. In contrast, there was no significant difference between the groups in terms of the SCASI F1a, F1b and F1 scores ($p>0.05$).

The distribution of the risk statement related to gender, child care providers and education levels of the mothers is shown in Table 5. The ratio of being at-risk in reference to the SCASI F1b was significantly higher in the children looked after by caregivers than the children looked after by their mothers and relatives ($p<0.05$). There was not found to be a difference in the F1a, F1 and F2 scores arising from the child care providers ($p>0.05$).

Evaluating the SCASI risk statement and education levels of mothers, a difference was found in terms of the SCASI F2 scores between the groups. The ratio of children-at-risk was significantly higher in the group of mothers who had only graduated from primary or high school than the mothers who had graduated from university ($p<0.05$). There was not found to be a difference in the F1a, F1 and F2 scores as a result of the education levels of the mothers ($p>0.05$).

On the other hand, there was not found to be any difference between the groups' respective SCASI subpart and total scores as a result of gender and the existence of siblings ($p>0.05$) (Table 5).

Discussion

During the critical developmental period of the first two years, regular check-ups and recording of the infant's development are very important in terms of ensuring that there is early intervention in the case of any delay in development stages. For assessment of development, the available tools include clinical observation or parental reporting. It is thought that the participation of parents in developmental tests is especial helpful for increasing awareness, improving the observation of children and solidifying cooperation with the individual conducting the test [8, 16].

In addition to the methods of only observing a child, there are now alternative methods including

family observations. The Parents' Evaluation of Developmental Status (PEDS) [24] and Ages and Stages Questionnaire (ASQ) [25] are based on the knowledge and concerns of parents in monitoring developmental levels, and are recommended for use by pediatricians [15]. Still, it is not possible to fully observe the developmental skills of the child in the health system. As observational evaluations cannot be used to evaluate all skills, there are many other items in this method, and a significant portion of the test is spent on explanations. During observation of the child's skills, there are many difficulties frequently encountered due to limited time, an inappropriate environment or shyness of the child. Especially in clinics with high numbers of patients, using these tools becomes even more difficult [26]. Additionally, due to parental report-based methods, it is thought that awareness of whether the children gain the appropriate skills for their respective age periods will increase in families.

In our study, the SCASI based on parental reporting was used to assess the developmental stages in infants. As it does not require special education before administration, takes a short time and is easily applied, it is an appropriate screening test for regular use by health workers during routine examinations. The short duration allows for certain key points to be used to inform the parents.

According to the results of the study, there was no significant difference observed in the development of male and female infants. This finding is in accordance with the results of studies which emphasized that there is no significant difference in development levels between the sexes [27-30].

Furthermore, the correlation of siblings with skills involving awareness was assessed, and it appeared that the awareness skills of infants with siblings were better than those without siblings. This result is in accordance with previous studies [31] which show that the presence of siblings ensures a stimulating environment, and as a result, the development of siblings is positively affected in terms of cognition [32, 33].

In situations where working mothers return to work early after child birth, the care of children is commonly undertaken by grandparents, other relatives or caregivers [34]. There are many studies which conclude that mothers returning early to work causes negative results in terms of the development of cognitive, social, emotional and behavioural skills at advancing ages [35-38]. It is thought that the mothers returning to work early negatively affects the bonding process experienced by the infant, and this affects the child's cognitive and behavioural development [39]. A study showed that children of mothers who begin working 4 years after birth have lower levels of hyperactivity, aggressive behaviour and anxiety levels than the children whose mothers begin working earlier [20].

Of the children included in this study, 79.9% were cared for only by the mother, 11.0% by relatives, 8.2% by a caregiver and 0.9% attended crèche. According to our study, when social content skills in terms of communication levels and total points were compared in infants cared for by mothers and relatives, it appears that those cared for by relatives had higher points compared to those only looked after by mothers. This situation may be explained by the fact that children looked after only by mothers may receive less social stimulation and verbal communication during the day. Relatives who take responsibility for caring for children ensure more intense social interaction, together with the mother and father, which may contribute to the development of skills related to communication.

In our study, when infants were attended to by caregivers as compared with those cared for by mothers and relatives, the former appeared to be at more risk in terms of limited word usage. There are insufficient studies on this topic to reach a decisive conclusion; however, one of the most important factors in a child's language development appears to be the environment, with a verbally rich and stimulating environment created by adults supporting more effective language development [40, 41]. This result could be explained by mothers and relatives supporting better language development by presenting more stimulation as compared to caregivers.

Studies have shown a strong correlation between maternal educational levels and a child's cognitive development [42], with this correlation being clearer after the first nine months [43]. In our study, it was concluded that mothers who did not graduate from university were in the lower section in terms of their capacity to ensure that awareness skills of infants were cultivated, forming a higher risk group than infants of mothers who graduated from university. As educational levels increase, mothers have more active verbal communication with infants, using more educational strategies such as frequent talking, making up stories, asking questions and providing positive feedback [44]. Additionally, the effort and expectation

that educated mothers have for their children's education is correlated with children's improved cognitive development and academic success in the future [45].

Our study indicated that according to total SCASI scores, 6.1% of infants were in the risk group. As developmental problems in the early childhood period are common in both Turkey and globally, this rate does not comply with the results showing that developmental issues may occur among nearly one out of every four children [46, 47]. This may be due to our study not including infants at risk in terms of development. The risk percentage for word use skills was 9.8, with the risk percentage of 4.0 for awareness skills. In the general population, awareness skills reflect a structural trait and are gained in the early developmental period. As a result, delays observed in this area are generally considered to be more distinctive as compared to those of the word use area. When the initial health situation of the infants forming our sample group is considered, an attempt was made to exclude situations that may have caused developmental delay. The study included infants applying for healthy child check-ups with no known medical condition. As the child's general health status [48], nutrition [49], iron deficiency [50] and family socio-economic situation [51] may affect assessment of development, the sample for the research was taken from healthy children after considering these conditions.

While the rate of women with education of high school and above is 10.7% for the country in general [52], in our study, 80.5% of mothers had graduated from university, 17.1% were high school graduates and 2.4% were primary school graduates. This data shows that the sample group contained families in the upper education level in the country.

The Limitations of the Study

Our sample was comprised of a relatively small group in terms of maternal educational levels and children at risk of developmental regression. This situation means that the results cannot be generalized to society, and this is one of the important limitations of the study. Taking account of the norms in Turkey, the strong points of the study are the use of an advanced current screening test and assessment of child development by the same individual.

Conclusions

Currently, problems have been experienced in terms of appropriately informing and supporting infants and their families without any diagnosis within the health system. The results of identifying infants at risk in terms of development shows that there is need of more support for the infant in terms of their development and the corresponding direction of the family. The more widespread use of a regular applicable survey for developmental screening, especially during routine check-ups, is of great importance for the various stages of diagnosis and for establishing direction of treatment. Consequently, there is a need for more comprehensive studies in this area.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing

The authors disclosed that they did not receive any grant during conduction or writing of this study.

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The European Research Journal
http://www.eurj.org
Original Article
e-ISSN: 2149-3189
DOI: 10.18621/eurj.273835

Inlay butterfly cartilage tympanoplasty for large central perforations

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ABSTRACT

Objective. The aim of this study is to evaluate the results of inlay butterfly tympanoplasty technique in large central tympanic membrane perforations. ***Methods.*** The files of the patients who underwent inlay butterfly cartilage tympanoplasty for large central tympanic membrane perforations were reviewed. Patients were followed with otoscopy and audiometry. Preoperative and postoperative pure-tone audiometry results were calculated and analyzed to evaluate the hearing gain. The grafting procedure was considered successful if no perforations were observed during postoperative microscopic or endoscopic evaluation during the follow-up period. ***Results.*** Twenty-nine patients with large central tympanic membrane perforation who were operated by inlay butterfly tympanoplastytechnique were included in the study. Twenty-one (72.4%) ears were operated with endoscope and 8 (27.6%) under microscope. The mean duration of surgery was 30.5±3.77 minutes. The mean follow-up time was 69.6±21.6 (range; 24-112 weeks). Graft take rate was 89.7% (26/29). ***Conclusion.*** Inlay butterfly cartilage tympanoplasty is a minimal invasive and effective technique for repairing large central tympanic membrane perforations.

Eur Res J 2017;3(3):259-263

Keywords: tympanoplasty, butterfly, cartilage, central perforations, take rate

Introduction

Since the first application of the tympanoplasty, various graft materials and techniques have been used to repair tympanic membrane perforations [1]. Both the underlay and overlay techniques using the temporalis muscle fascia or perichondrium have been regarded as popular and reliable methods [2]. The inlay butterfly cartilage tympanoplasty (IBT) was first described by Eavey in 1998 for the repair of small central perforations [3]. In this technique, tragal cartilage is used as a graft and placed transcanally. It has been shown that transcanal IBT technique is more advantageous when compared to onlay and underlay tympanoplasty because of the possibility of performing under local anesthesia, reducing the need for postoperative follow-up and care, and being a faster and cheaper procedure [4]. Since than IBT technique has been used to repair small central tympanic membrane perforations and has successful

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Received: December 6, 2016; Accepted: June 13, 2017; Published Online: July 28, 2017

results. However, there are not enough studies on the use of the butterfly cartilage tympanoplasty technique in large tympanic membrane perforations. In this study our aim is to evaluate the results of inlay butterfly tympanoplasty technique in large central tympanic membrane perforations.

Methods

This retrospective study was performed by analyzing the clinical records of 29 patients who underwent transcanal IBT for large central tympanic membrane perforation between 2013 and 2016 in a tertiary hospital. The study was approved by Adiyaman University Institutional Review Board with the number: 2016/6-7. Patients who had no ear discharge within the last three months, no signs of inflammation or infection of the middle ear mucosa, and no signs of inflammation or infection of mastoid cells, as evaluated with temporal bone computed tomography were included study. Patients with insufficient data and follow-up period were excluded from study. All the operations were performed by the same surgeon (MK). The absence of an intact ossicular chain was an exclusion criterion. Preoperative and postoperative pure-tone audiometry results at 0.5, 1, 2 and 4 kHz were calculated and analyzed to evaluate hearing improvement. The grafting procedure was considered successful if no perforations were observed in postoperative microscopic or endoscopic evaluation during the follow-up period. Perforations covering more than 2/3 of the tympanic membrane were considered as large tympanic membrane perforations. The time between the local anesthetic infiltration and the packing of the external ear canal with gelfoam following grafting was recorded as the durationof surgery.

Surgical technique

Figure 1 shows the main steps of surgery. The procedure is performed under general anesthesia. Patients are prepared in a sterile fashion. The meatal surface of the tragus is injected with 1 to 3 mL of the

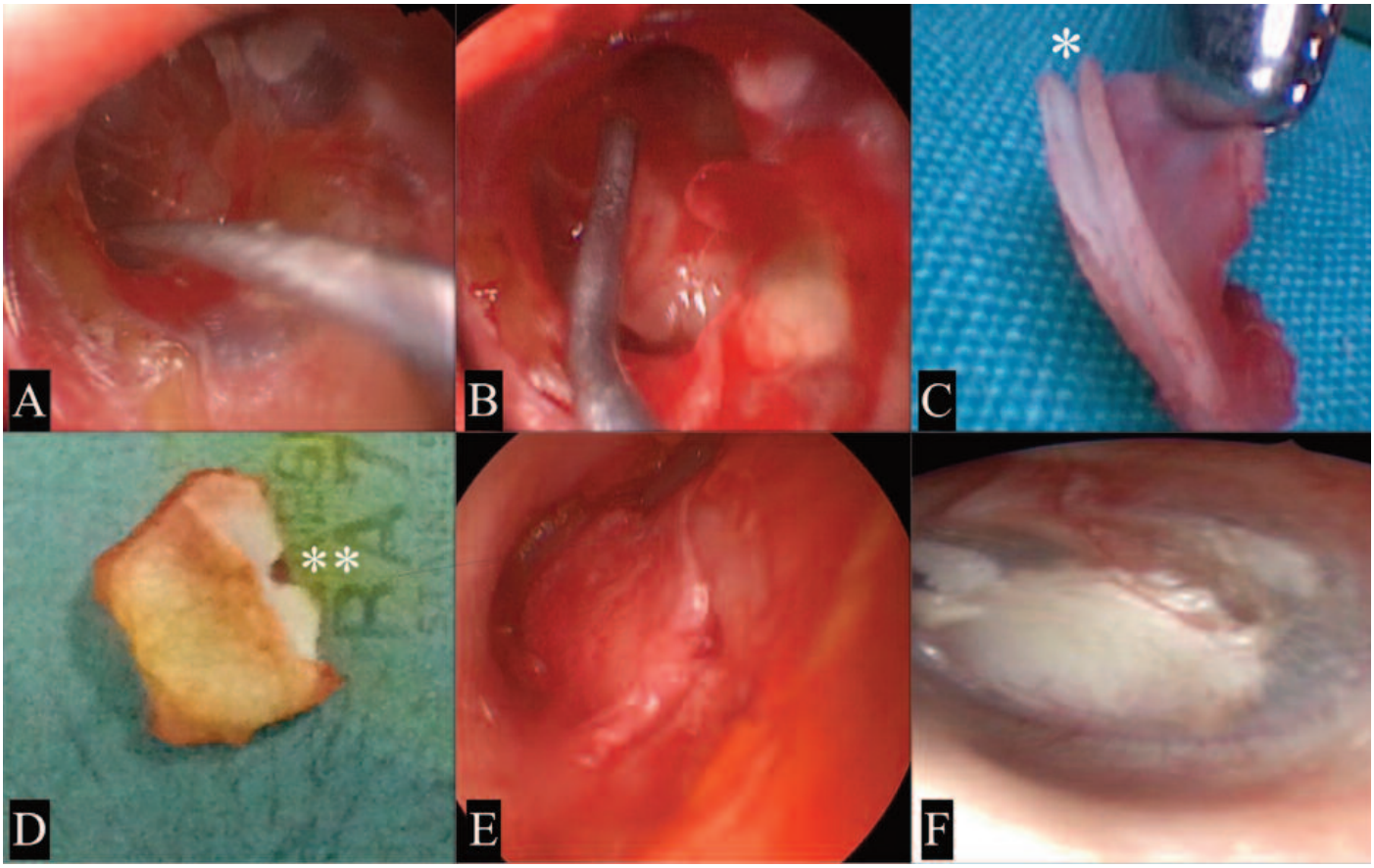

Figure 1. The main steps of inlay butterfly cartilage tympanoplasty. Initial perforation (A), the tympanic membrane remnant is refreshed and dimensions of the perforation are measured (B), a butterfly shaped tragal cartilage graft with preserved perichondrium (C), a triangular notch created in the cartilage to insert the graft laterally to the malleus (D), aspect of the tympanic membrane at the end of the procedure (E), Post-operative view of the tympanic membrane (F). *=circumferential notch around the graft, **=triangular notch

Table 1. Descriptive characteristics and clinical findings of the patients (n=29)

Sex (male / female)	16 (55.2%) / 13 (44.8%)
Age (year)	31.1±16.5
Operation side (right / left)	17 (58.6%) / 12 (41.4%)
Mean follow-up (month)	69.6±21.6
Graft intake success rate	26 (89.7%)
Preoperative ABG	22.7± 6.96
Postoperative ABG	8.9±4.5

Data were shown as mean±SD or n (%). ABG= air-bone gap

local anesthetic contained 40 mg of lidocaine HCl and 0.025 mg of epinephrine (Jetokain Ampule; Adeka Medical Inc., Samsun, Turkey). The graft is harvested by preserving the perichondrium on both surfaces of the tragal cartilage. Graft is prepared by measuring with a hook slightly larger than the perforation. The perichondrium must exceed the cartilage and a circumferential notch is opened all-around graft. The tympanic membrane remnant is refreshed with a pick, under the annulus edge and around the malleus, which needed to be stripped in some cases. The cartilage is then inserted throughout the perforation, yielding a butterfly configuration, with one wing remaining in the lateral position and the other lying medially to the perforated ear drum. In perforations which the malleus is exposed, a triangular notch is created in the cartilage and the graft is positioned laterally to the malleus. In such cases tympanic membrane remnants must be cleaned from malleus to prevent iatrogenic cholesteatoma. No packing is placed in the middle ear. The external ear canal is then packed with gelfoam and an ear wick is placed in the external meatus.

