Acta Medica Marisiensis

Volume 63 | Number 3 | 2017

Contents

Acta Medica Marisiensis

Acta Medica Marisiensis (ISSN: 2068-3324) is the official publication of the University of Medicine and Pharmacy of Tirgu-Mures, being published by University Press, Tîrgu Mureş.

The journal publishes high-quality articles on various subjects related to research and medical practice from the all the medical and pharmaceutical fields, ranging from basic to clinical research and corresponding to different article types such as: reviews, original articles, case reports, case series, letter to editor or brief reports. The journal also publishes short information or editorial notes in relation to different aspects of the medical and academic life.

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The Dipper Status – Do We Really Need to Fight for It?

Marcel Perian*
Editor

The idea of 24 hours blood pressure monitoring started more than 20 years ago [1] using semi-automatic devices, but even today we still need to inflate a cuff and listen to the Korotkoff sounds or to assess the maximum expansion of the artery when the pressure inside the cuff equals the mean blood pressure (the oscillometric method, used in actual ABPM devices). It is generally accepted that ABPM is a useful technique to evaluate the efficiency of the antihypertensive therapy, but there are more and more discussions about the relation between non-dipper status and cardiovascular risk.

The "dipper" status is defined by the European Society of Cardiology as a minimum of 10% decrease in the blood pressure [BP] during the night [2]. Patients without this BP dip are considered "non-dippers", although more dipping profiles exist today (mild dippers, extreme dippers, reverse dippers, etc), even if those patterns are not easily reproducible [3]. Several published articles are suggesting that the non-dipper status is associated with increased cardiovascular risk [4, 5] especially in chronic renal disease patients [6] and some authors recommended changing the medication timing, in order to convert non-dipper patients to dipping status.

The HALT study showed that doxazosin administration at bedtime decreased the nighttime blood pressure by 12mm Hg in non-dippers and by 18mm Hg in risers ($p<0.05$) [7]. A dipper status can sometime be obtained using other medication too (dihydropyridines or ACEI by ex.). Diuretics will reduce the likelihood of nocturnal dipping, because the patient will be often going to the bathroom. Choosing a long-acting ACE inhibitor (perindopril, for example) can give good results, too. Unfortunately, people with severe chronic renal disease, with high cardiovascular risk, seem to be less able to convert to a dipper status [8]. There are also studies that show there is no significant effect related to bedtime dosing [9, 10]. Moreover, "inducing" a dipper status, without changing the original condition which led to the non-dipper status might not be very effective. Sleep disorders, obstructive sleep apnea are often associated with elevated blood pressure levels and the effective treatment of apnea often converts a non-dipper to a dipper status [11].

The use of commonly available ABPM devices has a few drawbacks, the patient may feel uncomfortable, especially during the night, the cuff inflation wakes him and it can lead to increased BP. In patients with arrhythmias, such as atrial fibrillation for example, we will often find inaccurate measurements and that is the reason why patients with AF have been excluded from all trials using ABPM and from validation protocols of ABPM devices [12].

So, if we have to answer to the question "Do we really need a dipper status", except for patients with chronic renal disease, where we have studies suggesting that there is a clear benefit, we really do not know. For the moment, The European Society of Cardiology does not suggest different therapies among dipping profiles. Moreover, it seems that a non-dipper status is not associated with structural cardiac alterations [13].

Conflict of interest

None to declare.

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Acta Medica Marisiensis 2017;63(3):104-109 DOI: 10.1515/amma-2017-0026

REVIEW

The Wnt Signalling Pathways: A Short Review and Specific Roles in Bone Biochemistry

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As musculoskeletal diseases become an emerging healthcare problem worldwide, profound and comprehensive research has been focused on the biochemistry of bone metabolism in the past decades. Wnt signalling, one of the novel described pathways influencing bone metabolism from the early stages of tissue development, has been recently in the centre of attention. Several Wnt ligands are implied in bone forming pathways via canonical (β-catenin dependent) and non-canonical (β-catenin independent) signalling. Osteoporosis, a catabolic bone disease, has its pathologic background related, inter alia, to alterations in the Wnt signalling, thus key modulators of these pathways became one of the most promising targets in the treatment of osteoporosis. Antibodies inhibiting the activity of endogenous Wnt pathway inhibitors (sclerostin, dickkopf) are recently under clinical trials. The current article offers a brief review of the Wnt signalling pathways, its implication in bone metabolism and fate, and the therapeutic possibilities of osteoporosis through Wnt signalling.

Keywords: Wnt signalling, bone metabolism, osteoporosis, autophagy, antibodies

Received 14 June 2017 / Accepted 10 July 2017

Introduction

Osteoporosis is characterised by a loss in both bone mass and resistance, as a result of a disruption between bone mineral mass and perturbation of its microarchitecture, emerging in pronounced bone fragility. The pathophysiology of osteoporosis has a complex and multifactorial aetiological background, skeletal fragility having its origin from an inappropriate skeletal development or pronounced bone resorption as a result of microarchitecture deterioration, or inadequate remodelling after resorption [1,2]. Musculoskeletal diseases represent one of the leading global economic burdens, affecting more than five million people in Europe and hundreds of millions worldwide, mainly postmenopausal women [3].

Although Wnt signalling is a cellular mechanism known only since a few decades, yet, the Wnt related processes are highly conserved universal pathways, dating from the appearance of the first metazoan species, around 650 million years ago, in the Precambrian Era [4]. Structurally, the Wnt ligands are hydrophobic glycoproteins, comprising of approximately 350 amino acids, with 23-25 cysteine residues, providing the possibility to form intra-, or inter-molecular disulphide bonds [5,6]. Currently, three important Wnt signalling pathways are described, in the mechanisms of which several Wnt ligands, receptors, modulators and effectors are implied.

Hereby we present a short overview of the Wnt pathways, regulations, antagonists and therapeutic possibilities for osteoporosis targeting Wnt signalling-related molecules.

The Wnt/ β-catenin pathway

β-catenin (cadherin-associated protein, beta) plays a cardinal role in the Wnt signalling pathway and cellular mechanisms. In the "OFF" state of the canonical (Wnt/β-catenin) signalling the effector molecule is phosphorylated by a hetero-tetramer destruction complex, under the action of adenomatous polyposis coli (APC), axin, glycogen synthase kinase 3-beta (GSK-3β) and casein-kinase I-alpha (CK-Iα). The phosphorylation of β-catenin is realized on four different hydroxyl amino acid sites: at Ser^{33}, Ser^{37}, Thr^{41} by GSK-3β and in position Ser^{45} by CK-Iα. This way, the protein becomes a target for ubiquitination and a subsequent degradation inside a proteasome [7-9].

In the presence of Wnt ligands, Frizzled (Fz) and Lipoprotein receptor related protein (LRP) 5 or 6 receptors are stimulated and undergo hetero-dimerization. Fz are large receptors, comprised of seven transmembrane sites and a cysteine rich domain (CRD), which provides a docking site for the Wnt ligands. This interaction results in a subsequent phosphorylation of the LRP receptors, which in turn represents an adherence site for axin via dishevelled protein (Dsv). The phosphorylation process takes place simultaneously for both LRPs (by CK-Iγ and GSK-3β), and Dsv (by CK-Iε, CK-II). These conformational transformations also lead to an over-expressed activity of Dsv on the GBP/FRAT complex (GSK-3β binding protein) and antagonize the effect of the enzyme. Thus, the destruction complex undergoes modifications which lead to its disintegration. As a result, the intra-cytoplasmic β-catenin levels rise and with its transposition into the nucleus, the transcription of several genes occur [7-8, 10-11].

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Table I. Components of the Wnt signalling pathways [33]

Components and function	Family members
Wnt ligands	Wnt-1, Wnt-2, Wnt-2B, Wnt-3, Wnt-3A, Wnt-4, Wnt-5A, Wnt-5B, Wnt-6, Wnt-7A, Wnt-7B, Wnt-8A, Wnt-8B, Wnt-9A, Wnt-9B, Wnt-10A, Wnt-10B, Wnt-11, Wnt-16
Alternative ligands	Norrin and R-spondins
Extracellular modulators	Secreted Frizzled related proteins (sFRP), Wnt-inhibitory factor (WIF-1), Dickkopfs (DKK)
Frizzled receptors	Fz1-10
LRP receptors	Low-density lipoprotein receptor related proteins 5 and 6
Signalling intermediates	Dishevelleds: Dsv1, Dsv2, Dsv3
β-catenin destruction complex	Axin, adenomatous polyposis coli (APC), glycogen synthase kinase (GSK-3β), casein-kinase (CKIα), protein phosphatases (PP1 and PP2A)
Effector	β-catenin (Armadillo)
Transcription factors	T-cell factor (TCF-1, TCF-3, TCF-4), lymphoid enhancer factor (LEF-1)
Intracellular modulators	Nemo-like kinase (NLK), Groucho, Transducin-like enhancer of split (TLE), inhibitor of β-catenin and TCF4 (ICAT)

The Wnt/Ca^{2+} signalling pathway

Although β-catenin plays a primordial role in the Wnt signalling pathways, several alternative routes have been described. Considering that Fz receptors are G-protein coupled, this could lead to an interaction with second messenger molecules. The activation of Fz receptors by "non-canonical" Wnts enrolls enhanced activity of inositol-4,5-triphosphate (IP_3) and diacyl-glycerol (DAG). Elevated levels of these second messenger molecules result in higher intra-cytoplasmic calcium concentrations. Calcium in relation with calmodulin (CaM) activates the calcium-calmodulin-kinase II (CaMK-II). Calcium could also interact with DAG and IP_3. On one hand the calcium-DAG complex results in protein-kinase C activation, on the other hand, calcium-IP_3 complex interacts with calcineurin (Can). Subsequently, Can activates nuclear factor associated with T-cells (NFAT), resulting in gene expression in several tissues [12]. Recent findings describe that, despite the current standpoints – that the canonical and non-canonical pathways do not interact and have antagonistic effects – there is a relation between these signalling routes, acting in an interdependent way. Considering that β-catenin, a 92 kDa protein, has limited or no pass through the nuclear envelope due to its size, this limitation could be

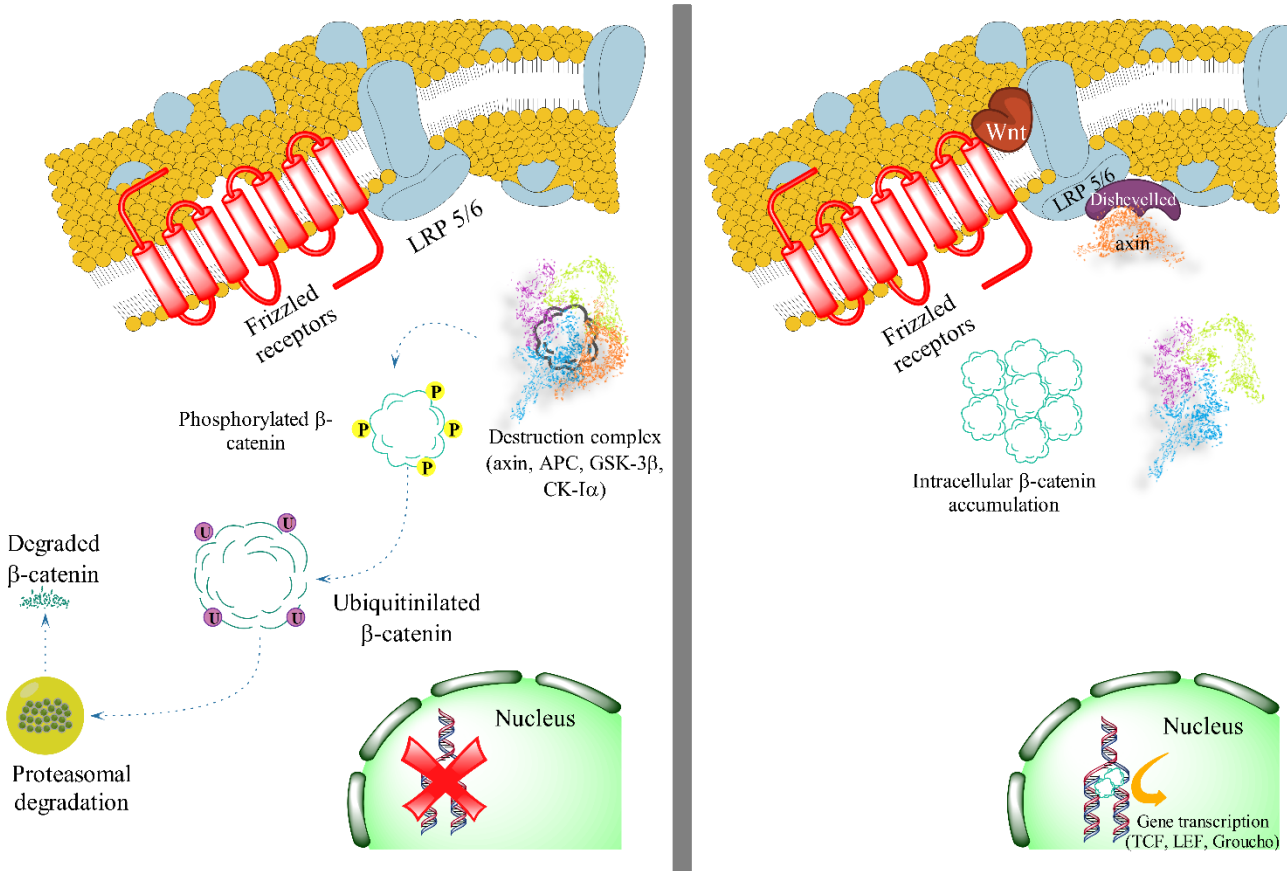

Fig. 1. β-catenin dependent Wnt signalling – left: in the absence of Wnt ligands the degradation of β-cathenin occurs inside a destruction complex; right: contrarily, in the presence of Wnt ligands, the intra-cytoplasmic accumulation of β-cathenin results in gene transcription. Abbreviations: LRP – low-density lipoprotein receptor related proteins, APC – adenomatous polyposis coli, CKIα – casein kinase Iα, GSK-3β – glycogen synthase kinase 3β, Dsv: dishevelled, TCF – T-cell factor, LEF – lymphoid enhancer binding factor

overpassed by the interrelation of Wnt/β-catenin – Wnt/Ca^{2+} pathways. The increase of intra-cytosolic Ca^{2+} could possibly lead to nuclear barrier depolarization and subsequent β-catenin translocation into the nucleus [13]. The relation between these is also emphasized by the activation of Nemo-like kinase (NLK) by Ca^{2+} signalling, which hereinafter phosphorylates T-cell factor (TCF), thus acting like an inhibitor for the β-catenin activity [7].