Statistical Analysis

SPSS 21 software was used for statistics. Distribution property (Normality) was performed with Kolmogov Smirnov test. Descriptive statistics were used and values were expressed as mean±SD or n (%). Normal distribution data were analyzed with an Independent t test (Student t test). p value <0.05 was evaluated as statistically significant.

Results

Twenty-nine patients with large central tympanic membrane perforation who were operated by IBT technique were included in the study. Sixteen (55.2%) patients were male and 13 (44.8%) were female. The mean age of the patients was 31.1±16.5 (8-67 years) years. Seven (24.1%) patients were children and 22 (75.9%) patients were adult patients. Seventeen (58.6%) patients were operated on the right ear and 12 (41.4%) on the left ear. Twenty-one (72.4%) of the surgeries were performed by endoscope and 8 (27.6%) under the microscope. The mean duration of surgery was 30.5±3.77 minutes. The mean follow-up time was 69.6±21.6 (range; 24-112 weeks). The graft take rate was 89.7% (26/29) (Table 1). Of the unsuccessful three cases, in one patient total graft extrusion was observed, and in the other 2 cases perforation diameter became smaller than the preoperative diameter, and annular perforations were observed around the grafts. The mean preoperative air-bone gap was 22.7±6.96 dB, with an average postoperative 8.9±4.5 dB gap. A significant decrease in the mean air-bone gap from the preoperative period to the last follow-up was found (p<0.001) (Figure 2). No complications or adverse reactions were noted in the patients.

Discussion

Tympanoplasty operations are one of the most common surgical procedures in the middle ear surgery. The goal of tympanoplasty is to repair the perforated ear drum, to create a middle ear free of infection and to improve hearing ability of the patient. For this purpose, many materials such as fascia, skin, venous vein wall, perichondrium, fat, dura have been used [5]. Classical tympanoplasty techniques have a success rate between 80% and 95%, but can cause some problems during the postoperative period. A wider incision is made in order to harvest a graft. The elevation of the tympano-meatal flap temporarily deteriorates the anatomy of the middle ear cavity and the external ear canal and requires more postoperative care [6, 7]. These procedures prolong the duration of

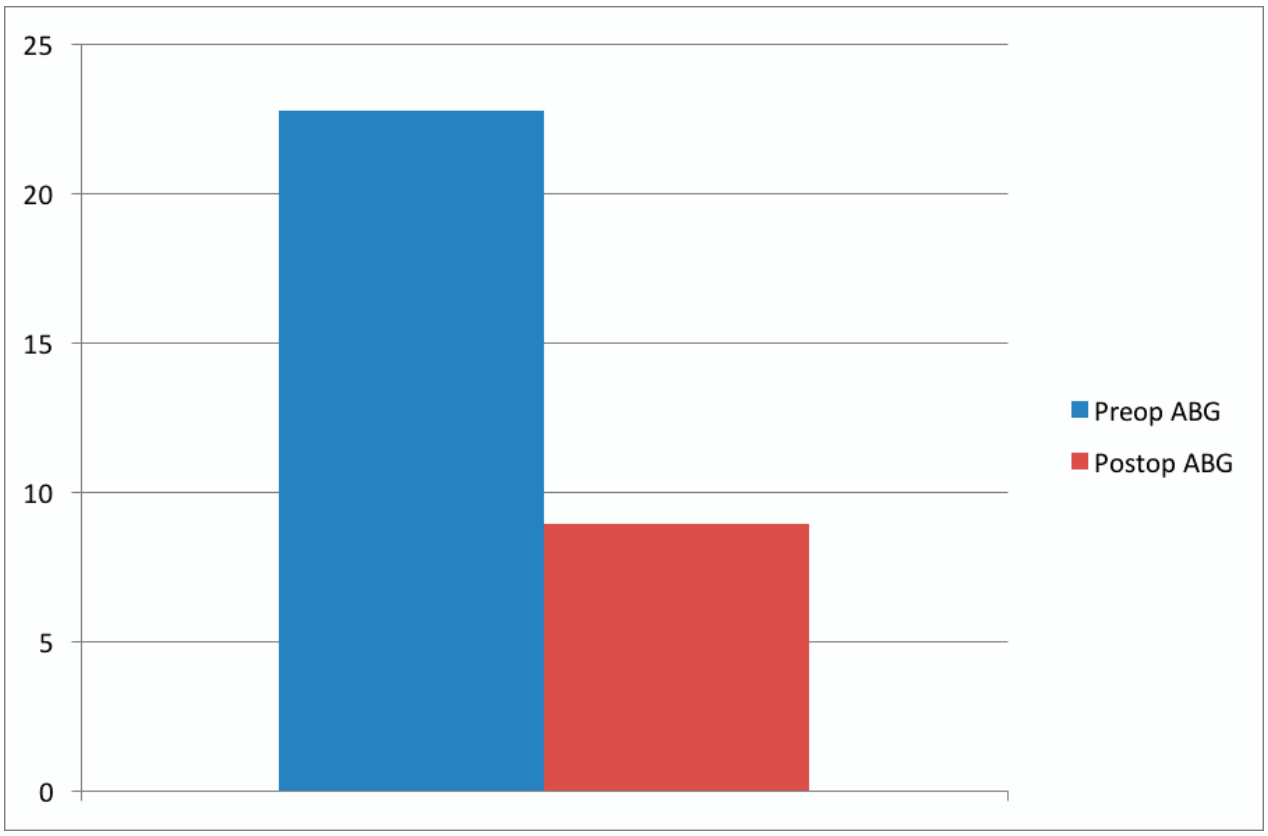

Figure 2. Comparison of preoperative and postoperative air-bone gap. ABG= air-bone gap, Preop=preoperative, Postop=postoperative

the surgery and hospitalization and the patients are susceptible to infection. Moreover, malleus head or damage to the ossicular chain may occur during operation [7].

IBT, which has been widely applied in the last two decades, is a comfortable and reliable method for both surgeon and patient and the results are also satisfactory. The main advantages of the butterfly technique are its reliability, avoidance of tympano-meatal flap elevation, which prolongs surgical time, and minimal postoperative discomfort [9]. Another important advantage of this technique is that the only incision is made on the tragus. There are many studies conducted in the literature in which the anatomical success rates range from 43% to 100% [3, 9-12]. Eavey [3] achieved 100% graft success with this technique in the pediatric age group. Lubianca-Neto [13] conducted a study in 2000, applying this technique under local anesthesia in adult patients and achieving success of 90%. Mauri et al. [14] compared the results of the IBT technique with the underlay tympanoplasty and suggested that there was no difference between the two techniques in terms of perforation repair and audiometric outcomes.

IBT related studies generally refer to successful outcomes of IBT techniques in patients with small central perforations. However, there is not enough study about the success of IBT technique in larger tympanic membrane perforations. Alain et al. [4] performed butterfly cartilage tympanoplasty in patients with total and subtotal tympanic membrane perforations and reported a graft success rate of 88%. Angeli et al. [15] and Fishman et al. [16] reported that graft success was 86% and 84%, respectively, in large tympanic membrane perforations with classic tympanoplasty techniques using fascia and dermis grafts. Moreover, IBT is discussed as a rapid and time saving procedure. Kim et al. [12] compared patients who underwent inlay butterfly cartilage tympanoplasty and patients who underwent conventional underlay tympanoplasty using the temporalis fascia. They reported that there was no statistically significant difference between two groups considering the surgical success rate, the mean air-bone gap decrease but they reported a significantly shorter operation duration in IBT group. The mean operation duration was 25.6±8.5 minutes in IBT group and 48.6±19.5 in conventional temporalis facia group. In our study, the mean operation duration was 30.5±3.77 minutes and the operation duration is

similar to the studies which were performed with IBT in the literature.

In this study, we presented our results with IBT technique in large central perforations. We demonstrated that the IBT technique is feasible and achieves good results. The graft take rate was 89.7% at the end of the follow-up and the mean operation duration was 30.5±3.77 minutes. The graft intake success rate is similar to the graft success rates of patients with large tympanic membrane perforations, which were performed with other methods in the literature and operation duration is shorter than conventional tympanoplasty methods.

Conclusions

Inlay butterfly cartilage tympanoplasty is a minimal invasiveand effective technique for repairing large central tympanic membrane perforations. Using IBT is shorter in duration than other methods and has less surgical and postoperative morbidity. Moreover, the duration of hospitalization was shorter and the success rates were found to be similar to the standard methods in our study. Therefore, IBT technique can be a feasible treatment option for large central tympanic membrane perforations.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing

The authors disclosed that they did not receive any grant during conduction or writing of this study.

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The European Research Journal
http://www.eurj.org
Original Article
e-ISSN: 2149-3189
DOI: 10.18621/eurj.287838

Management of the complications of pubovaginal sling surgery

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ABSTRACT

Objective. To present complications of pubovaginal sling surgery (PVS) and their managements. ***Methods.*** A total of 21 patients who underwent PVS in 4 different tertiary refferal centers between June 2014 and May 2016 were reviewed retrospectively. Demographic characteristics of the patients, previous surgical history, daily pad use, Urogenital Distress Inventory (UDI-6) and Incontinence Impact Questionnaire (IIQ) were recorded. Also, the management of the complications were stated. ***Results.*** The mean age of the patients were 54.9±12.32 (range, 36-76) years. Six (28.5%) patients had a history of previous incontinence surgery and 15 (71.4%) patients were naïve. There were no intraoperative complications.The mean daily pad use was decreased from 4.04±0.95 to 0.95±0.86 (p=0.0001). While mean preoperative UDI-6 scores were decreased from 6.66±2.09 to 2.09±1.22 (p=0.0001) at postoperative sixth month; preoperative mean IIQ-7 scores decreased from 16.8±1.16 to 5.09±4.21 (p=0.0001) at postoperative sixth month. One (4.76%) patient had abdominal hernia at postoperative 2nd year, three (14.28%) patients had an increased post-micturational residual (150-200ml) and two (9.52%) patients had urinary retention. ***Conclusion.*** Although the efficiency of PVS is high; one should aware of complications. The successful management of the complications will increase efficiency and patient satisfaction.

Eur Res J 2017;3(3):264-268

Keywords: Complication, pubovaginal sling, rectus fascia, stress urinary incontinence

Introduction

Urinary incontinence is an important health problem with a prevalence rate of 15-50% and stress urinary incontinence (SUI) represents the most common type of incontinence in women [1]. Today mid-urethral slings (MUS) are the most preferred method for the surgical treatment of female SUI

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Received: January 24, 2017; Accepted: May 27, 2017; Published Online: June 1, 2017

worldwide. MUS becomes very popular due to lesser invasive nature of the procedure and higher efficiency. After Food and Drug Administration (FDA) announcement on 2008 and 2011 the interest on MUS replaced with concern either from the patients or physicians. The number of pubovaginal slings (PVS) which have been performed in academic centers found to increased especially FDA notifications regarding mesh [2].

PVS and Burch colposuspension were the former leading surgical procedures for women with SUI. PVS was mainly indicated for intrinsic sphincter deficiency. However, it can be used in all SUI regardless of the underlying pathology [3]. The efficiency of PVS has been largely studied in previous series. In a recent review the success of PVS was found to be between 47% and 90% [4]. Autologous, allograft, xenograft or synthetic materials can be used for PVS. While all of these materials have its own advantages and disadvantages the autologous rectus fascia sling is the most preferred material for PVS [5].

Although PVS is an effective surgical method; complications including urinary retention and voiding difficulties including urgency and urge incontinence can be devastating [3, 6, 7]. In this study we aimed to review complications after autologous rectus fascia PVS and their managements.

Methods

Between June 2014 and January 2016, a total of 21 patients who underwent PVS in four different referral center were investigated retrospectively. All of the patients either had intrinsic sphincter deficiency (ISD) or recurrent stress urinary incontinence. The patients with mixed urinary incontinence or neurogenic bladder were not included in the study. Urodynamics study was performed to all patients preoperatively, and an abdominal leak point pressure threshold of 60 cm H_2O was considered as an indicator of ISD.

The age, previous surgical history and daily pad use of the patients were recorded. The patients completed ''Urogenital Distress Inventory 6 (UDI-6)'' and ''Incontinence Impact Questionnaire (IIQ-7)'' at preoperative and postoperative 6th month. In addition, complications of the PVS surgeries, and management of the complications were stated.

Operation Technique

The patient was placed in lithotomy position and a 18-F Foley catheter was implanted.

1. Graft Retrieval

A 6-7 cm long phannenstiel incision was performed 2 cm above the symphisis pubis for retrieving an autologous fascia. Rectus fascia was marked for to have a graft of 8 cm x 2 cm with transverse or longitudinal incision (Figure 1). Rectus fascia was elevated from one edge, and separated from the underlying rectus muscle with the aid of a scalpel or electrocautery to harvest graft material. The graft material was spread on the sterile drape, and it was cleaned from overlying fat, and perifascial tissues. Fixation sutures were passed through both ends of the graft with 1-0 PDS or 1-0 polypropylene sutures (Figure 2).

2. Vaginal Approach

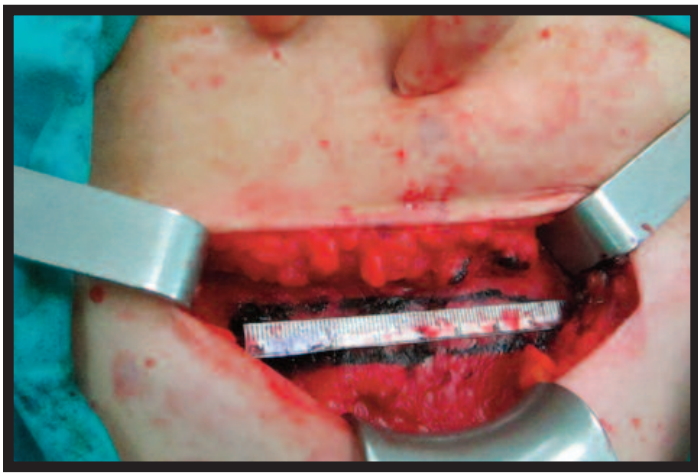

Figure 1. Rectus fascia was marked for to have a graft of 8 cm x 2 cm

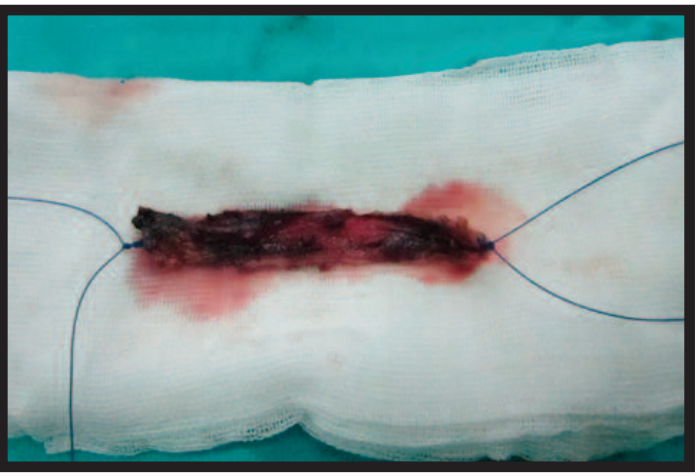

Figure 2. Fixation sutures were passed through both ends of the graft with 1-0 PDS.