Wnt/JNK pathway

The Wnt/JNK (Jun-N-terminal kinase) or PCP (planar cell polarity) pathway involves also the action of Dsv proteins, mainly its PDZ, DEP domains, which is capable to activate on a downstream route rhoA or cdc42 (cell division cycle protein). Wnt activation of Fz receptors results in a complex formation between Dsv and Daam-1 (Dishevelled associated activator of morphogenesis 1). This complex subsequently activates Rho-GTPases via WGEF (Rho-guanine exchange factor). This leads to the activation of ROCK (rho-associated kinase), resulting in phosphorylation of the myosin light chain, thus creating the interaction between actin and myosin, leading to the disruption of the cytoskeleton's integrity. Alternatively, the activation of Dsv DEP domain results in Rac-GTPase activation. These GTPase type proteins subsequently activate JNKs [7,14]. Among these JNK1 and JNK2 are ubiquitously present, whereas JNK3 is expressed mainly in the brain. Various types of proteins are targets for the JNKs, mainly transcription factors, which are responsible for cell proliferation or cell apoptosis (ATF2 – activating transcription factor 2; Bcl-2 protein – B-cell lypohoma-2; BAD – Bcl-2 associated death promoter) [15]. Interactions between the JNK and β-catenin pathways have also been described. Activation of Rac1-GTPase and JNK2 could result in the phosphorylation of the free intra-cytoplasmic β-catenin, thus intensifying its translocation in the nucleus [9].

Regulation of the Wnt signalling pathways

Although β-catenin plays a primordial role in Wnt signalling, several ligands with regulatory activity are known to influence the previously described pathways. Norrins, are proteins with a cysteine-knot motif, with special affinity to Fz4 receptors and LRP5 as co-receptors, and activate the canonical Wnt signalling pathways. The activity of Norrins was described in the vascular development in the eye, ear, brain and female reproductive organs [16].

The proteins of the R-spondins (RSPO) family have 234 to 272 amino acids and present the following domains in their structure: an N-terminal putative signal sequence for secretion, a thrombospondin type I repeat (TSR) domain, a variable length domain, rich in basic amino acids and a furine-like CRD. RSPOs are also considered as Wnt agonists, as they activate the β-catenin signalling pathway, however RSPOs may have different receptors as Wnts [17]. Recently, the presence of leucine-rich repeat containing G-protein-coupled receptors (LGR) has been described. The activation of these receptors could lead to an enhanced and more potent activity, showing synergy with the β-catenin and PCP pathways [18]. RSPOs are potential ligands for the Kremen (Krm) receptors, acting like competitive an-

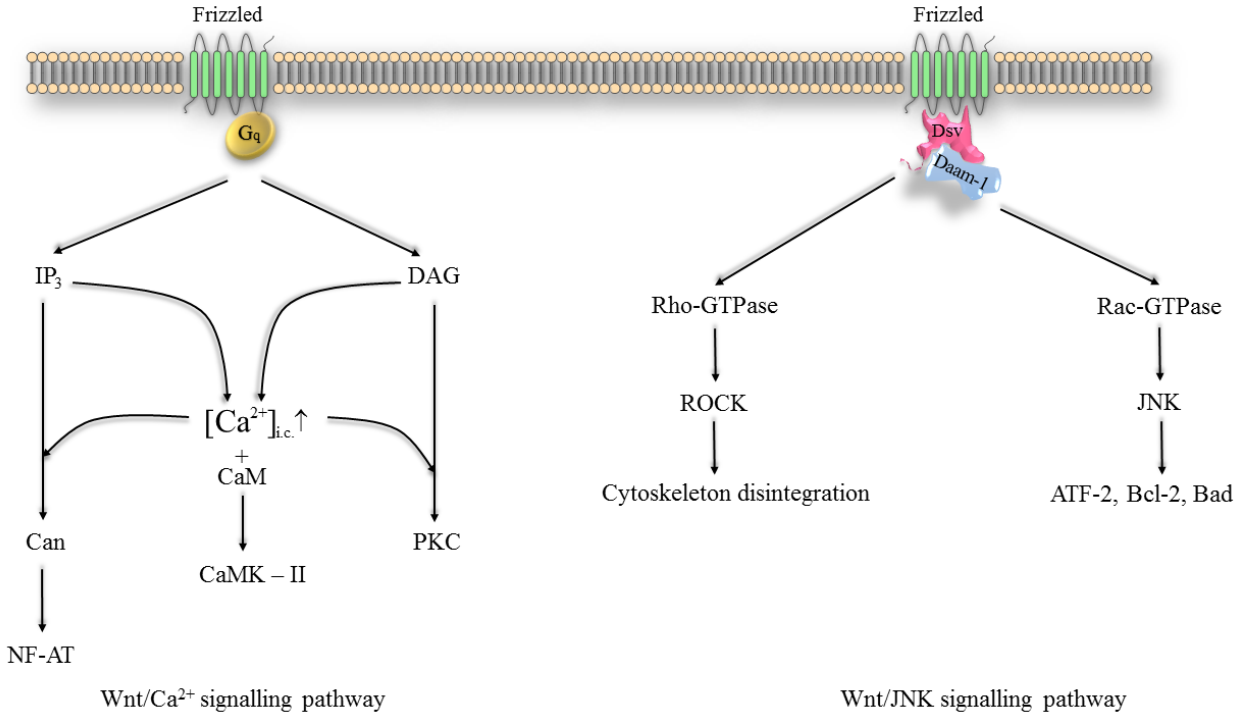

Fig. 2. Brief overview of the "non-canonical" Wnt signalling pathways. Abbreviations used: IP3 – inositol triphosphate, DAG – diacylglycerol, Can – calcineurin, PKC – protein kinase C, CaM – calmodulin, CaMK-II – calcium calmodulin kinase II, NF-AT – nuclear factor associated with T-cells, Dsv – dishevelled, Daam-1 - Dishevelled associated activator of morphogenesis 1, ROCK – rho associated kinase, JNK – Jun-N terminal kinase, ATF-2 – activating transcription factor 2, Bcl-2 – B cell lymphoma, Bad – Bcl-2 associated death promoter

tagonist for Dickkopfs (DKK), therefore inhibiting the formation of DKK/Krm complex [9].

Soluble Frizzled-related proteins (sFRP) are antagonists of the Wnt signalling pathways. They form complexes not only with Wnts, but also form non-functional complexes with Fz receptors. This results in an inhibitory mechanism for the β-catenin and/or PCP pathways. It has also been described that sFRPs do not always act like antagonists, as in some cases activation of Wnt signalling could be promoted. This could be explained in such a way that sFRPs have low- and high-affinity binding sites for the Wnt proteins. When binding to the low-affinity site, sFRPs behave as inhibitors, contrarily, binding to the high-affinity site they would behave as activators [19].

WIF (Wnt inhibiting factor), unlike sFRPs binds only to the Wnt proteins, exerting an inhibitory effect on the canonical, as well as on the non-canonical pathway. Beside this, WIF has the potential to modulate and regulate extracellular proteins which are implicated in Wnt signalling.

Members of the Dickkopfs (DKK) family are considered as specific Wnt pathway inhibitors. DKKs are competitive antagonists of the LRP receptors and in this way inhibit the formation of the Fz-LRP complex. It is also believed that DKKs could disrupt the Wnt-Fz complex. Besides, DKKs have the potential to bind to Krm receptors, behaving as agonists. Krm receptors provide the internalization of the LRP receptors and prohibit the possibility of Fz-LRP receptor formation [9].

Other inhibitory mechanisms in the Wnt signalling are represented by Shisa proteins, which suppress Fz receptor docking to the cell membrane, by trapping the receptors inside the endoplasmic reticulum. Cerberus, Nodal, bone morphogenetic protein (BMP), insulin-like growth factor binding protein (IGFBP-4) inhibit Wnt and both Fz and LRP receptors [9].

Wnt ligands in bone metabolism and fate

Several Wnt ligands are implied in osteoblastogenesis. Wnt1 was shown to enhance β-catenin mediated osteoblast formation, by an endogenous phosphorylation of GSK-3β via G-protein coupled Fz receptors and LRP 5 or LRP 6 co-receptors, which leads to an increased β-catenin activity. Furthermore, it was elucidated that the enhanced activity of the Wnt canonical pathway results in an expressed Runx2 downstream regulation [20]. The downstream osteoblastogenetic and mineralization enhancing properties of Wnt1 were also described in Wisp1$^{-/-}$ mice. Wisp1/CCN4 (Wnt1-inducible signalling pathway protein 1/connective tissue growth factor) induces mesenchymal stem cell differentiation and osteoblast proliferation and maturation in the detriment of chondrocyte differentiation. It was also described that Wisp1 interacts with BMP-2 having a positive effect on osteogenesis. Wisp1 proved to be a key regulator of bone turnover and Wnt signalling, resulting a significantly lower bone mass, with diminished biomechanical strength in Wisp-1 knockout mice [21-23]. Wollnik et al. recently reported that different mutations, hypofunctional alleles of Wnt1 may lead to osteogenesis imperfecta, or low-bone-mass phenotype which predisposes to an early-onset of osteoporosis. These altered molecular changes in Wnt1 protein result in a loss of activity over LRP5-receptor mediated β-catenin signalling [24]. Similar mutations were identified by other research groups in families with hereditary early-onset osteoporosis and osteogenesis imperfecta [25]. Okamoto et al. demonstrated the importance of non-canonical Wnts during osteoblastogenesis. In calvarial cell cultures increased levels of mRNA expression of Wnt5a and Wnt10b were observed, but not Wnt7b. Furthermore, it was described that Wnt5a, as a non-canonical Wnt ligand, induces the expression of LRP5 and LRP6 receptors during osteoblast differentiation, thus being implied in Wnt/β-catenin dependent, canonical stimuli of osteoblasts [26]. Contrarily, Chen et al. described that Wnt7b is involved in bone formation via mTORC1 (mammalian target of rapamycin complex 1) activation. The bone formation activity of Wnt7b was assessed by the measurement of serum osteocalcin levels, which appeared to be significantly higher in comparison to the control group. Also, CTX-I (C-terminal telopeptide) levels showed a similarity with the control group in two months old mice [27]. Wnt11 and Wnt16 are also key regulators of bone homeostasis. Wnt11 acts through the activation of R-spondin2, a known activator of the Wnt signalling. Moreover, Friedman et al. demonstrated enhanced alkaline phosphatase activity in murine pre-osteoblasts under Wnt11^{+} over-expression. Wnt11 also proved to be a potent enhancer of BMP2-induced osteoblast maturation and mineralization [28]. Wnt16, known as an activator for both canonical and non-canonical Wnt signalling, has a great role in bone thickness and porosity modulation, interestingly not affecting in a significant manner osteoblast differentiation, proliferation and maturation, contributing modestly to bone homeostasis. Altering the RANKL/RANK/OPG triad, Wnt16 contributes to an OPG over-expression, potentiating the inhibition of RANKL-induced osteoclastogenesis, interfering with osteoblast-to-osteoclast cross talking [29].

Wnt signalling and autophagy in bone

Autophagy is one of the cell's novel described fate mechanisms, involved in tissue regeneration and recycling of molecules of key importance in cell metabolism (macromolecules and eventually organelles). Onal et al. reported that autophagy suppression in osteocytes results in decreased bone mass and bone turnover in mice and proves to contribute to the state of oxidative stress, thus resulting in a process similar to ageing in bone tissue and skeleton. Sustaining evidence was published by Nollet et al., demonstrating that impaired autophagy in osteoblasts and the environmental oxidative stress may lead to an enhanced osteoclastogenesis via TNFSF-11 activation (RANKL)

and leading to osteoporotic-like phenotype [30,31]. Autophagy negatively alters the Wnt/β-catenin cascade by promoting the degradation of all three isoforms (Dsv1, Dsv2 and Dsv3) of docking protein, Dishevelled. This attenuation in the Wnt signalling was reported by Gao et al. in autophagy-deficient cells ($Atg5^{-/-}$ and $Atg7^{-/-}$). It was also shown that inappropriate autophagy mechanisms lead to a slower turnover rate of all Dsv members [32].

Molecular targets in Wnt signalling

Sclerostin (Wise/SOST) is an osteocyte-expressed glycoprotein, inhibiting the bone forming activity of osteoblasts, by interrupting the Wnt signalling. SOST selectively inhibits the interaction between the Fz and LRP5 or LRP6 receptors by binding through a PNAIG motif to the receptor complex and disrupts its functionality. From the beginning of the millennium SOST has been one of the promising targets for the treatment of osteoporosis. As SOST is not ubiquitously expressed, its effects are only limited to bone metabolism; thus its inhibition using targeted monoclonal antibody therapy, will not result in systemic effects as in the case of other antagonists of different Wnt inhibitors [34,35]. Sclerostin being a physiological antagonist of osteoblast operated bone matrix formation, osteoporosis can be treated by its neutralization using monoclonal antibodies. Several antibodies were already developed and are currently in clinical studies, such as blosozumab, which is described as an immunoglobulin G4 (IgG4) protein, or romosozumab, formally known as AMG785 [36]. Blosozumab is a humanized monoclonal antibody targeted against sclerostin, which in phase II clinical trials produced significant changes in postmenopausal women, mostly in lumbar spinal and hip bone mass density (BMD). Romosozumab, similarly to blosozumab is a monoclonal antibody that inhibits the effects of the sclerostin, increases osteoblast mediated bone formation and decreases the intensity of bone resorption. In a study made by Cosman et al., after 12 weeks of treatment with romosozumab followed by another 12 months with denosumab (monoclonal antibody targeted against RANKL), postmenopausal women with osteoporosis had a 73% lower risk of vertebral fracture in comparison to the control group. Romosozumab increased BMD T score of the lumbar spine and the hip bone after 6 months of treatment, while after 12 months the increase was even more significant [37]. In another study made by Sugiyama et al. made upon romosozumab and blosozumab, there were no significant changes in the areal BMD of the radius bone which reflects that the forearm is not exposed to high levels of mechanical strain under normal physical activity and therefore the anti-sclerostin antibodies could not increase the strength and density of the bones which are not exposed to stress and strains within the skeleton. The treatment with anti-sclerostin antibodies also has its limitations, in both cases postmenopausal women's increased areal BMD returned to the pre-treatment levels within a year despite the continued therapy with romosozumab or blosozumab [38].