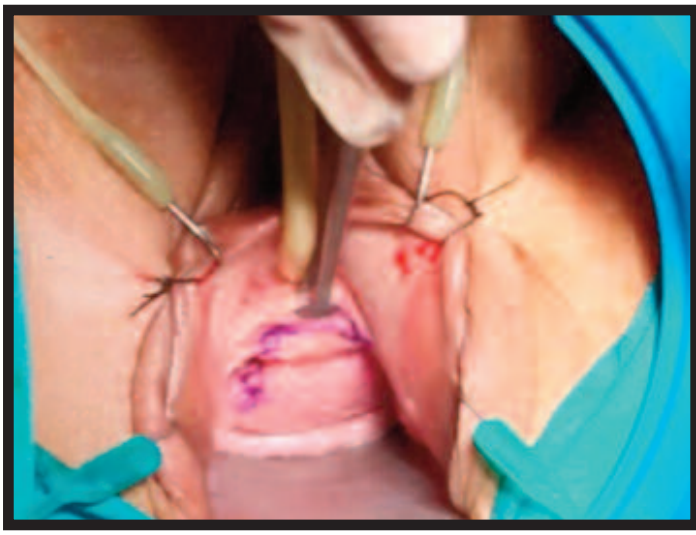

Figure 3. An inverted "U" incision.

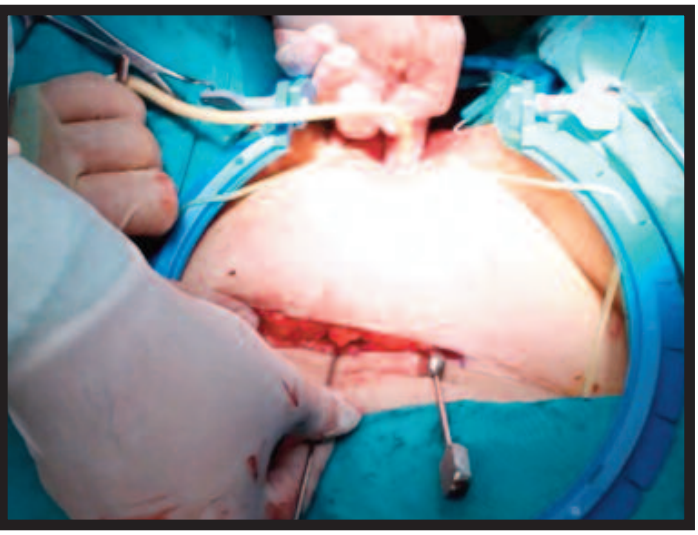

Figure 4. Stamey needles were inserted through abdominal incision, and were brought out of the vaginal incision.

Initially, 0.9% saline was injected into vaginal epithelium to achieve periurethral hydrodistension, and to facilitate dissection of tissues. An inverted U incision was performed so as to advance easily to the urethra, up to the level of bladder neck, endopelvic fascia, and then retropubic space (Figure 3). Immediately below the external meatus was retracted with an Allis clamp, and an incision was made starting from 2 cm below the external meatus up to the vaginal epithelium using a 15 G scalpel. With the aid of Metzenbaum scissors, and Allis clamps, subepithelial layer of vagina was dissected for to create vaginal epithelial flaps.

After creation of adequate-sized lateral flaps, further dissection to the ischiopubic rami was performed which facilitated perforation of endopelvic fascia. The bladder was always completely evacuated so as to prevent perforation of the bladder with Stamey needles. Beneath the ischiopubic ramus, and superior to the dissected area, metzenbaum scissors were held with its tips pointing towards the ipsilateral shoulder, and advanced strictly medially to perforate endovesical fascia. Retropubic space was dissected bluntly with finger and passage between retropubic, and infrapubic spaces was achieved.

3. Placement of PVS

Stamey needles were inserted through abdominal incision, and carefully advanced just underneath the symphysis pubis, then it was passed lateral to the bladder, and brought out of the vaginal incision (Figure 4). After passing the needles through the both incisions, cystoscopy was performed using firstly 30o , and then 70o optical lenses to confirm lack of inadvertent bladder perforation with needles. Then the sutures which were placed at the edge of the graft were passed through needle holes of Stamey needles, and midpart of the graft was held with a clamp to prevent slipping of the midline of the sling. The sutures were pulled backward together with the needle, and brought out through the inserion points of the needles, and held with hemostatic clamps.

In the area of vaginal dissection, graft material was fixed to the periurethral area with 4-0 polyglican sutures from two points. Following hemostasis, vaginal incision was closed with 2-0 polyglycan sutures. Then both sutures were pulled up through the abdominal incision. Proximal urethra was inspected with cystoscopy using 30-degree optical lenses to determine if adequate closure was achieved. If adequate closure was realized, then sutures from left, and right side were tied to each other. In most of the cases two fingers could be snugly inserted between the knot, and the rectus fascia, which meant adequate urethral closure. Then abdominal incision was closed, and estrogen containing sponges were placed into vagina.

Statistical Analysis

Average, frequency and percentage values were given as descriptive statistics. SPSS for Windows Version 11.5 (Chicago, Illinois) was used for analyses. The Mann-Whitney U test and chi-squared test were used to determine the difference within groups as for continuous and categorical variables, respectively. A $p<0.05$ value was accepted as significant.

Results

There was no intraoperative complication. The mean age of the women was 54.9±12.32 (range; 36-76) years. Six (28.5%) patients had a history of previous anti-incontinence surgery and 15 (71.4%) were naïve for surgery. The mean pad use was decreased from 4.04±0.95 to 0.95±0.86 (p=0.0001) at postoperative follow up. There was a significant improvement in comparison of preoperative and postoperative UDI-6 and IIQ-7 scores. The mean preoperative UDI-6 score was decreased from 6.66±2.09 to 2.09±1.22 (p=0.0001) at postoperative sixth month and the mean preoperative IIQ-7 score was decreased from 16.8±1.16 to 5.09±4.21 (p=0.0001) at postoperative sixth month.

Thirteen (62%) patients out of 21 had no complications. One (4.76%) patient had abdominal hernia at postoperative 2nd year and hernia was repaired by a general surgeon. Recurrent SUI was developed in one (4.76%) naïve woman and in one (4.76%) woman with a history of trans obturator tape. Both patients were scheduled for urethral injection. Two (9.52%) patients had urgency incontinence and their symptoms improved after prescription of antimuscarinics.

Three (14.28%) patients had an increased post-micturational residual (PMR) (150-200 ml). In one of them the abdominal sutures were cut at early postoperative period and she was able to void and there was no recurrence for SUI. The other two patients had decreased PMR at the 3rd month follow-up (50-100ml).

Two (9.52%) patients had urinary retention. While one patient was able to void after 5 weeks course catheterization, the other woman managed with clean intermittent catheterization (CIC). Initially this woman underwent urethrolysis. The operation was extremely difficult due to the scar tissue surrounding the bladder neck. She continued to have CIC after failure of urethrolysis. Afterwards she underwent sacral neuromodulation and the frequency of CIC was decrased from 6 to 3 times daily. She was able to void an amount of 150 ml in an obstructive pattern at uroflowmetry. An another course of urethrolysis was planed to the patient patient but she become pregnant and the sacral neuromodulation was turned off.

Discussion

The surgical outcomes following surgery has been well established in the literature. Due to discrepancies in definition of a successful operation, the success rates varies between 47% and 90% [4]. In the present study the objective success which was defined as no leakage on pelvic examination found as 90.4% and there was a significant improvement in scores of UDI-6 and IIQ-7 when compared preoperatively and postoperatively. While confirmation of no SUI on physical examination is one of the important goals of the operation; patient satisfaction is another important aspect of success. Correction of SUI does not always indicate a good outcome. As an example a woman with a de novo urge incontinence may have even more bother than her previous condition. Therefore, it is essential to achieve good results in all aspects of lower urinary tract symptoms including continence, storage and voiding.

Failure or recurrence of PVS is a very difficult condition and there is no agreement on the next step in the literature [8-12]. Patients may find it difficult to have another major surgery in that situation. We offered urethral injections to our patients with recurrence as a minimal invasive alternative to avoid any further complications.

PVS surgery is prone to produce complications such as retention, dysfunctional voiding, urgency and wound problems. The rate of overall complications is reported between 3% and 46% in most of the large series [8-12]. Also, in the largest series in the literature, Blavias Blaivas and Chaikin [13] reported less than 1% of complications requiring catheterization or urethrolysis. In the present study the overall complication rate was 38%. These complications included abdominal hernia in one, urgency urinary incontinence in two, increased PMR in three and complete urinary retention in two patients. The reported rate of complications can differ in various studies similar to the variations in definition of success. In most of the studies transient retention or increased PMR was not mentioned as a complication. This can be the reason why the overall complication rate is high.

The rectus fascia is the most common autologous material used for PVS. In the present study we only chosen the rectus fascia for sling. Although it is a more convenient way of harvesting fascia than fascia lata, wound infections, seroma or hernia can occur in a rate of 0.8-1% [13, 14]. In our study there are not infectious complications of the rectus fascia. However, in one patient abdominal hernia developed two years

after the surgery and surgical repair was employed by a general surgeon.

Athanopolos *et al.* [12] reported a total complication rate of 29.2% out of 264 patients. The rate of complications was high in sister trail. The complication rates found in this study was in accordance with the other reported series. Of course the surgical experience is the most important factor for complications. While experienced surgeons such as Blaivas and Chaikin [13] reported prolonged retention rates of %1 this can be higher up to in other series.
Although PVS has been recognized as a rescue surgery for those with an unsuccessful attempt of incontinence surgery, the majority of our cases were naïve patients. The common reasons for PVS as the primary surgery were: i) surgeons preferences, ii) patient preferences. Today MUS has been the treatment of choice in most of the forms of SUI and PVS is reserved for the recurrent cases. In the present study 71.4%) of the patients were naïve patients for SUI

De novo urgency is one of the frequent complications of PVS surgery. Although the underlying pathology is not fully understood bladder outlet obstruction and nerve damage caused by the sling may have a role in development of de novo urgency [3]. In our series two (9.52%) women of the patients had de novo urgency. This rate is consistent with the other series published [3, 6, 10]. These patients were treated with anticholinergics. Before initiation of the treatment they were checked for PMR and afterwards they were followed up cautiously. The response rate after anticholinergics in de novo urgency. Of course, urinary retention is the one of the most devastating complications of PVS surgery. If necessary urethrolysis should be accomplished ASAP, the delay can result in no a retention but PMR [15].
The autologous PVS can be done either with the rectus fascia or fascia lata. In most of the circumstances rectus fascia is the first choice because of the familiarity of the abdomen anatomy for the urologist. Multi-center contribution to the present study represents one of its strengths. However the small number of patients and limited follow-up are the major drawbacks of this study.

Conclusions

In conclusion, PVS surgery carries increased risk of complications. The risk of complications should be counterbalanced with the benefits of this surgery. The patients should be informed about the possible complications of this surgery in the long run.

Informed consent

Written informed consent was obtained from the patient for the publication photographes used in this study.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing

The authors disclosed that they did not receive any grant during conduction or writing of this study.

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The European Research Journal
http://www.eurj.org
Case Report
e-ISSN: 2149-3189
DOI: 10.18621/eurj.337348

Human papillomavirus causing tonsillar hypertrophy in non-cancerous tonsil

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ABSTRACT

Human papillomavirus (HPV) is associated with the development of most anogenital carcinomas, including cervical cancer and, has more recently been suggested to be a risk factor for a subset of head and neck squamous cell carcinoma. A 15-year-old female presented with complaints of chronically progressive pain on swallowing. Examination revealed bilateral Grade III tonsillar enlargement with multiple warty appearances over the surface of the tonsils. She underwent tonsillectomy and histopathologic study revealed tonsillar crypts lined by stratified squamous epithelium with focal koilocytic changes. Polymerase chain reaction study detected HPV 11 DNA. The presence of HPV in the oral cavity and upper respiratory tract mucosa is of great importance, since several studies have demonstrated an association of HPV with a great variety of benign and malignant lesions. A rare case of benign papillomatosis of the tonsil is presented in this study. There is a need for long-term follow-up due to the possibility and risk factors for malignant transformation.

Eur Res J 2017;3(3):269-272

Keywords: Human papillomavirus, chronic tonsillitis, tonsillectomy, benign papillomatosis

Introduction

Human papillomavirus (HPV) is associated with the development of most anogenital carcinomas, including cervical cancer and, has more recently been suggested to be a risk factor for a subset of head and neck squamous cell carcinoma (HNSCC) [1]. Even though HPV is well known to be related to several diseases with significant morbidity and mortality, few researchers have attempted to determine the frequency that HPV colonizes the tonsils [2]. The prevalence of HPV in normal oral mucosa ranges from 0.6% to 81% [3].

HPV may be postulated to play a role in the pathogenesis of palatine tonsillar cancer, not only due to its morphological similarities to cervical cancer but also because the mucosal squamous epithelium, similar to that of the uterine cervix, is easily exposed to viral infection [3]. HPV infection is prevalent in the oropharyngeal mucosal regions, and the tonsil is the most commonly affected anatomical region in the oropharynx, but the presence of HPV infection associated with benign lesions of the tonsils are a relatively rare phenomenon.

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Received: September 9, 2017; Accepted: September 28, 2017; Published Online: October 2, 2017

A rare case of benign papillomatosis of the tonsil is presented in this study.

Case Presentations

A 15-year-old female presented to the outpatient department of Otorhinolaryngology and Head and Neck surgery of a tertiary care center in South India with complaints of chronically progressive pain on swallowing, recurrent intermittent sore throat and mild difficulty in swallowing which was slowly progressive for 3 years. The patient gave no history of change in voice, difficulty in breathing, snoring, mouth breathing or painful neck swelling. The patient had been prescribed several courses of antibiotics and analgesics by various local general practitioners over the course of this three-year period.

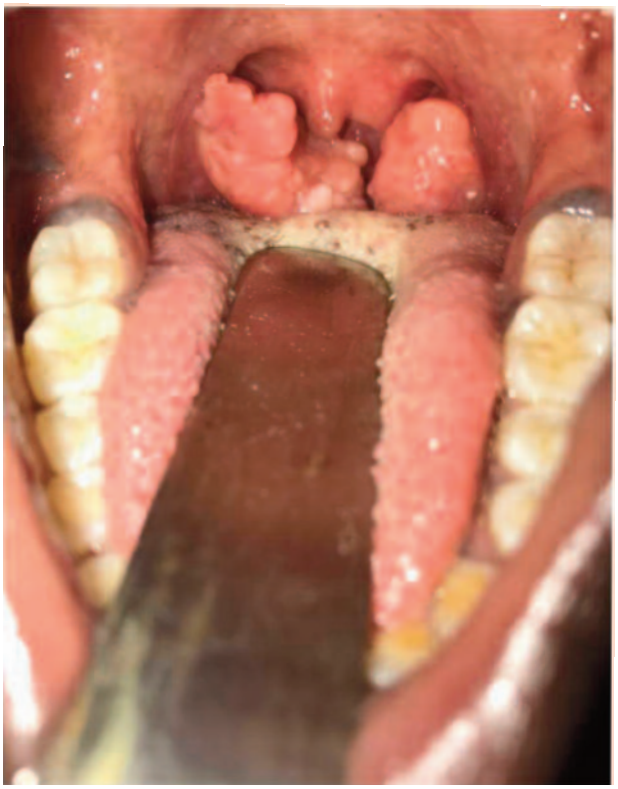

Figure 1. Examination of oropharynx revealing bilateral Grade III tonsillar enlargement with multiple warty appearances over the surface of the tonsils

General examination was unremarkable. On local examination, she had bilateral Grade III tonsillar enlargement with multiple warty appearances over the surface of the tonsils. (Figure 1) She also had bilateral non-tender jugulodigastric nodes. Subsequently she underwent tonsillectomy and the specimen (Figure 2) was sent for histopathologic examination and Polymerase chain reaction (PCR) study as well. Histopathologic study revealed tonsillar crypts lined by stratified squamous epithelium with focal koilocytic changes (Figure 3). PCR study detected HPV 11 DNA. The patient was given prophylactic HPV vaccination during follow up. The patient was asymptomatic at 1 year follow-up and examination did not reveal any recurrences in the tonsillar fossa.

Discussion

The HPV virion is a double-stranded, circular DNA genome comprised of eight open reading frames with early (E) and late (L) genes. The L1 gene encodes for the major capsid protein and is well preserved across numerous genotypes. All known HPV types are exclusively epitheliotropic and depend on epithelial differentiation for completion of their life cycle. Infection and virus uncoating begins in the basal layer where expression of early (E) genes occurs, which maintains the viral genome and retards terminal differentiation of basal cells stimulating cell-cycle progression. In the mid or upper epithelial layers, expression of late (L) genes occurs allowing amplification and packaging of virions into infectious particles. When infection by a virus occurs, the cell-mediated arm of the immune system is activated leading to production of IL-2 that serves as a critical messenger in the stimulation of helper and cytotoxic T cells [4, 5].

It is known that smoking, starting sexual activity and giving birth at an early age, having numerous sexual partners, and using oral contraceptives is associated with a higher risk for HPV infection in women [6]. Several nonsexual modes of transmission can be proposed for pediatric HPV infections, including vertical transmission, horizontal transmission, and autoinoculation. Vertical transmission is divided into 3 subtypes: periconceptual (time around fertilization), prenatal (during pregnancy), and perinatal (during birth and immediately thereafter) transmissions. HPV transmission could also be the result of close contacts of the fetus with infected cervical and vaginal cells of the mother during delivery. In horizontal transmission, children might acquire HPV infection from breast milk during breastfeeding, from householders, and from friends via kissing and digital contacts. Although the consequences of vertical and horizontal transmission

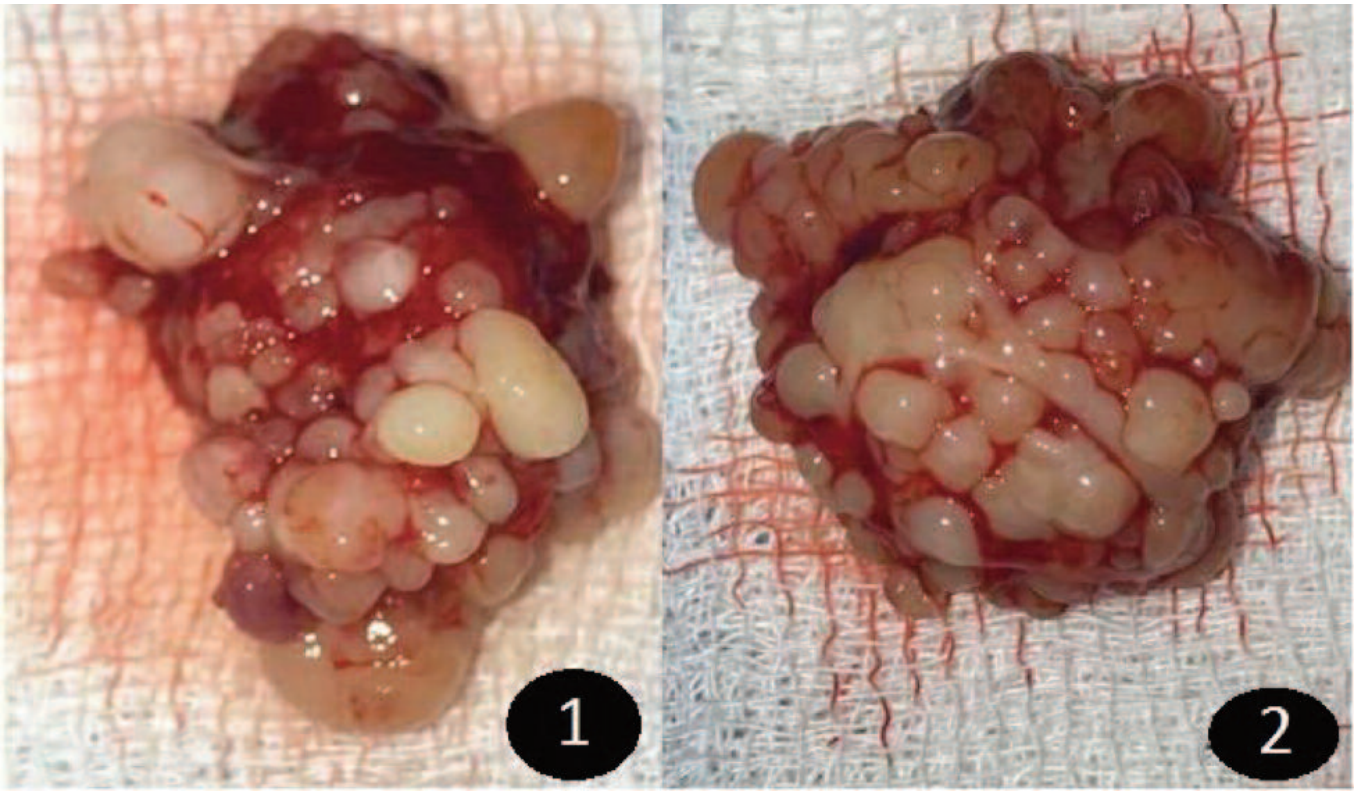

Figure 2. Post-tonsillectomy specimens showing multiple warty appearances over the surface of the tonsils

in early childhood HPV infection are still unknown, HPV infection may induce HPV-specific humoral tolerance. In this case, prophylactic HPV vaccines should be administered at birth [7].

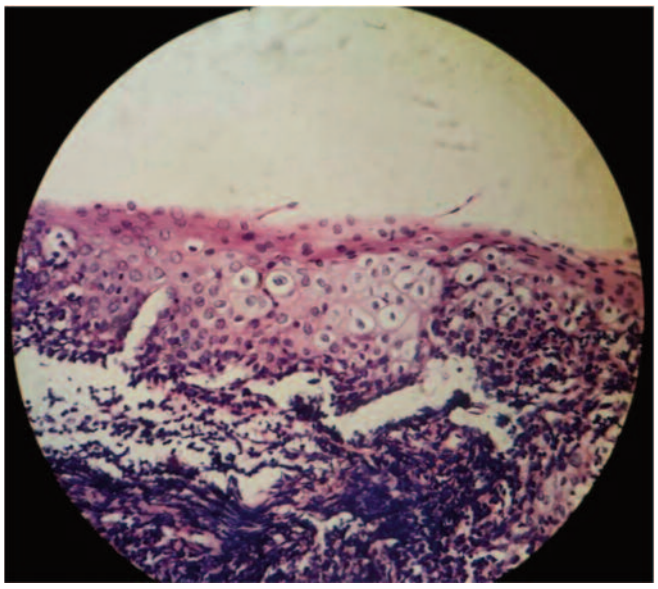

Figure 3. Section from crypt showing stratified squamous epithelium with koilocytic change [HE, 40X]

The exact mechanism of HPV infection in non-genital regions remains uncertain, but the easy access to the tonsillar crypts and the favorable micro environmental factors of the crypts may be causes of the high prevalence of HPV in non-genital regions. HPV DNA is detected predominantly in the epithelium lining the tonsillar crypt by in situ hybridization [8].

The presence of HPV in the oral cavity and upper respiratory tract mucosa is of great importance, since several studies have demonstrated an association of HPV with a great variety of benign and malignant lesions. Some of the most frequent benign lesions of the oral mucosa related to HPV are oral papilloma, focal epithelial hyperplasia and leucoplakias. Frequency of HPV in head and neck cancer patients varies between 8 and 50%; it is believed that its detection may be dependent on several factors including the molecular technique utilized (Southern blot hybridization, PCR, in situ hybridization), the treatment of the sample material (fresh, frozen, paraffin embedded), ethnic and geographical differences, and the anatomic site of the lesion [9].

HPV DNA can be detected in tissue specimens by various methods, including in situ hybridization, HPV DNA assays, and HPV nucleic acid detection with target amplification. As a result of its increased sensitivity, PCR has become a popular method for molecular diagnosis of HPV infection. Furthermore, PCR for HPV DNA can be designed to amplify a single HPV genotype or a broad spectrum of HPV

types based on the primers used. Type-specific PCR is labor-intensive and expensive leaving broad-spectrum amplification better suited for molecular screening of HPV infection [10].

The detection of HPV DNA in tumor-free tonsils has been reported in only a few studies, and others failed to detect the viral DNA in tonsillitis samples. The overall frequency of HPV DNA in normal tonsillar mucosa or benign tonsillar lesions is 8.5%, consisting of 70% with HPV-16 and 30% with HPV-6/11. HPV DNA is seldom detectable in normal tonsillar exfoliated cells, suggesting that HPV DNA either locates in the crypt epithelial cells or normal tonsils do not harbor HPV DNA [11].

Conclusion

Benign papillomatosis of the tonsils is rare and further studies with a long-term follow-up are warranted to clarify whether or not this condition is related to the development of HPV-related cancer. The appropriate modality of treatment of this condition also needs to be developed, although in our case surgery was effective and long-term follow-up has not revealed a recurrence.

Informed consent

Written informed consent was obtained from the patient for the publication of this case report.

Conflict of interest

The authors declared that there are no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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The European Research Journal

http://www.eurj.org

Case Report

e-ISSN: 2149-3189

DOI: 10.18621/eurj.295991

Brain function - not size - is important

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ABSTRACT

Some incidental findings on cranial imaging may render every one speechless. Brain plasticity in childhood and slow expansion of arachnoid cyst may be possible explanations of the discordance between the clinical and radiological findings. Here, we presented an asymptomatic, incidentally detected arachnoid cyst accompanied by compression of most parts of brain, displacing the motor cortex to the occipital parts of the existing brain tissue. The case was discussed in the light of the rare patients in the literature.

Eur Res J 2017;3(3):273-275

Keywords: Plasticity, arachnoid cyst, brain function

Introduction

Brain plasticity, ability of the brain to change - physically, functionally, and chemically - throughout life, is one of the fully unexplained conditions of the human brain. Advanced brain plasticity in childhood may facilitate cognitive reorganization. Arachnoid cysts are benign congenital lesions that develop during the formation of the endomeninx. In this paper we presented a case of displaced motor cortex, as evident from functional magnetic resonance imaging, due to a giant asymptomatic arachnoid cyst that was detected incidentally.

Case Presentation

A 38-year-old right-handed man was admitted to the outpatient clinic with the complaint of headache that was localized to frontal region and unresponsive to the medical treatment. He was primary school graduated worker, married with two children. He had no nausea or vomiting. His neurological examination was intact. Computed tomography and magnetic resonance imaging of the brain revealed giant arachnoid cysts on the convexity of frontal lobe, occupying 17.5x8x9 cm in dimension on the right side, and 12x8x5.5 cm on the left (Figure 1). Temporal lobes were almost completely erased and volumes of the parietal lobes were reduced. Only occipital lobes were developed. Neuropsychological examination with frontal attention and memory tests revealed mild cognitive decline. His general mental capacity was limited. Speech centre showed left-sided dominance,

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Received: March 2, 2017; Accepted: July 28, 2017; Published Online: July 29, 2017

shown by activation sides on functional magnetic resonance imaging (Figure 2). There is no change with his neurological status at the end of 2-year follow-up.

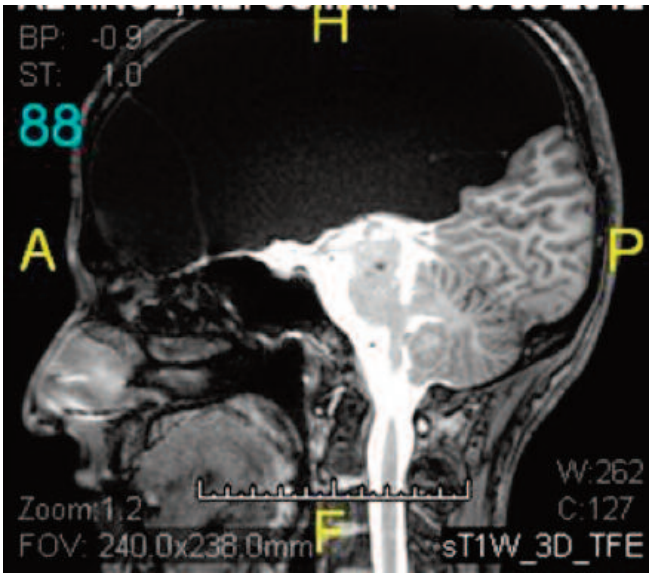

Figure 1. T2 weighted sagittal section of MRI of the brain revealed giant arachnoid cysts on the convexity of frontal lobe.

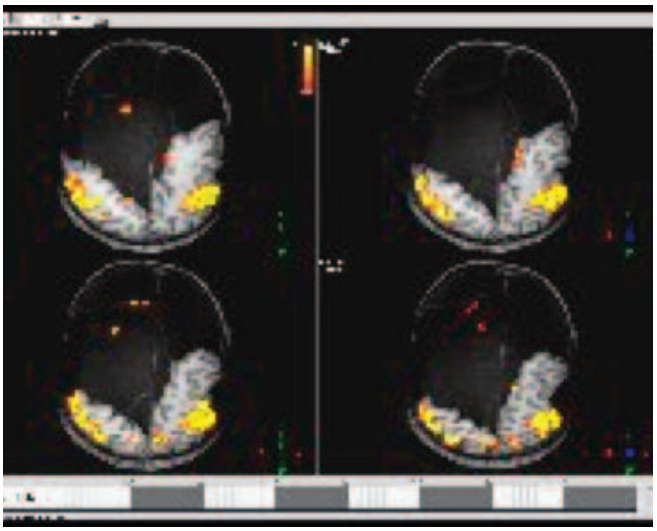

Figure 2. Speech centre showed left-sided dominance, shown by activation sides on functional MRI.

Discussion

Arachnoid cysts are cavities with content similar to cerebrospinal fluid. They are often found incidentally in 0.1-0.4% of the general population and they make up approximately 1% of all intracranial space-occupying lesions [1]. A critical point is the extent of the compression capacity of human brain by space-occupying brain lesions. Rare case illustrations, in the literature, showed that giant arachnoid cysts displaced cortical and subcortical structures which were still compatible with normal brain function [1-3]. Motor cortex can be displaced due to a giant asymptomatic arachnoid cyst, as in our case. We showed this with functional magnetic resonance imaging.

There was no deformation at the skull of our patient so this showed us that this arachnoid cyst hadn't created a high intracranial pressure and its growth was parallel to the developing brain. Nickel *et al.* [2] and Artico *et al.* [4] pointed that the continuum of the compression of the patients' brain in early adulthood, during which complete skull ossification had occurred. This was controversial to the findings in our patient.