DKK-1 is the most studied protein of the four members of the DKK family (DKK-1 to DKK-4). DKK-1 is a secreted antagonist of the Wnt/β-catenin signalling, which inhibits the Wnt-induced stabilization of β-catenin and β-catenin/Tcf-dependent transcription by binding to LRP 5 or 6. Besides this blocking mechanism, DKK-1 also has effects on the Wnt/LRP6 signalling by disrupting it and promoting endocytosis via Krm-1 and Krm-2. BHQ880 is an anti-DKK-1 monoclonal IgG1 isotype antibody developed for the potential treatment of multiple myeloma (MM) produced osteolytic bone fractures, where the neutralizing effect of the antibodies could readjust the disrupted balance between bone formation and bone resorption. The drug encoded BHQ880, in vitro increased the osteoblast differentiation and serum human osteocalcin levels, neutralized the negative effects of MM cells on osteoblastogenesis, reduced interleukin-6 (IL-6) secretion, and in addition up-regulated β-catenin levels and down-regulated nuclear factor-κ B (NF-κ B) activities in bone marrow stromal cells [39,40]. In a preclinical model study published by Goldstein et al, BHQ880 reduced DKK-1 serum levels in treated mice with orthotopically implanted patient-derived osteosarcoma tumours, slowed the growth of the tumours and inhibited metastasis by neutralizing the DKK-1 protein, which is overexpressed by these cells. The positive effects over the tumorous cells were correlated with increased β-catenin levels and increased expression of the bone differentiation marker osteopontin, which altogether suggests that Wnt signalling has antitumorigenic effects [40].

PF-04840082 is a humanized prototype anti-DKK-1 IgG2 isotype antibody for the potential treatment of osteoporosis that binds the physiological antagonist of the Wnt/LRP5 signal pathway, therefore increasing bone mass and BMD by activating osteoblasts. In 2010 Betts et al. published a study about the pharmacokinetic/pharmacodynamic modelling of the antibody in concordance to dose and effect that shows significant decreasing in free DKK-1 concentrations in rats and cynomolgus monkeys, with a proper safety and efficiency interval that can be transposed to humans, making it a potentially effective biological drug after furthermore preclinical and clinical studies [41].

Many antibodies that bind and neutralize either sclerostin or DKK-1 are in preclinical or clinical trials making them a potential treatment for postmenopausal osteoporosis and MM induced disease in the future. Anti-sclerostin and anti-DKK-1 antibodies have the potential to improve BMD T score, fracture healing via increased bone formation. However anti-sclerostin treatment alone is currently not enough, patients presented a relapse in BMD levels after a year despite the still undergoing therapy with the antibodies [38,42]. Therefore Florio et al. hypothesized that blocking both proteins at once with one antibody, this could further increase Wnt signalling, resulting in a

more robust effect on bone formation and repair. Their results show that the simultaneous inhibition of sclerostin and DKK-1 by the newly engineered bi-specific heterodimer IgG antibody promotes synergistic bone formation in mice. The inhibition alone of sclerostin leads to increased DKK-1 levels, which act as a negative regulator upon the bone formation, reducing the administered anti-sclerostin antibody's effects. The inhibition of either one of them may stimulate compensatory mechanisms that would rise the other antagonist levels in order to return the Wnt signalling in a steady state, making the dual inhibition a more proper treatment of osteoporosis in the future [42].

Conflict of interest

None to declare.

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Acta Medica Marisiensis 2017;63(3):110-114 DOI: 10.1515/amma-2017-0030

REVIEW

Can a Person with Attention Deficit Hyperactivity Disorder be an Athlete?

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Attention-deficit–hyperactivity disorder (ADHD) is a common neuropsychiatric disorder that impairs academic, social and occupational functioning in children, adolescents and adults. It is characterized by excessive activity, restlessness, and nervousness. The disease occurs in general at children before the age of 7 and usually is not easy to be detected, due to various symptoms. When the diagnosis is established the physician can prescribe two types of drugs, stimulants: amphetamine, dexamphetamine, lisdexamphetamine, methylphenidate, and non-stimulants such as: guanfacine, atomoxetine, and clonidine. So what can be done for a person who has ADHD, and wants to be an elite athlete? Due to the rules established by the World Anti-Doping Agency the stimulant drugs are prohibited in competition and if traces of a prohibited substance are detected in the sample of blood of the athlete his access to competition can be blocked from 2-4 years, from that date of the incident. Fortunately for some athletes the disease was acute in childhood but as they grew up the symptoms were reminiscent and they could concentrate at the sporting task that was supposed to be achieved. What about those athletes that still have the symptoms? Well, they can be treated with the non-stimulant drugs, but their doctor must monthly verify if the list of prohibited drugs has been changed. In conclusion we can say that ADHD can be an impediment, but with the help of parents, teachers, and physicians the athlete can achieve very good performances.

Keywords: attention-deficit–hyperactivity disorder, athlete, sports, doping

Received 21 June 2017 / Accepted 17 August 2017

Introduction

Attention deficit hyperactivity disorder (ADHD) is a syndrome of neurodevelopmental type characterized by excessive activity, restlessness, nervousness and problems in paying attention. Usually these symptoms appear before the age of 7 and can create problems in different social settings such as school, home or recreational activities. Children affected by ADHD have trouble staying focused; are distracted easily or get bored when working on a certain task, appear not to listen when spoken to, have difficulty in remembering things and following instructions; do not pay attention to details, have trouble finishing projects and staying organized, frequently losing or misplacing homework, books, toys, or other items. The most obvious sign of ADHD is hyperactivity; while many children are quite active naturally, children with ADHD are always moving, trying to do several things at once, bouncing around from one activity to the next. The impulsivity of children with ADHD can also cause problems with self-control, as they have the tendency to interrupt conversations, invade other people's intimate space, ask irrelevant questions in class and make tactless observations [1,2].

ADHD can be classified in 3 subtypes by symptoms [3]:

– 1 A symptoms only: ADHD, Predominantly Hyperactive Type
– 1 B symptoms only: ADHD, Predominantly Inattentive Type
– Both 1 A and 1 B symptoms: ADHD, Combined Type

The fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), classifies ADHD based on the presence of particular symptoms. Each symptom is classified as being either from the "Inattention" (1A) or "Hyperactivity - Impulsiveness" (1B) group [3]. Symptoms are listed by group in Table I.

Children with ADHD are at greater risk than other children for substance abuse and juvenile delinquency; however, early diagnosis and treatment with psycho-education as well as drug therapy may decrease the negative outcomes of ADHD, including the rate of adult antisocial personality and of conduct disorders [4].

Is important to discover the disease as soon as possible, because children with undiagnosed ADHD remain untreated, and the feedback from the people that are close to them, parents, teachers is important, because they need the help from the people with whom they are in contact with [5,6].

Children with ADHD when feel criticized, they tend to control less their symptoms [7, 8].

Studies have shown that children with ADHD who cannot control their behavior are exposed to the probability of persistence of the behavior [9-11].

The exact cause of ADHD isn't known, but it seems that heredity is the most common cause of ADHD, as there are genetic characteristics that seem to be passed on. However, like many other illnesses, ADHD probably results from a combination of factors, as possible environmental factors,

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Table I. Symptoms used in the DSM-IV diagnostic criteria for classifying ADHD diagnoses by group [3]

Inattention (1A)	Hyperactivity (1B)	Impulsiveness (1B)
1. Has trouble keeping attention on certain tasks. 2. Doesn't give enough attention to details and makes careless mistakes in schoolwork and other activities. 3. Doesn't follow instructions and fails to finish certain tasks. 4. Doesn't seem to listen when spoken to. 5. Avoids, dislikes, or doesn't want to do things that take a lot of mental effort for a long period of time. 6. Has trouble in organizing daily activities. 7. Loses things for tasks or other activities. 8. Is easily distracted by external stimuli during activities. 9. Forgets daily activities.	1. Leaves seat in classroom or in other situations in which remaining seated is expected. 2. Runs and climbs excessively in situations in which it is inappropriate. 3. Plays with hands or feet or squirms in seat. 4. Has trouble playing or enjoying leisure activities quietly. 5. Is always ''on the go'' or often acts as if ''driven by a motor.'' 6. Talks excessively.	1. Answers to questions before questions have been finished. 2. Interrupts or intrudes on others. 3. Has trouble waiting one's turn.

brain injuries, nutrition and the social environment might contribute to ADHD [12].

Beside medical treatment, Cognitive Behavioral treatment (CBT) has outcomes in adolescents and also in adults. If we are talking about adults there are results which are showing that CBT has great effects on the symptoms of ADHD, with the reduction of anxiety and depression. It has been observed that the ADHD symptoms tend to be reduced after CBT, while anxiety and depression, may have an impact in decreasing the ADHD symptoms, lowering the effect of CBT [13].

In children, CBT is a protocol which can be used to individuals who do not respond to stimulant medication, or the stimulant medication cannot be tolerated by them; it is also a protocol which validated at adults could help adolescents with ADHD. The CBT protocol may also decrease the doses of medications prescribed for the children in need [14]. Psychosocial management includes the following interventions: behavioral parent training (BPT), adolescent-parent training in solving problems, training teachers to manage kids with ADHD and also all the three interventions mentioned above combined [15]. CBT may be also used for treating: anxiety [16], autism [17], and for children exposed to violence [18].

Diagnosis

Clinical interview with the parents, the child or adolescent and any significant family members is essential to establish a diagnosis of ADHD by verifying the functionally impairing symptoms of inattention, impulsivity and hyperactivity. There are also other tools that can be used to help the physician in establishing this diagnosis, such as: Connor Questionnaire, NICQH Vanderbilt Assessment Tool, DM5- criteria, SNAP IV and Pelham Scale [19].

We consider adhering to DSM IV diagnosis is important because it can be established which component is the most pointed out and by establishing this component, a better diagnosis can be achieved, the physician knowing which component is the most sweeping and needs more attention in the treatment of the person with ADHD. DSM-5 and DSM IV are both used in diagnosis of mental diseases.

Management of ADHD

There are a few factors that are important before establishing the way the patient will be treated, as treatment depends on the: age, gender, the way the patient acts at home, work, school. Medication and also non medication treatments are available, but studies show that medication has superior results [20].

ADHD can be treated using medication or therapy, but a combination of both is often the best choice. There are five types of medication licensed for the treatment of ADHD: methylphenidate, dexamphetamine, lisdexamphetamine, atomoxetine and guanfacine [21]. The chemical structures of the drugs are presented in Figure 1.

Amphetamine was the first medicine that was used for treating ADHD; it has been introduced on the pharmaceutical market 50 years ago, but nowadays is no longer used because of serious addiction risks [1].

Methylphenidate (methyl phenyl (piperidin-2-yl) acetate) is the most commonly used medication for ADHD; it is a central stimulant of phenethylamine and piperidine classes. Its mechanism of action involves the inhibition of catecholamine reuptake, primarily inhibiting the dopamine reuptake, leading to increased concentrations of neurotransmitters within the synaptic cleft. The medication can be taken as either immediate-release tablets (small doses taken two to three times a day), or as modified-release tablets (taken once a day in the morning, and they release the dose throughout the day). The ADHD patient may also have other co-morbidities such as depression, in

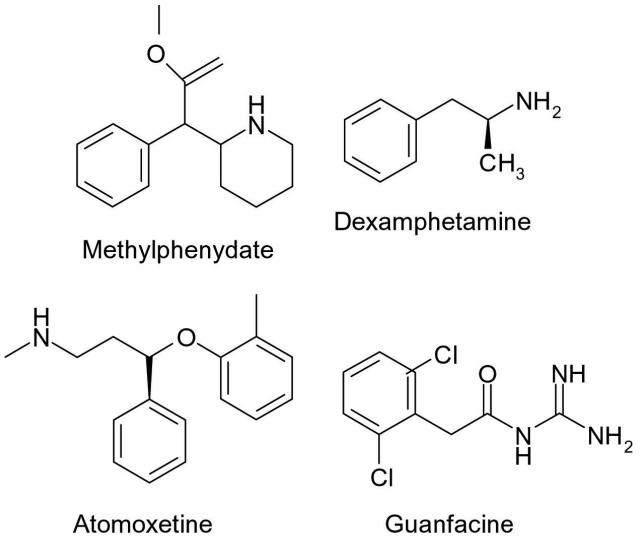

Fig. 1. Chemical characteristics of the drugs currently used in therapy in the treatment of ADHD

some studies it has been observed that methylphenidate has a good effect if the patient has depression and ADHD [1, 21].

Dexamphetamine ((2*S*)-1-phenylpropan-2-amine) is the dextrorotatory enantiomer of amphetamine, its mechanism of action is related to the activation of a trace amine receptor, increasing monoamine and excitatory neurotransmitter activity in the brain, with the most pronounced effects of catecholamine neurotransmitters like norepinephrine and dopamine [22].

Lisdexamphetamine ((2*S*)-2,6-diamino-*N*-[(2*S*)-1-phenylpropan-2-yl]hexanamide) is a very similar medication to dexamphetamine and has the same mechanism of action, its chemical structure consists of dextroamphetamine coupled with an essential amino acid L-lysine; lisdexamfetamine is the prodrug of dextroamphetamine as *in vivo* suffers the cleavage of the lysine portion of the molecule [23].

Atomoxetine ((3*R*)-*N*-Methyl-3-(2-methylphenoxy)-3-phenylpropan-1-amine) works differently to other ADHD medications; it is known as a selective noradrenaline reuptake inhibitor (SNRI). Its primary advantage over the standard stimulant treatments for ADHD is that its abuse potential is low. Atomoxetine has the advantage of dosage, because the patient has to take only one pill daily. It has been proven that atomoxetine is noninferior to immediately release methylphenidate but if we compare it with extended release methylphenidate it has been concluded that it is less effective [24].

Guanfacine (*N*-(diaminomethylidene)-2-(2,6-dichlorophenyl)acetamide) is a sympathomimetic drug, being a α_{2A}-receptor agonist, very similar to clonidine. It can be used if the other treatments do not work well; it also has some advantages compared to clonidine, because guanfacine has a longer half life, and some side effects such as sedation and changing the blood pressure are not as powerful like in the case of patients using clonidine [25].

ADHD in Sport

An athlete with ADHD faces certain unique challenges. The scientific literature has traditionally focused on the cognitive and psychological aspects of ADHD, and only a few studies comment on sport performance.

So the question is can a person with ADHD be an athlete?

Well, it depends on the medicine he is using, for example some athletes used amphetamines and they were banned from competition; but if they demonstrated that they had ADHD and they were using those medicine for treating their condition their suspension was lifted; but in the Code it is mentioned that if a athlete is suffering from a disease and needs to use a medicine which is forbidden to be taken, he may use it but only to help him treat his disease. As it was mentioned before there are two classes of substances that can be used in ADHD: those which are stimulants and the others that have not any stimulant effects. So why are the athletes using the stimulant ones when they could use the other class? The representative of the stimulant class are all banned and can be found on the prohibited list at the section prohibited substances, section S6 Stimulants, which are prohibited only in competition, so if a person has ADHD and uses amphetamine he can use it but only when he is not competing, when he competes he may use substances from the class of non-stimulants: atomoxetine, clonidine, guanfacine.