The subtle neuropsychological impairment, being evident only in the attention and memory domains of the frontal lobe function, may be due to the arachnoid cyst. However, this interpretation cannot be made with absolute certainty since the patient himself did not report any difficulty in his daily life.

In a series of five patients with non-symptomatic arachnoid cysts in the left temporal fossa, the authors concluded that arachnoid cysts did not disturb the normal asymmetry of hemisphere language organization despite delicate locations adjacent to the left inferior frontal gyrus because of the absence of aphasic disturbance in their patients [5]. The topography of language activation did not differ significantly from the one in the healthy controls, although magnetic resonance morphometry of the patients' brains revealed reduction of cortical thicknesses in the left hemispheric grey matter adjacent to the cysts. In our case, the language activation sides were placed to occipital lobe.

Conclusion

The discordance between clinical and radiological findings were evident in this giant asymptomatic, incidentally detected arachnoid cyst which was accompanied by compression of most parts of brain, displacing the motor cortex to the occipital parts of the existing brain tissue. Slow expansion of this intracranial cyst may be a possible explanation of this discordance.

Informed consent

Written informed consent was obtained from the patient for the publication of this case report.

Conflict of interest

The authors declared that there are no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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The European Research Journal
http://www.eurj.org
Case Report
e-ISSN: 2149-3189
DOI: 10.18621/eurj.336041

A prenatally diagnosed gastric duplication cyst connecting the pancreatic duct and paraspinal region

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ABSTRACT

The gastrointestinal duplication of stomach is uncommon congenital anomalies of gastrointestinal tract. Herein a rare case of gastric duplication with newborn is being reported. A 14-day-old term female baby was admitted to our clinic with intermittent bilious vomiting and abdominal distention. In evaluation, abdominal cystic mass was detected in the left upper quadrant. The cyst which was attached to both the pancreatic duct and spinal cord was removed totally by laparotomy. A histologic examination showed gastric duplication cyst. The postoperative course was uneventful. There were no any complications at the 8-month follow-up period. This rare entity should be included in the differential diagnosis of cystic masses of the gastrointestinal tract and treated surgically by complete resection due to the risk of malignant transformation and other complications. This is the first case report that the gastric duplication cyst has a connection with both the pancreatic duct and spinal cord.

Eur Res J 2017;3(3):276-280

Keywords: Gastric duplication, cyst, newborn

Introduction

Alimentary tract duplications comprise a wide range of mass lesions throughout the gastrointestinal tract. Gastric duplications (GD) are often cystic and asymptomatic, and can reach very large sizes. They can have a wide range clinical presentations due to mass effect and pressure on the surrounding structures ranging from abdominal pain to perforation [1]. Although there are cases of GDs connecting with the pancreatic duct in the literature, to the best of our knowledge a GD communicating both with pancreas and spinal cord have not been reported up to date.

Herein, we present a case of a newborn with a prenatally detected GD cyst.

Case Presentation

A 14-day-old term female infant presented to the emergency department with intermittent vomiting and abdominal distention. In her history, it was demonstrated that she was diagnosed with a homogenous cystic mass (3 × 4 × 4 cm) on the upper

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Received: August 24, 2017; Accepted: October 3, 2017; Published Online: October 30, 2017

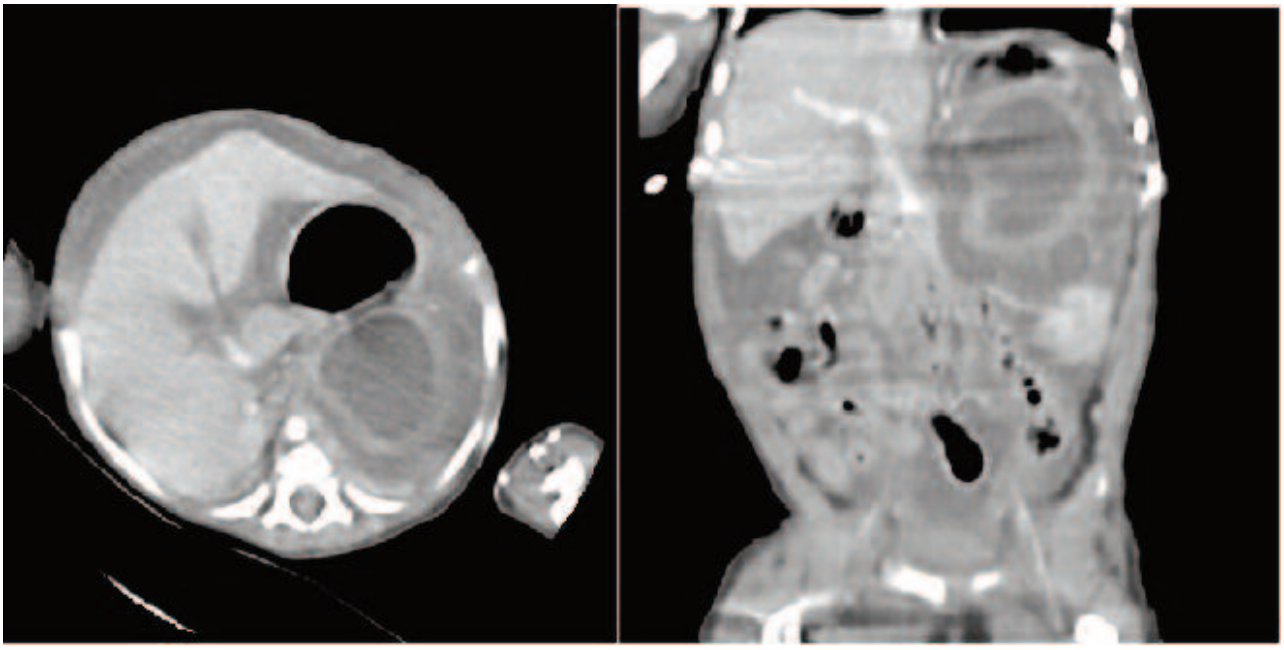

Figure 1. CT demonstrates an intraabdominal cyst on upper left quadrant.

left area in the abdomen. A general physical examination revealed a temperature of 38° C, pulse rate of 110 per minute and respiratory rate of 35 per minute. The general condition of the infant was poor and she was intubated and a nasogastric tube was inserted. The drainage fluid was bile stained with an amount of 50 mL per day. On abdominal examination, there was a marked abdominal distention without rebound tenderness and the bowel sounds were normo-active.

On follow-up, the plain abdominal radiographs were normal and ultrasonography (US) of the abdomen demonstrated 7 × 8 × 6 cm homogenous cystic mass with millimetric echogenities arising from the upper pole of the left adrenal gland extending into the paraumblical area lateralizing the left kidney and spleen.

The abdominal tomography (CT) showed a big encapsulated cystic lesion which was located inferior-posterior of great curvature of stomach (Figure 1). It was extending from the left upper suprarenal side to the para-umbilical area without a dilated stomach and

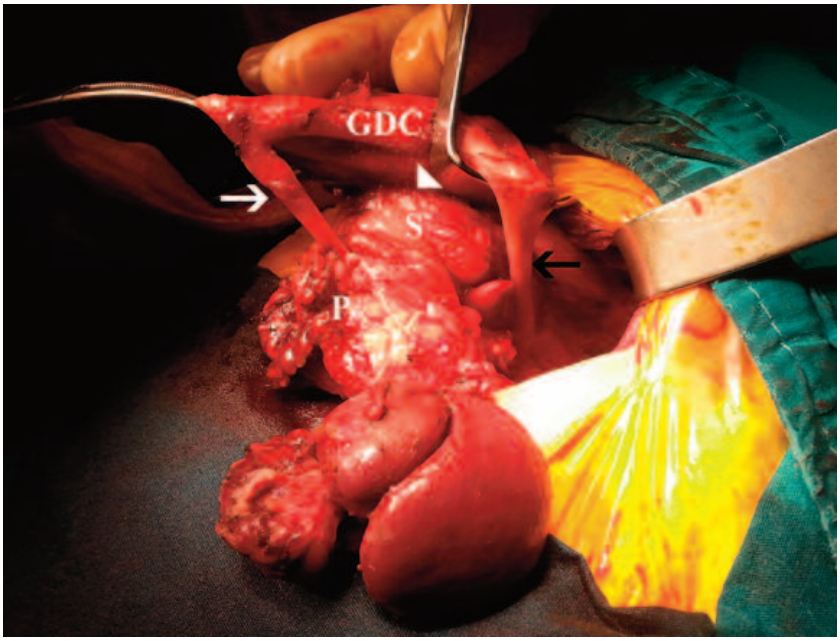

Figure 2. The intraoperative photograph shows the gastric duplication cyst (GDC) which has two connections with both the pancreas (P, white arrow) and the spinal cord (black arrow). Cyst also attached to the stomach (S, white triangle).

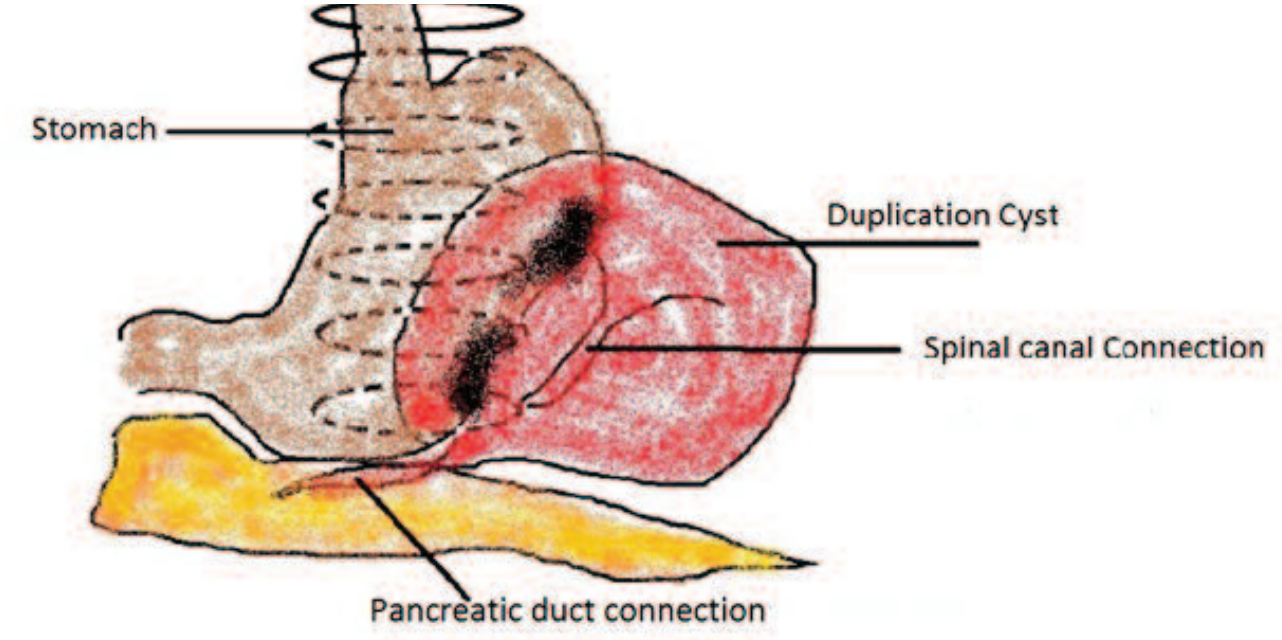

Figure 3. The illustrated image shows common wall with the stomach at two points and connections with pancreatic duct and spinal canal.

pancreatic ducts. An urgent exploratory laparotomy was planned after preparations.

On laparotomy, a cystic mass of about 6 × 10 cm was noticed, and it was considered to be a GD cyst. The cyst was firmly adhered to the spleen and attached to the posterior wall of the stomach. The cyst was shown to share a common wall with the stomach at two points. GD cyst had also a connection with both the spinal canal and the pancreatic duct. The wiev of the operation and illustrated image are showed (Figures 2 and 3). About 100 ml of dark brown fluid was aspirated. Both connections were ligated and transected. The cyst was dissected from spleen's and stomach's adhesion and mucosectomy of the two points was performed.

Postoperative course was uneventful. She was discharged on postoperative fifth day. In the eight months of the follow - up period, the patient was healthy.

Histopathologically, on the examination by light microscopy, the sections stained with hematoxylin-eosin revealed the layers of gastric wall that is mucosa, sub mucosa, muscularispropria and serosa (Figure 4a). Periodic acid – Schiff-alien blue staining showed us that, the most of the mucosal cells were gastric cell and the some of them were cells containing apical intestinal mucus (Figure 4b). The most mucosal cells had positivity of CK7 immunohistochemical staining (Figure 4c), but some cells were positive with CK20 (Figure 4d).

Discussion

The duplication cysts can be occurring along the whole alimentary tract [2]. Although the most involved tract is ileum (30-35%) the GD represent only 4 to 5 % of all duplications [3]. GD, most of which are cystic duplication (80%) and have not communicated with lumen of the stomach. The remainder structures are tubular duplication which associated with gastric lumen [4]. In general, patients typically present at neonatal period with symptoms of gastric outlet obstruction and palpable abdominal mass which may be seen on antenatal ultrasonography [1, 3]. In determining of GD cyst, US and CT are an effective diagnostic modality.

Our case with antenatal diagnosis of intra-abdominal cystic lesion presented with postnatal gastric outlet obstruction. Based on US and CT scan, we didn't make the preoperative diagnosis of a duplication cyst. Although it is difficult to diagnose GD cyst preoperatively, imaging studies have provided some evidence informative. It is stated that the characteristic "double wall sign," delineating the acrogenic inner mucosa from the hypo echoic outer rim of muscle can be used to identify on US [5]. Additionally, a contrast-enhanced CT scan can also be demonstrated GD as a thick-walled cystic lesion with enhancement of the inner lining [4].