There are other methods used for treating ADHD that do not have the same usefulness as the medication, but they may help the patient, such as: diet, using the herbal treatments (Ginkgo Biloba, Panax quinquefolius), yoga and homeopathy [26].

Team physicians should understand the effects of the medications commonly prescribed for ADHD on athletes during daily life and also while the athletes are engaged in athletic activities. Unfortunately, there are some people that are using the rules in their advantage, and they use medicine for ADHD, and are also participating at Olympic Competitions, as the stimulating drugs may induce advantages comparing over other competitors.

Recent studies have shown that an 80 dB noise (white noise) may improve the attention of the ADHD patient, it has been observed that if the patients were asked to repeat a word even if they had or not ADHD the results were the same, in both cases it has been observed the improvement of the capacity of remembering a word for both categories [26].

Studies regarding the influence of physical activity in the ADHD symptoms, showed that aerobic exercise seem to decrease hyperactivity, inattention, and impulsivity, among other symptoms. Another physical therapy includes yoga, which could improve behavioral symptoms, and psychological function. Physical exercise is good for children with ADHD because the release of dopamine which can decrease inattention symptoms. Practicing more sports seems to improve the anxiety and depression symptoms which may occur in children with ADHD [27].

In the list of athletes serving a period of ineligibility as a result of a rule violation we found that only a few were caught using amphetamine (4 athletes out of 296), and no positive results were detected for methylphenidate [28].

Athletes that proved that ADHD is not a barrier for performances

In some studies it has been shown that the lack of physical activity relates with the depressed affect. Physical activity can conduct to improvement of the ADHD, while the lack of physical activity (sedentarism) did not have a good outcome [29].

The study of Gapin et al shows that physical activity is related to ADHD; they measured the total move score (TMS), total execution time (TET), and the results showed an improvement to those parameters, in children which made physical activity [30].

Due to the issues that ADHD includes, children who want to be part of the other children activities present

low performance in doing them and some children with ADHD choose to have their own physical activity. Unfortunately some of them do not understand the purpose of the physical activity. For having better results the children with ADHD they should be helped by the physical education teachers, and also they should discuss with adults (teachers, parents) about the leisure counseling [31].

Michael Phelps was diagnosed with ADHD at the age of nine when his mother decided to take him to a pediatrician because he was more active than the kids at his age, and he couldn't concentrate to the activities in school. His mother, also a teacher, has received notification from the teachers that her son had a problem. When the athlete was diagnosed with ADHD, he also received pills that were making him concentrate to the tasks he was receiving, and with the help of his mother, his results at the school improved. At the age of 12 he stopped taking medicine for his disease, and at the same age the good results in sport appeared. Being energetic was an advantage, and he was doing remarkably well at swimming. His mother used his love for swimming to help him learn things: if the pool has 500 meters and the swimmer's speed is 3 m/second, in what time will he arrive at the finish line? [32].

Michael Phelps is now no longer using medicine for ADHD, but since the age of 15 he had incredible results at all the swimming competitions that he participated in, winning many gold medals and he is the youngest world record holder. His results are incredible, he participated at 4 Olympic Games and won at every Olympic Game at least one gold medal (since 2004 Athens) [32]. He is one of the ADHD athletes that can help us say that having ADHD is not stopping us from achieving our purposes in life.

Another example can be Michael Jordan who excelled at basketball since he was in high school, but due to his height he could not reach the high school basket team. After a summer of hard working, beneath the skills that he got, he also reached the height of 190 cm. The height and the skills brought him a place in the team. He channeled all his energy into playing basketball and achieved the goals that he wanted, becoming one of the greatest basketball players of all time, with records that were not beaten still. He is also one of the athletes that struggled with ADHD during his childhood [33].

Justin Gatlin, an American sprinter, was the co-holder of the world record at 100 meters. He declared that competing is important to him, because beneath the competitors he has to struggle against ADD, and every competition in which he attended is a victory against ADHD, a disease with which he fought during all his life [34]. Unfortunately Justin was tested positive in 2004 and he was banned to compete for two years. The banning came due to the medication he took for his disease, which is not permitted in competitions. [34]

Cammi Granato was a Canadian hockey player who won the gold medal at the Olympics in Nagano 1998, and silver in Salt Lake City in 2002; she discovered her disease only in 2003, and concluded that the disease she had helped her in the sport she practiced because she didn't need too much thinking, she only needed to react at what happened on the ice. At first she thought that she was lazy and that was the issue that prevented her from doing her daily tasks, but after discovering the disease she concentrated more on the problem that she had and put her life in order [34].

Chris Kaman was a great basketball player who also had to handle with the ADHD symptoms. As a kid he had issues in staying focused, and had bad grades in school, but after growing up and taking his medication, things became better for him and he managed to play for the Los Angeles Clippers, doing the best thing he was good at: play basketball [34].

Conclusions

There are several options in treating ADHD, but using methylphenidate, amphetamine or lisdexamphetamine may create an advantage for the athlete using them. Many athletes are willing to take forbidden substances, but the athletes should be careful in using the substances, because their future and their career may be affected in a bad way by breaking the rules. The question remains if the athlete can use these substances out of the competition, knowing that they present a great risk of addiction.

The decision to use medications during sporting activities should be made on an individual basis. All athletes with ADHD are likely to benefit from general behavioral skills oriented treatments, which include time management, stress management, organizational skills and problem solving skills training.

If we are talking about athletes that may experience ADHD at an adult age and also practicing sport, why not use other medication that does not affect the sportsman reputation and can be taken, in and out of competition, without any risk that the athlete might be excluded of the competition. We do not say that the person who is suffering from ADHD and it is also practicing sports should not take their medicine, we stand for using the right medicine for them, the one that does not affect in a cheating way their results, but also helps them fighting with the disease.

The majority of athletes which were diagnosed with ADHD also have great performances in the sport they practice. Sport performance is a therapy for some kids, because they use their energy into something that they like and enjoy and they are also rewarded; their self-esteem is growing, helping them getting over some of the symptoms of ADHD and concentrate on sport. Studies have shown that practicing more sports can help the children with ADHD, while practicing high level sport is also a solution for the ADHD kids, who can use their energy in making something good for them, for their self-esteem and for their parents.

There are quite a few athletes that already succeeded in sport while also having ADHD, so the answer to the ques-

tion that was put at the beginning of the study is yes, an ADHD person may be a great athlete.

We must also say that becoming a great athlete and having ADHD is not always an advantage; it depends on the symptoms that the ADHD child has and also how severe is the disease. Physical activity may improve the ADHD symptoms but it will not cure it.