Many varied theories have been claimed regarding the origin of GD cyst, like recanalization defect of alimentary tract, adhesions between endoderm and

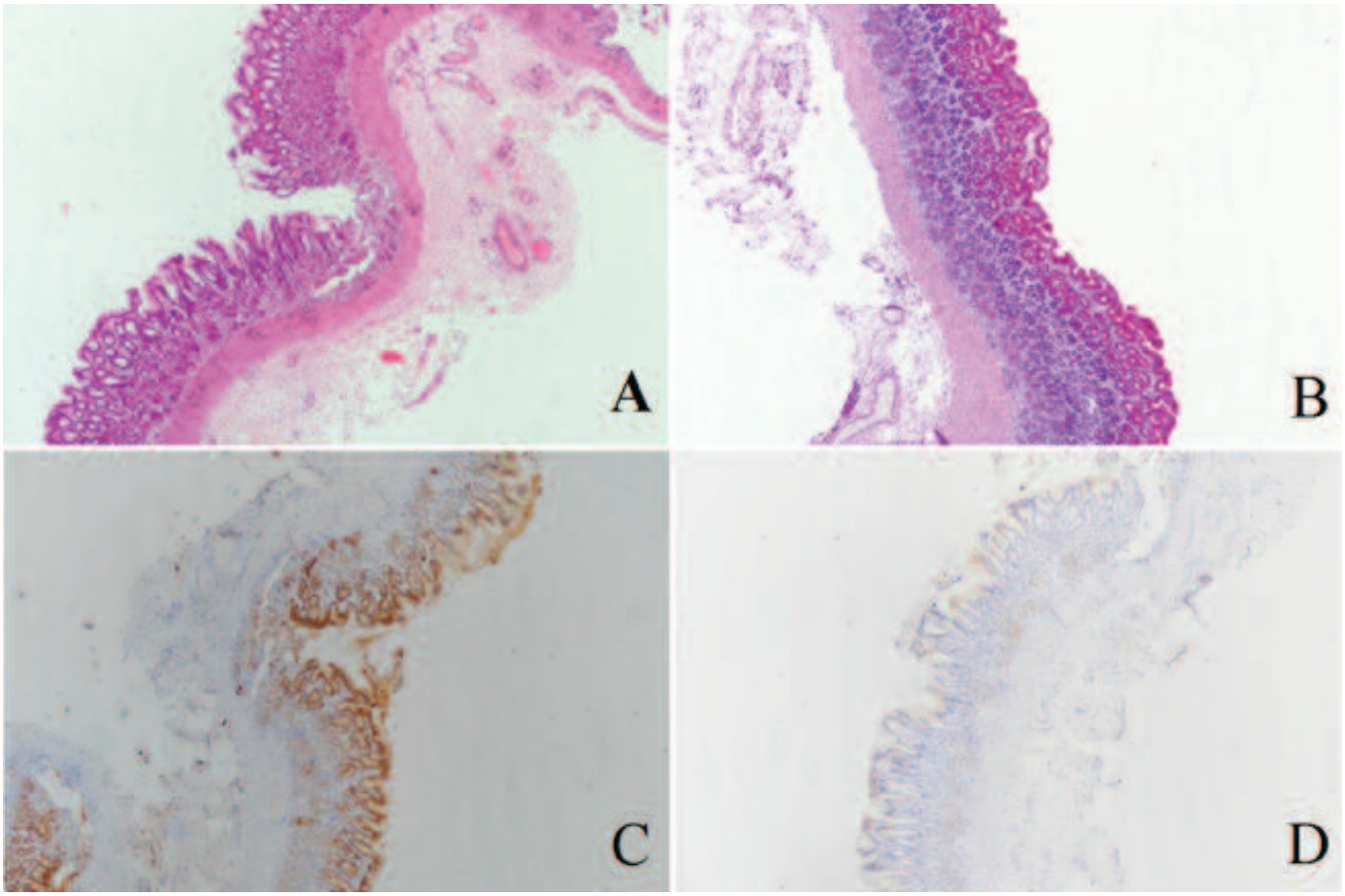

Figure 4. Histopathological appearance; (a) Gastric duplication wall comprised of all layers; mucosa, submucosa, muscularispropria and serosa (Hematoxylin and Eosin, X40), (b) Mucosa was predominantly of gastric type (pink colored) with some cells containing apical intestinal mucus (blue colored) (Periodic acid-Schiff-alcian blue staining, X40), (c) CK7 positive mucosal epithelium (CK7 immunohistochemical staining, X40) and (d) CK20 positive mucosal areas (CK20 immunohistochemical staining, X40).

neuroectoderm, theory of split notochord and abortive attempts of twinning [1-3]. Most commonly accepted theory is "split notochord syndrome" to explain the development of this congenital anomaly. In this theory, persistence of ectoendodermal connection that has developed at an early embryonic stage causes diverticular elongation of the gut and leads to the formation of duplication cysts [6]. In previously reported gastro duodenal duplications with communication to the pancreatic ducts, it has been proposed that the dorsal traction onto the portion of the foregut that gives rise to the dorsal pancreatic bud might represent the initial trigger for formation of the cyst [6]. Indeed, as previously reported, connections between GD cyst and pancreas well described. To our knowledge, the present case is the first case report of a connection with both the pancreas and spinal cord as well. It also is a valuable case to prove split notochord theory.

The management of GD is essentially surgical. In GD cyst, the treatment procedure is resection without injury to the lumen of the stomach. No communicated GD cysts are classically treated by complete excision of cyst and resection of common wall between stomach and cyst with mucosal stripping. In the communicating GD cyst drainage and marsupialization of the cyst have been suggested. Gastrostomy or even a feeding jejunostomy may be helpful according to patient position [1, 4]. Furthermore, leaving the residual tissue of cyst wall is given potential risk for inflammatory complications and malignant transformation [2, 4, 7]. In presented case we performed totally cystic excision with mucosal stripping at two points in common wall.

In asymptomatic or untreated patients, such as infection, gastrointestinal bleeding, ulceration or perforation may be occur because of ectopic gastric mucosa, also may lead to pancreatitis and mimic a pancreatic pseudo cyst due to ectopic pancreatic tissue [4]. In addition, duplication cysts have the potential risk for neoplastic transformation especially adenocarcinomas which has been reported [4, 8, 9].

In histopathological evaluation, most of GD cyst contains gastric mucosa with smooth muscles. In addition, up to 10% gastric duplications may contain ectopic pancreatic tissue [2, 4, 7]. In our case cyst surface was covered with gastric mucosa, and smooth muscles in the wall.

Conclusion

This rare entity should be included in the differential diagnosis of cystic masses of the gastrointestinal tract. US and CT facilitates the diagnosis. This case emphasizes that the GD cyst should be treated surgically by complete resection due to the risk of malignant transformation and other complications. According to the literature and with the results of the present report, this is the first report to demonstrate a communication between both the pancreas and spinal cord, and it would help to explain the development of GD cyst by the split notochord theory.

Informed consent

Written informed consent was obtained from the patient for the publication of this case report.

Conflict of interest

The authors declared that there are no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Acknowledgement

This paper was presented previously at the 34th Congress of Turkish Pediatric Surgeons, October 26-30, 2016, Kyrenia, Cyprus (TRNC).

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The European Research Journal
http://www.eurj.org
Case Report
e-ISSN: 2149-3189
DOI: 10.18621/eurj.315130

Superior vena cava syndrome after creation of arteriovenous fistula

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ABSTRACT

Patients with end-stage renal disease require vascular access for hemodialysis. Repeated insertions and long-term duration of permanent central venous catheters may cause venous stenosis and thrombosis in central veins, which may result in superior vena cava syndrome. A 38-year-old male patient with end-stage renal disease was admitted with complaint of dyspnea, edema of the face, neck and bilateral upper extremities. We had created a radiocephalic arteriovenous fistula at the level of left wrist two weeks ago, he had a permanent catheter in the right internal jugular vein for 2 months. The Doppler ultrasound and venographic examinations of central veins showed that the permanent catheter in the right internal jugular vein had caused the edema of the neck, face and bilateral upper extremities by narrowing the superior vena cava. The catheter was removed and the patient was anticoagulated. The patient's symptoms of shortness of breath were alleviated on hospital discharge and the swelling of the upper extremities diminished after one week. The hemodialysis patients should be directed to arteriovenous fistula surgery instead of central venous catheterization as soon as possible to avoid superior vena cava syndrome due to central venous catheters.

Eur Res J 2017;3(3):281-284

Keywords: Superior vena cava syndrome, central venous catheter, central venous stenosis, arteriovenous fistula, end-stage renal disease

Introduction

The obstruction of superior vena cava by thrombosis or external compression may cause superior vena cava syndrome (SVCS) [1]. The most common reason of external compression is intrathoracic neoplasm [2] whereas implantable central venous access devices and permanent catheters are the most common reasons of venous thrombosis [1, 2]. When the obstruction begins within a chronical process, the collateral veins improve and the obstruction symptoms may be absent. However, the acute obstruction does not allow compensatory changes and results into SVCS which is defined as critical congestion that leads to edema on the face, neck, upper extremities and even to cerebral edema and herniation [2]. Vascular access for hemodialysis with a temporary or a permanent catheter is a well-

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Received: May 21, 2017; Accepted: August 8, 2017; Published Online: August 12, 2017

known etiological risk factor for thrombosis. During insertion of the catheter, vascular endothelium is traumatized and it causes inflammation and coagulation finally it may cause intravascular thrombosis [3].

Arteriovenous fistula (AVF), as described by William Hunter in 1757 is an anomalous communication of the artery and the vein. An AVF may be related to trauma or surgical procedures also it may be congenital whereas it is created for vascular access in hemodialysis patients on purpose [4, 5]. However proximal vessel dilatation, pseudoaneurysm formation, rupture of fistula, cardiac overload and cardiac failure are some of the complications of AVFs [4].

Case Presentation

A 38-year-old male hemodialysis patient was hospitalized for creation of an AVF for long-term vascular access. He had a permanent catheter in the right internal jugular vein and a history of repeated central venous catheterization of bilateral subclavian and internal jugular veins. The size of the cephalic vein and the radial artery were sufficient at the level of wrist of the left upper extremity in the ultrasonographic imaging. A radiocephalic AVF was created with an end-to-side anastomosis technique with 7/0 polypropylene suture under local anesthesia. There was thrill on the location of created AVF. Two weeks after the operation the patient presented with progressive dyspnea and edema on the face, neck and bilateral upper extremities (Figure 1).

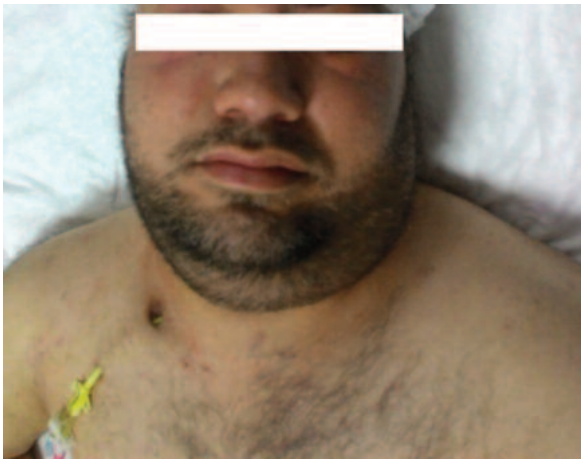

Figure 1. The edema at the face and neck. It can be seen especially at around the eyes.

The upper extremity veins were dilated bilaterally. He had tachypnea, tachycardia and crepitate rales at both lungs on admission. Doppler ultrasound and venography was performed. According to the doppler ultrasound findings, AVF and the related cephalic vein was patent and 272 ml per minute flow measured. However venography showed that there was a narrowing starting from right internal jugular vein and continuing to the superior vena cava, due to the permanent catheter at this side (Figure 2).

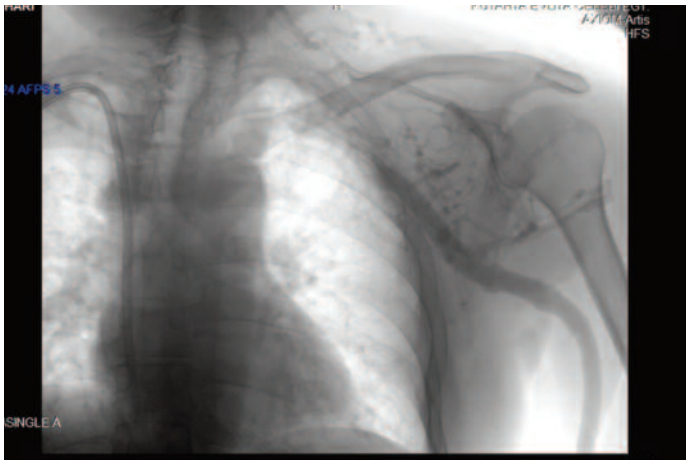

Figure 2. Permanent hemodialysis catheter, narrowed superior vena cava and presence of collateral veins.

Also mild stenosis could be seen at left side and collateral veins possibly due to the previous catheter insertions (Figure 3).

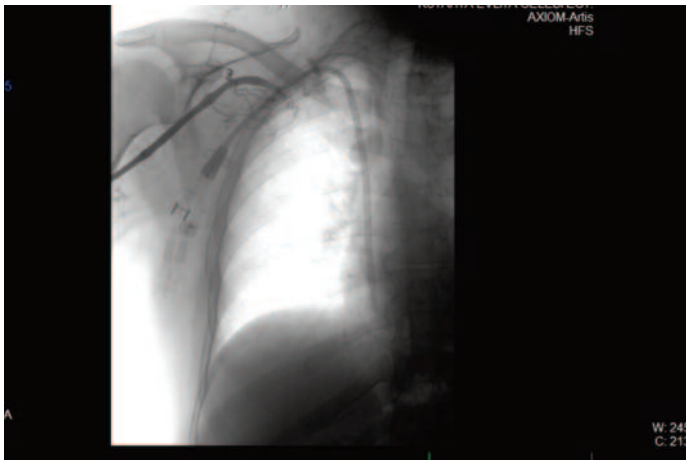

Figure 3. Venogram shows collateral veins with severe stenosis of the subclavian vein.

The flow were more than enough and the vessels had stenosis. These both conditions together caused the complaints of the patient. The permanent catheter in the right internal juguler vein was removed and right femoral vein catheterized with temporary catheter. Anti-aggregant and anticoagulant treatment

was applied. Daily dosage of per oral 100 mg acetylsalicylic acid (ASA) and 100 IU/kg subcutaneous enoxaparin sodium therapy was given. For long term anticoagulant therapy warfarin sodium was applied per oral according to the international normalized ratio (INR) adjusted level. After the removal of the catheter there has been a decrease in the swelling dramatically thus we decided to apply only medical treatment and avoid any further intervention. At discharge after three days he did not have any dyspneic symptoms more and after one week the edema on the face, neck and upper extremities was nearly totally diminished (Figure 4).

Figure 4. The edema diminished competely.

Discussion

Catheter-related SVCS is not a rare complication. It may develop because of hypercoagulability or stenosis / thrombosis in the superior vena cava. The reason is important because the treatment is shaped according to the reason. Removal of the catheter, anticoagulant, even thrombolytic treatment, percutaneous intervention could be the suitable treatment [6, 7]. In our case patient relieved by removing the catheter so no any other procedure needed.

Central venous catheters are related to thrombosis, infections, bacteremia, SVCS and pneumonia [8]. There are many kinds of vascular access devices but the complications related to hemodialysis catheters (HD) are more often. Particularly permanent catheters have a long term duration and a great internal lumen width which may facilitate thrombosis and stenosis of the vessel. There are also patient related factors such as venipuncture trauma, vein caliber, thromboembolic event history, malignancy and hypercoagulative diseases [8].

According to the recommendations of American College of Chest Physicians for the treatment of catheter-associated thrombosis; catheter should be removed and the patient should be undergone anticoagulant treatment at least for three months [8]. But if the catheter is functional and cannot be removed anticoagulant treatment should be continued as long as catheter remains [8].

By the technological developments new treatment methods such as thrombus aspiration, mechanical thrombolysis, transluminal balloon angioplasty and venous stenting may be applied in appropriate patients [9]. Our patient's compliations were significally reduced after removing the catheter and the AV fistula was still working well. So percutaneous intervention not considered.

Conclusion

Patients with end-stage renal disease are at risk of SVCS and pulmonary thromboembolism due to catheter-related thrombosis. These are life-threatening complications. Also repeated vascular interventions may be harmful for central venous structures even if there is not intravascular clotting. We think that hemodialysis patients should be directed to AVF creation as soon as possible and the patients should be prepared for an AVF from the time of diagnosis for avoiding multiple catheter insertions. Particularly autogenous AVF is the most comfortable and safe way of hemodialysis vascular access.

Informed consent

Written informed consent was obtained from the patient for the publication of this case report.

Conflict of interest

The authors declared that there are no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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The European Research Journal
http://www.eurj.org
Case Report
e-ISSN: 2149-3189
DOI: 10.18621/eurj.304373

Severe bone marrow suppression following single-dose methotrexate treatment for ectopic pregnancy

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ABSTRACT

Methotrexate is associated with multisystem side effects which depend on the dosage, route of administration and length of treatment. Bone marrow suppression is a very rare but one of the most mortal complications. A case of severe bone marrow suppression following single dose methotrexate for ectopic pregnancy was presented in this study. A 33-year-old woman, diagnosed as ectopic pregnancy, was given single-dose intramuscular methotrexate of 50 mg/m². On 10th day patient was admitted with lethargy, irritability, vomiting, hematemesis, diarrhea, oral mucositis and skin rash. Laboratory findings revealed severe bone marrow suppression. Supportive treatment together with antibacterial/ antifungal drugs and granulocyte colony stimulating factors were administered. On the 3rd day of treatment, mucositis and oral intake began to improve. After 1 week complete blood count was normal. Methotrexate may have mortal clinical consequences even with very low doses. Therefore, a careful patient history for patient selection and closer clinical follow-up are essential.

Eur Res J 2017;3(3):285-289

Keywords: Bone marrow suppression; ectopic pregnancy, methotrexate, side effects, single-dose regimen

Introduction

Ectopic pregnancy is defined as implantation of blastocyst somewhere other than uterine cavity with an incidence of 1-2% [1]. The treatment modalities of ectopic pregnancy vary from expectant management to radical surgery. Methotrexate (MTX) is the most commonly used agent in medical treatment of ectopic pregnancy [2].

MTX, which is an antifolate cytotoxic drug inhibiting cellular proliferation, can be used as a chemotherapeutic or immunosuppressive agent in several conditions such as leukemia, lymphomas, lung, breast and bladder cancer, multiple sclerosis, rheumatoid arthritis, Crohn's disease. In daily gynecologic practice, it is most commonly used in

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Received: April 6, 2017; Accepted: August 24, 2017; Published Online: August 29, 2017

treatment of ectopic pregnancy and gestational trophoblastic neoplasia [3]. Although the indication of MTX treatment has a broad spectrum, it has several side effects some of which are mortal.

During MTX treatment, mucositis which is generally associated with gastrointestinal symptoms such as nausea and vomiting, and hematological side effects are quite common. In addition to these, pneumonia, pleural effusion, pericarditis and pericardial effusion arising from serositis could be encountered during treatment. However, bone marrow suppression is a very rare but one of the most mortal complications [3, 4]. The side effects of MTX depend on the dosage, route of administration and length of treatment period.

In this study, we presented a case of severe bone marrow suppression following single dose of MTX treatment for ectopic pregnancy.

Case Presentation

A 33-year- old, gravida 2 parity 2 women with a history of cesarean section and with no other medical or gynecologic history was admitted to emergency room with secondary amenorrhea and vaginal bleeding. In general physical examination, there was minimal tenderness on right adnexal area. In gynecologic assessment, there was minimal vaginal bleeding and painful cervical movements. In transvaginal ultrasonography, endometrial thickness was 8 mm and there was 15 x 13 mm hyperechogenic mass not including fetal nod which is coherent with ectopic pregnancy at right adnexal area. Serum beta-HCG level was 1,374 mIU/ml. During clinical follow up, the serum beta-HCG value at 48th hour was 1,532 mIU/ml. In detailed laboratory assessment it was found that white blood count (WBC): 4,200/mm^3, neutrophil: 2,300 x 1,000/mm^3), platelet (PLT): 295,000/mm^3, hemoglobin (Hb): 11.4 g/dl, hematocrit (HCT): 33.7 (%), MCV: 92.2 fL, vitamin B_{12}: 327 pg/mL, folic acid: 21 , alanine aminotransferase: 18 IU/L, aspartate aminotransferase: 16 IU/L, creatinine: 0.8 mg/dL, urea: 17 mg/dl, INR: 1.3 and APTT: 36 min. No pathological finding was observed in chest x-ray. She was hospitalized with the diagnosis of ectopic pregnancy. Since it is not recommended to use antibiotics in patients without any risk factors, we did not give any antibiotics. The patient, who did not have any contraindications for MTX treatment, was given single-dose intramuscular MTX of 50 mg/m^2 for ectopic pregnancy. After 24 hours following MTX dose she developed severe nausea and vomiting unresponsive to metoclopramide and ranitidine hydrochloride.

On the 1st, 4th and 7th days of MTX treatment, beta-HCG was 1,560, 1,645 and 1,905 mIU/ml, respectively. Because of increasing serum level of beta-HCG, a second single dose of MTX was planned but because of patient's severe nausea and vomiting unresponsive to medical treatment and development of acute abdomen without any clinical and/or sonographic findings of acute hemorrhage, diagnostic laparoscopy was performed. In abdominal exploration, a 20 x 10 mm ectopic mass was observed in the ampulla of right uterine tube and right salpingectomy was performed. On the 1st postoperative day, patient was discharged without any early complications. After two days, patient was admitted to hospital with fever, severe vomiting accompanied by hematemesis, diarrhea, lethargy, irritability and confusion. Patient's vital findings were as follows: arterial blood pressure was 100/70 mmHg, heart rate was 80 beats per minute and body temperature was 38^{o}C. In physical examination the skin was dry. There were multiple eruptions particularly located on the trunk and pigmentation of face, subconjunctival hemorrhage and diffuse oral mucositis disturbing oral intake. She was suffering from oliguria and hematuria. The reason of hematemesis and diarrhea are explained by diffuse mucositis affecting gastrointestinal tract including oral mucosa, which consequently results in dehydration. As the severity of diffuse mucositis increases dehydration worsens gradually and at the end symptoms like lethargy, irritability and confusion arise. Mucositis is not only associated with gastrointestinal tract. Also, urinary tract mucosa is affected in case of MTX toxicity. Together with mucositis affecting urinary tract severe dehydration are most possible underlying reasons for hematuria. Respiratory and cardiovascular system examination did not reveal any pathological findings. Abdominal examination revealed diffuse abdominal tenderness accompanied by hyperactive bowel sounds.

In laboratory assessment it was found that WBC:1,200/mm^3, neutrophil: 300x1,000/mm^3, PLT: 200,000/mm^3, Hb: 10.3 g/dl, HCT: 31%, alanine aminotransferase: 17 IU/L, aspartate aminotransferase: 13 IU/L, creatinine: 0.9 mg/dL, urea: 11 mg/dl, INR: 1.1 and APTT: 32 min, CRP: 10 mg/L. No pathological finding was observed in electrocardiography and chest x-ray. She was

hospitalized with the diagnosis of suspected bone marrow suppression.

Since, the patient's oral intake was disturbed intravenous fluid, total parenteral nutrition was given as supportive treatment. Urine and gaita samples were collected for direct examination. After collecting samples for cultures, prophylactic antibiotherapy protocol composed of ciprofloxacin 400 mg; 2x1, amoxicillin/clavulanic acid 1 gr; 2x1, fluconazole 150 mg; 1x1 were started. As palliative treatment folinic acid 50 mg; 1x1, antifungal and analgesic containing oral suspensions were added. Direct examination of urine and gaita and cultures did not reveal any pathological findings. For neutropenia, filgrastim (recombinant methionyl human granulocyte-colony stimulating factor) 300 mcg; 1x1 was given for 4 days. The complete blood count and CRP values of the patient during filgrastim treatment were given in Table 1. As the patient's monocyte count tended to increase patient's mucositis clinic was began to resolve and patient's oral intake returned to normal.

The reason for using monocyte count in clinical follow up is that our patient was mainly suffering from diffuse mucositis affecting all gastrointestinal tract including oral mucosa and monocyte count closely correlates with the recovery of oral lesions. Therefore, we mainly followed monocyte count to determine clinical recovery of most of the symtoms which were associated with oral intake and dehydration.

The patient was discharged from hospital and after one week following hospital discharge, laboratory findings of patient were completely normal.

Discussion

The treatment modalities currently being used in clinical management of ectopic pregnancy are composed of expectant management, medical treatment and surgical approach. Today in medical treatment of ectopic pregnancy, considering its cost effectiveness and opportunity of fertility preservation, MTX has a well-proven role if patient is selected appropriately.

MTX can be administered as single-dose, double-dose or multi-dose therapy. Stovall et al. [5] were the first researchers to explore the consequences of MTX therapy in treatment of ectopic pregnancy and they reported a cure rate of 96.7% with single-dose regimen. Their single-dose therapy and monitoring protocol is being currently used worldwide by clinicians. Following administration of a single-dose intramuscular MTX of 50 mg/m^2, serum beta-HCG levels are monitored by measurement of its blood levels on the 1st, 4th and 7th days and weekly until complete recovery. Determination of ≥ 15% of decrement in serum beta-HCG levels between 4th and 7th days of MTX administration is accepted as successful with a positive predictive value of 93%, a sensitivity of 93% and a specificity of 84.2% [6]. If the expected decline does not occur, another MTX dose of 50 mg/m^2 can be given intramuscularly [7]. It was reported that nearly 20% of patients require additional MTX dose(s) to reach the expected serum beta-HCG levels.

However, to have successful results with MTX treatment appropriate patient selection is essential. Treatment failure is not uncommon if the upper limit of pretreatment serum beta-HCG level exceeds 3,000-5,000 IU/L and/or if there is a gestational sac larger than 3-4 cm in size and/or a fetal node with cardiac activity [3, 7].

In our case the patient who did not have any contraindications for MTX therapy with a pretreatment serum beta-HCG level of 1,532 IU/L was given a single-dose intramuscular MTX of 50 mg/m^2.

Table 1. Complete blood count and CRP values during filgrastim treatment.

Day of treatment	Hb (g/dl)	MCV (fL)	Monocyte (/mm^3)	NEU (x1000/mm^3)	WBC (/mm^3)	PLT (/mm^3)	CRP (mg/L)
1st	9.8	87.4	0.21	700	1,600	100,000	14
2nd	9.3	86.5	0.18	500	1,700	70,000	15
3rd	8.3	82.4	0.25	900	1,830	50,000	14
4th	8.1	88.4	0.37	1,100	2,100	180,000	17

Hb=hemoglobin, MCV=mean corpuscular volume, WBC= white blood count, NEU= neutrophil, PLT=platelet, CRP=C-reactive protein

Serum beta-HCG level was monitored on the 1st, 4th and 7th days of MTX administration. Serial serum beta-HCG measurements revealed an increase whereas it was expected to decrease. Because of increasing serum beta-HCG levels a second-dose MTX was planned. But since the patient had severe nausea and vomiting unresponsive to treatment and developed acute abdomen, laparoscopic exploration was preferred instead of giving a second-dose MTX.

MTX, which competes with folinic acid functioning in DNA synthesis and cell proliferation, prevents division of cytotrophoblasts resulting in regression of ectopic pregnancy tissue [8]. Although, life-threatening complications of MTX treatment are not very common, because of its non-specific effects on cell division, MTX toxicity may cause severe clinical consequences. Common side effects were reported to be observed in less than 10% of cases [9]. MTX particularly affects rapidly dividing cells of hematological, gastrointestinal and dermatological systems. As a consequence, most frequently reported signs and symptoms are neutropenia, generalized myelosuppression, nausea, vomiting, diarrhea, gastrointestinal inflammation, generalized erythema, rash, photosensitivity and alopecia [3]. Although, it was reported that side effects were dose-dependent and increased as the duration of treatment increased, in the present case the side effects were developed following only a single-dose MTX of 50 mg/m^2. She suffered from generalized myelosuppression, ulcerative stomatitis, gastrointestinal inflammation and generalized erythema.

The underlying mechanism of toxicity may be methylenetetrahydrofolate reductase (MTHFR) 2 mutations resulting in hypersensitivity of MTX, gene polymorphism in the folate metabolic pathway, decreased renal excretion due to any reason such as dehydration, ingestion of cola containing drinks and coincidental use of some drugs [9-11].

After our patient developed the side effects associated with MTX her family members gave us the information that there were people in their village who had once used MTX for several reasons such as malignancies and rheumatologic diseases and developed mortality and morbidity because of associated complications following treatment. For that reason, in the present case we thought that the probable cause of toxicity may have a genetic basis.
In this case, we gave folinic acid and neupogen together with antibiotic and antifungal agents. Our patient relieved clinical signs and symptoms of toxicity on the 4th day of treatment and she became normal as her oral intake increased.

In the literature, we came across only one patient who developed life-threatening neutropenia following single-dose MTX therapy for ectopic pregnancy [12]. In spite of the fact that side effects were reported to be as rare as 2%, its consequences may be life-threatening and even may occur with low doses of MTX for non-malignant conditions [3]. Strategies, like measuring blood levels of MTX during treatment, considering plasma kinetics of MTX may be helpful in preventing its toxicity and improving safety [13].

Conclusion

In conclusion, since MTX metabolism is prone to be affected by several conditions and its blood levels can vary because of any intervening factors, a careful patient history for a better patient selection and closer clinical follow-up are essential. It should be better to keep in mind that although, its side effects are quite rare they may have mortal clinical consequences even with very low doses given for treatment of benign conditions.

Informed consent

Written informed consent was obtained from the patient for the publication of this case report.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of interest

The authors declared that there are no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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The European Research Journal

http://www.eurj.org

Case Report

e-ISSN: 2149-3189

DOI: 10.18621/eurj.310619

Successful management of bupropion poisoning: Possible benefit of acidosis treatment

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ABSTRACT

Bupropion is used as an antidepressant and smoking cessation aid. It has been described in the literature as a norepinephrine-dopamine reuptake inhibitor and is also a nicotinic antagonist. Bupropion itself is not a common cause of intoxication cases seen in Emergency Department (ED). The most important side effect is an increase in risk for epileptic seizures. We aimed to present the management of a 29-year-old female patient in ED with epileptic seizures and acidosis due to bupropion intoxication, who had taken 30 bupropion 150 mg tablets and 16 fluoxetine 20 mg tablets for suicidal purpose. According to her blood gas counts sodium bicarbonate therapy was begun in ED and after having response to the treatment was continued in intensive care unit. She was discharged three days later without any other seizures and complications. Although bupropion intoxication is rare it can cause severe metabolic acidosis and epileptic seizures. There is no specific antidote therapy to bupropion so symptomatic therapy still have benefit.

Eur Res J 2017;3(3):290-294

Keywords: Bupropion intoxication, fluoxetine intoxication, seizure, acidosis

Introduction

Bupropion is a unicyclic aminoketone antidepressant, which is lipophilic at a high rate. Bupropion is a selective reuptake inhibitor of noradrenaline, dopamine and serotonin [1, 2]. Bupropion is currently used in the treatment of major depression and smoking cessation [3]. In addition to being effective in depression treatment. Bupropion was approved by the Food and Drug Administration (FDA) in 1997 for smoking cessation treatment, and was the first non-nicotine pharmacological agent [3]. Bupropion lowers the seizure threshold [1, 4]. Therefore, bupropion can cause seizures at therapeutic doses. Seizures are thought to be the most likely side effects in bupropion overdose [5]. In cases of overdose of bupropion, acidosis treatment may be effective [6]. The main recommendation in most reported cases is that early recognition and treatment of shock and metabolic acidosis together with the intensive care therapy of the neurological effects are essential. Our recent experience of a 29-year-old female who had ingested 4500 mg of bupropion and expected to have a fatal outcome, showed that early gastric lavage with serum physiologic solution, oral activated charcoal and intravenous sodium bicarbonate treatment contributed to survival.

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Received: May 5, 2017; Accepted: October 18, 2017; Published Online: October 25, 2017

Case Presentation

A 29-year-old female was found unconscious at home by her husband. While being brought to hospital, she experienced a generalized tonic clonic seizure in the ambulance. 10 mg midazolam was given in ambulance Intravenously. The history revealed that 2.5 months previously, the patient had consulted a psychiatrist because of complaints of introversion, insomnia and lack of enjoyment of life and treatment was started of slow-release bupropion 150 mg/day and fluoxetine 20 mg/day. It was reported that 2 hours before the patient was found unconscious at home by her husband, she had been normal. From the history supported by the empty medicine packages brought by her husband, it was determined that approximately 3 hours previously, the patient had taken 30 bupropion tablets plus 16 fluoxetine (20 mg) and 10 tablets containing alverine citrate and simethicone. On presentation, the blood pressure measurement was 100/60 mmHg, pulse was 143/min, temperature was 36.6˚C and SO2: 99%. ECG was consistent with sinus tachycardia. Gastric lavage and active charcoal were applied. The patient arrived in the Emergency Department (ED) at 05:02 and the first blood gas was taken at 05:11. Meanwhile, the patient experienced a generalized tonic clonic seizure. Diazepam IV 10 mg was applied at the dose of 0.15 mg/min/kg in bolus form and 18 mg/kg phenytoin. Blood gas results were determined as pH: 7.00 and HCO3: 10.6. As the patient had metabolic acidosis, firstly 100 meq NaHCO3 (2 meq/kg) was administered IV and a maintenance dose of 120 meq NaHCO3 IV infusion was administered in 6 hours. A total of 2000 cc 0.9% NaCl was administered to the patient.

During the treatment, the patient experienced a second seizure at 07:15 and a second 10 mg IV diazepam bolus was administered. Under observation in the ED, a third seizure occurred at 11:30. A total of five seizures had the patient experienced, three of those were in ED. Detailed seizure intervals are on Table 1. The patient was observed neurologically normal after seizures and any kind of respiratory distress did not occur. Blood gas values taken at 18:03 and subsequently were seen to be within the normal range (Figure 1). No other seizure occurred during follow-up of the patient.

No pathology was observed on computed brain tomography. No abnormality was determined in the full blood count or biochemical parameters. It was thought that the generalized tonic clonic seizures of the patients could be associated with the slow-release Bupropion and fluoxetine. The patient responded to the NaHCO3 treatment given in the ED and was admitted to the Internal Medicine Intensive Care Unit. On the 3rd day, the patient was discharged. During a 3-month follow-up period, the patient experienced no further seizures. On the magnetic resonance imaging (MRI) and electroencephalography (EEG) follow-up examinations, the results were normal. We obtained written informed consent from the patient.

Table 1. Seizure, location, happening time and blood gases results.

Seizure	Location	Time	Time to take Blood gases	pH
1st	Home	04:30 AM Patient was found fainted. According to her husband she has a tonic clonic seizure.	-	Unknown
2nd	Ambulance	04:48 AM According to ambulance health worker, she has a tonic clonic seizure.	-	Unknown
3rd	ED	05:11 AM Generalized tonic clonic seizure	07:11 AM	7.02
4th	ED	07:15 AM Generalized tonic clonic seizure	07:24 AM	7.17
5th	ED	11:30 AM Generalized tonic clonic seizure	11:43 AM	7.18

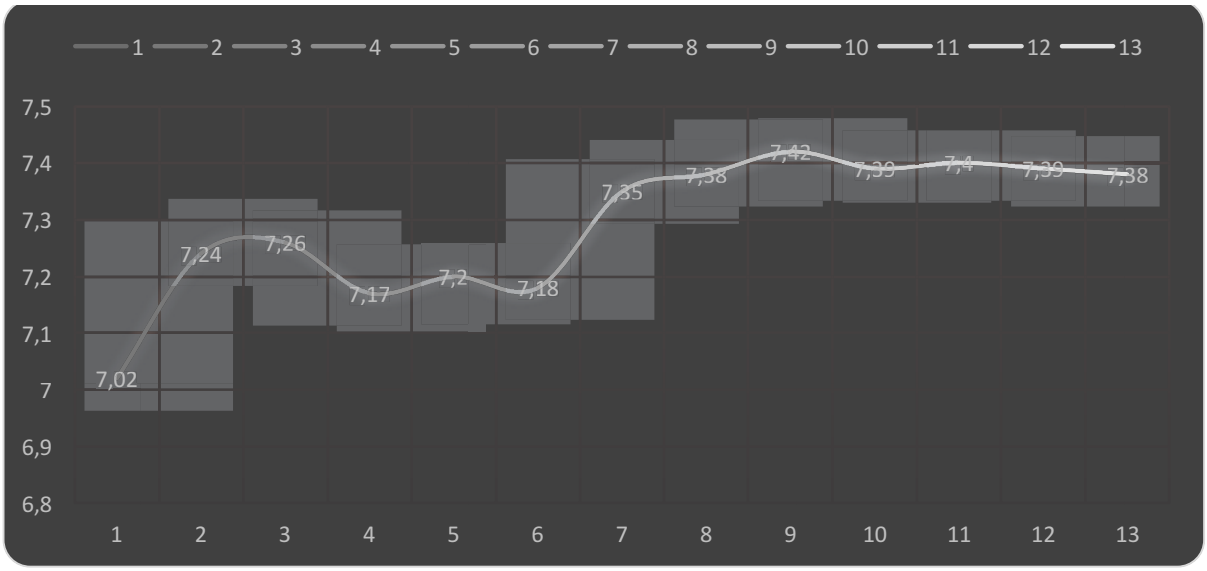

Figure 1. The pH (power of hydrogen) curve of the blood gases taken during the follow-up of the patient. The figure was formed from the results of 13 blood gases taken during follow-up.

Discussion

The transformation of bupropion to the active metabolite hydroxy bupropion has been shown to be related to cytochrome CYP2B6 [3]. There is a potential interaction between bupropion and drugs affecting CYP2B6 isoenzyme (carbamazepine, rifampicin) [3]. Bupropion also inhibits the anti-arrhythmic and anti-psychotic metabolizing CYP2D6 isoenzyme activity of some antidepressants (tricyclic and SSRI) [3, 7]. Care must be taken with the use of these drugs and bupropion together, and when they are used together the dose should be kept in the possible lowest dose. The current case had taken these two drugs together with the aim of committing suicide. As a result of a seizure, the patient was taken to the ED. Patients presenting at the ED because of seizure must be questioned in respect of medications used and overdose. In these types of cases, blood gas measurements are extremely helpful.

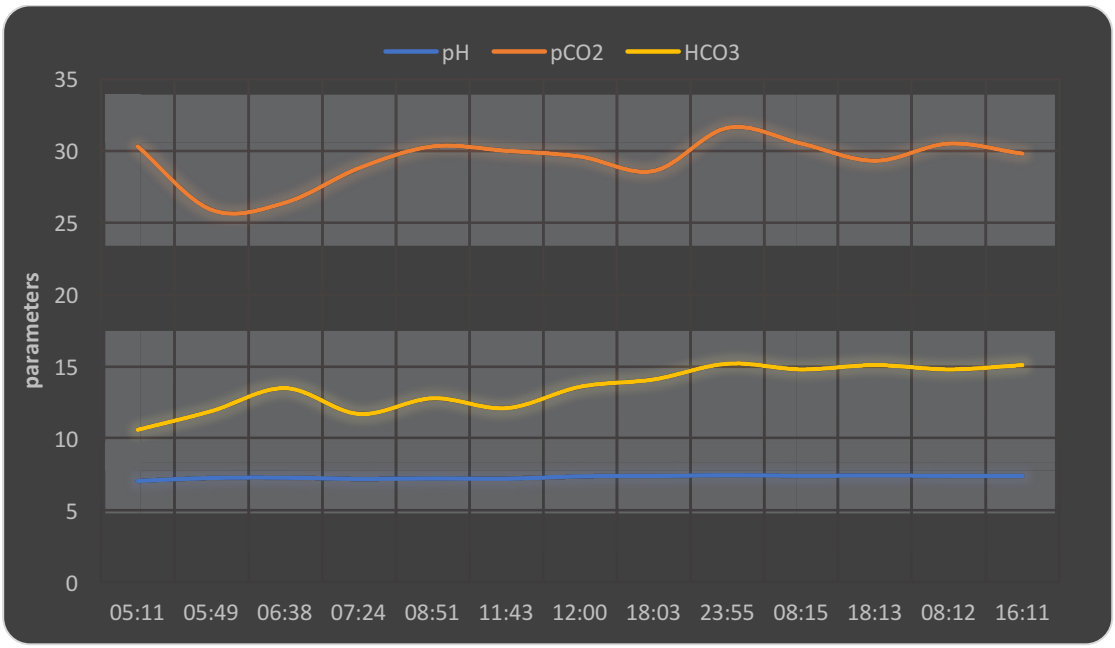

Figure 2. The times at which blood gas measurements were taken and the changes during the follow-up of the patient. The figure was formed from the results of 13 blood gas taken during follow-up. (pH: Power of hydrogen, pCO_2: partial pressure of carbon dioxide, HCO_3: bicarbonate.). Time at blood gases were measured; (First day; 05:11, 05:49, 06:38, 07:24, 08:51, 11:43, 12:00, 18:03, 23:55), (Second day; 08:15, 18:13), (Third day; 08:12, 16:11).

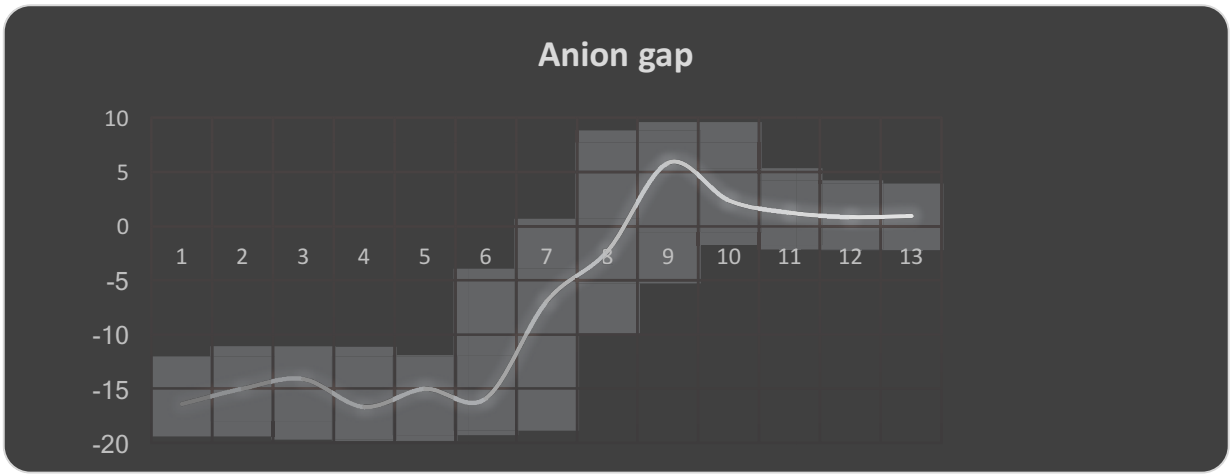

Figure 3. The base deficit curve in the blood gases taken during the follow-up of the patient. The figure was formed from the 13 blood gas results taken during follow-up.

The etiology of spontaneous seizures is not known completely. GABAergic mechanisms primarily have been invoked for the generalized inhibition [8]. Endogenous adenosine release and actions on purinergic receptors have been implicated as well [9]. One intriguing line of speculation involves modulation of ion channels by protons a notion supported by long-standing historical observations that neuronal activity and seizures can reduce brain pH [10].

Bicarbonate should be given at an arterial blood pH of ≤ 7.0. The amount given should be what is calculated to bring the pH up to 7.2. The urge to give bicarbonate to a patient with severe acidemia is apt to be all but irresistible. Metabolic acidosis is an acid-base disorder characterized by a primary consumption of body buffers including a fall in blood bicarbonate concentration. The optimum extracellular pH for all physiologic mechanisms and organ functions is 7.4. By contrast, intracellular pH is approximately 7.1 in virtually every tissue studied. Many diverse mechanisms are in place to maintain both extracellular and intracellular pH within this very narrow range. Deviations from normal pH will obviously decrease the efficiency of all reactions, although the degree will vary depending on the specific event. For example, whereas acidemia protects the central nervous system against seizures, it sensitizes the myocardium to arrhythmias. Because we do not measure intracellular pH, we have to use extracellular pH (arterial or venous) as a surrogate. Most authorities in acid-base physiology would give bicarbonate to a patient with an arterial pH < 7.1. In some patients, only a small amount of bicarbonate may be required [11].

In the current case, it was determined that all the seizures occurred when the pH value was < 7.2 (Figures 1 and 2). In cases of deep acidosis, the necessary treatments should be given without any loss of time. It is extremely important that repeated blood gas measurements are taken during follow-up. Not only the pH value, but also the pCO2, base deficit and HCO3 levels are helpful in the follow-up of treatment [3]. In cases of Bupropion overdose, acidosis treatment can be useful [6]. It was also seen in the current case that with an improvement in the base deficit, no further seizures occurred (Figure 3).

At approximately 3 hours after ingestion, bupropion reaches an effective plasma concentration and the elimination half-life is approximately 21 hours [1, 12]. Bupropion maximum plasma concentration of around 140 g/L is reached approximately 3 hours after oral ingestion of 150 mg. In a study by Spiller et al. [13] of 4 autopsy cases, the bupropion level was found to be 3.1-20 mg/L.

In terms of the patient prognosis, the time from taking the drug to arrival at the ED is extremely important. The current case presented at the ED after 3 hours. Bupropion is dependent on plasma proteins at the rate of 85% [3, 14]. The therapeutic treatment margin of bupropion is very narrow. In 21% of patients who have taken a high dose, seizure activity is observed [12]. As this side-effect is related to the pharmaceutical form of the drug and the dose, the slow-release form was produced in 2003 to be able to reduce this side-effect [7, 14]. It has been reported in literature that seizures are very rarely seen with this form. When necessary, follow-up should be made in intensive care units until stabilization of seizures is achieved. In the current case, follow-up was applied for 2 days in intensive care and for one day in the clinic. Intensive care stays preferred due to observe possible aspiration risk during metabolic acidosis. The patient at last was removed to clinic and then was

discharged the next day.

The most frequently seen side-effects of bupropion are insomnia (34%-42%), headache (26%) and mouth dryness (10%) [1, 14]. Rashes, nausea, excessive sweating, tinnitus and hypertension (especially in patients with underlying hypertension) are also seen [15]. The use of bupropion is contra-indicated in those using monoamine oxidase inhibitor (MAOI) and in those with anorexia or bulimia, head trauma or a family history of epileptic seizures [14]. In our case, we used to benzodiazepines for treatment seizures. Because; benzodiazepines are the first medications to be used and are the seizure of treatment. They function by stimulating GABA receptor subunits. This leads to the inhibition of chloride through the channel with neurotransmission induced hyperpolarization of resting membrane [16]. At high levels, benzodiazepines function in similar to phenytoin [16].

Due to the rapid and continuous release form of bupropion, there is a high rate of correlation between the dose and risk of seizure. In study of mice by Silverstone et al. [17], seizures occurred in 40 of 120 mice. There is the potential to reduce the seizure threshold associated with the dose by combined use with tricyclic antidepressants (TCA) and MAOI [17]. When prescribing these drugs, other drugs with which there can be an interaction must not be prescribed. In patients with the potential for suicide, care must be taken in the use for smoking cessation.

Conclusion

Although bupropion intoxication is a rarely encountered event, it can lead to severe metabolic acidosis and seizures. As there is no specific antidote treatment, a symptomatic approach provides benefit to these patients. It is important to monitor the patient's blood gases. Bicarbonate treatment is effective for patients who have experienced a seizure. In literature, benefit has been shown from lipid treatment in bupropion intoxication. With this case report, by showing that bicarbonate treatment could be useful in bupropion intoxication, it was aimed to make a contribution to literature. There is a need for further research to be able to fully explain the effects on the brain.

Informed consent

Written informed consent was obtained from the patient for the publication of this case report.

Conflict of interest

The authors declared that there are no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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