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Acta Medica Marisiensis 2017;63(3):115-120 DOI: 10.1515/amma-2017-0031

RESEARCH ARTICLE

Immersive VR in Phantom Limb Pain Therapy of Amputee Patients Due to Critical Limb Ischemia

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Introduction: Phantom limb pain (PLP) occurs in approximately 75% of patients who undergo limb amputation. In identifying the etiopathogenic mechanisms, multidisciplinary approaches are increasingly important in explaining the causality based on neurological and psychological factors. PLP has many negative effects on the amputee's physical and mental integrity, which is why a variety of treatments have been conceived, whose effectiveness is rather limited. **Objective:** The purpose of this study is to evaluate the effectiveness of an immersive virtual reality (IVR) intervention program using the mirror therapy principles in decreasing PLP. **Method**: Twenty participants suffering from PLP were randomly assigned to one of the two intervention groups: IVR and kinesiotherapy, respectively. Pre- and post-intervention measurements were performed both on pain level and on several psychological variables: depression and anxiety symptoms, pain catastrophizing, quality of life, body representation and coping strategies. **Results**: Preliminary data show a significant pain relief in patients in the IVR group compared to those in the kinesiotherapy group. Besides, significant improvement was found in the case of the patients in the IVR group, in terms of life quality improvement, reducing irrational pain catastrophizing-related thoughts and positive coping strategies (positive refocusing and reappraisal). There were no differences identified between the two groups in terms of anxiety and depression symptoms. Despite expectations, patients in the IVR group experienced a significant increase in one negative coping strategy: rumination. **Conclusions**: The results obtained are advocating the use of IVI intervention as a method phantom limb pain alleviation, with positive consequences on patients' life quality.

Keywords: immersive virtual reality, mirror box therapy, phantom limb pain, cognitive and emotional coping strategies

Received 23 July 2017 / Accepted 7 September 2017

Introduction

Limb amputation is a major loss and has a significant negative impact on the patient's well-being: from loss of independence and restriction of daily activities to isolation, mental health impairment and body image disturbances. In addition, the amputee may experience sensations in the severed limb, phantom limb pain and stump pain. In this study, we will deal with the phantom limb pain, defined as painful sensations referred to the absent limb [1, 2].

Several studies suggest that significant phantom limb pain is rare [3, 4, 5]. On the other hand, many other studies show that 60-80% of amputees experience phantom limb pain [6]. This lack of consistency between studies may be explained, on the one hand, by the fact that PLP incidence was assessed by taking into account only the population that has actively sought medical assistance for their phantom limb pain and, on the other hand, because this type of pain is often underreported by patients, who are generally convinced that pain in an amputated limb only exists in their minds. Even two years after limb loss, approximately 75% of amputees complain about pain in their phantom limb [7, 8].

Phantom limb pain is a complex phenomenon, with underlying theories evolving from mono-disciplinary approaches, based exclusively on neurological mechanisms, to complex approaches that include both neurological mechanisms and psychological factors. Therefore, the need for multidisciplinary interventions is becoming increasingly stringent [9].

Specialized literature lists two primary levels at which phantom limb pain relief interventions are performed: pharmacological administration and psychological, behavioural and physical intervention [10]. Pharmacological treatments use analgesics, antidepressants, neuroleptics, anticonvulsants, opioids, muscle relaxants etc. Although pharmacological interventions appear to be numerous, a systematic review of the studies in this field shows that there is limited evidence supporting their effectiveness in PLP management [11]. On the other hand, where the pain in the phantom limb is linked to several pathological processes, including cortical changes, an intervention program that includes physical, behavioural or psychological components may prove more beneficial. Psychological interventions address in particular the emotional factors involved in the occurrence and persistence of the pain in the phantom limb [12]. Cognitive-behavioural therapy and hypnotherapy appear to provide encouraging evidence to this effect [13]; however, in the absence of controlled clinical trials and the fact that most results are based on case studies only, there is no solid evidence supporting their effectiveness. On the other hand, behavioural interventions have been given a particular attention, partly owing to their

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affordability and reduced costs. Kinesiotherapy, a thereapy where patients make muscle toning movements, has proven to have only limited effectiveness, with positive results being rather recorded in patients with prosthetic limbs [10]. Therefore, new behavioural interventions need to be applied to patients who have not yet been prosthetized, but who are experiencing pain in their phantom limb. Mirror-box therapy is one of the most common interventions. The therapy uses the principle of visual illusion to change amputee's bodily representation and thus alleviate PLP.

The therapy uses a box containing a mirror. The amputee places his or her intact limb into the box, which allows him/her to view a reflection of his/her anatomical limb in the visual space occupied by his/her phantom limb, while moving the intact limb to release tension in the phantom limb. However, the mirror box presents a number of inherent limitations: the illusion is tentative, relaying on the patient's maintaining attentional focus on the reflected image as opposed to the moving anatomical limb; otherwise, a simple glance of the amputee at the missing limb would compromise the illusion. Besides, the patient is forced to remain in a restricted, fixed position and to move the anatomical limb in a very narrow space [14]. The interventions based on mirror therapy seem promising, although most of its positive results come from case studies or single-subject experiments [15, 16, 17].

The conclusion is that the literature highlights the need for both controlled clinical trials and for improvement in behavioural interventions, so as to overcome the limitations presented by the mirror therapy. Therefore, this study uses an immersive virtual reality intervention program (IVR) based on mirror-box visual illusion principles. By using the virtual space, the visual illusion is enhanced, because the number of disruptors is limited and the movement space is much wider [18].

In this context, our research aims at assessing the effectiveness of an IVR-based intervention program in reducing phantom limb pain. To this end, we have compared the effectiveness of this type of intervention with a standard behavioural program based on kinesiotherapy. Studies show the higher effectiveness of the interventional programs based on visual illusion compared to those based on physical exercise, for which reason the study did not include a placebo or a waiting list type of control group.

Moreover, in order to draw a comprehensive picture of the psychological mechanisms underlying the pain in the phantom limb, measurements of psychological variables were included: depression and anxiety symptoms, coping strategies, pain catastrophizing, body image representation. A series of studies show that depression symptomatology is associated with pain intensity [19] and that depression and anxiety symptoms persist several years after amputation [20]. Also, amputees tend to use negative cognitive and emotional coping strategies, which have a negative impact on the presence and intensity of pain [19, 21]. Furthermore, a number of studies have drawn attention to the negative effects of visual illusion and virtual reality therapies, seen as triggers of distorted bodily representation, given that the creation of an illusion of the amputated limb might exacerbate depression symptoms in amputees, causing them to experience persistent phantom limb pain [22].

Method

Design

This study is a controlled clinical trial, based on two study groups: an IVR intervention group and a kinesiotherapy intervention group. The twenty participants were randomly assigned to one or the other of the two groups. This study was approved by University of Medicine and Pharmacy "Iuliu Hatieganu " ethics committee.

Participants

The subjects were recruited from among patients who were admitted to the Surgery Clinic 2 in Cluj-Napoca between 2014 and 2017 and who had undergone lower limb amputation surgery. Twenty people were selected to participate in this clinical trial: 16 males (80%) and 4 females (20%). The participants were aged 49-87 years, most of them living in urban areas (85%), and they were distributed by the two intervention groups: the IVR group (n = 10) and the kinesiotherapy group (n = 10). Inclusion criterion: reported pain in the phantom limb. Exclusion criteria: neurological conditions or cognitive impairment.

Measurements

Pain. The *McGill Pain Questionnaire* (MPQ) [23] was used to evaluate pain in the phantom limb. The phantom limb pain subscale from the *Trinity Amputation and Prosthesis Experience Scale* (Gallagher, Desmond & MacLachlan, 2000) was also used.

Irrational cognitions. The Pain Catastrophizing Scale (PCS) [24], was used to assess irrational pain-related cognitions.

Body image. *Amputatee Body Image Scale* (ABIS) [25], was used to assess the relative presence of concern related to amputee's own body image.

Depression and anxiety symptoms. The *Hospital Depression and Anxiety Scale* (HADS) [26], was used to assess the severity of depression and anxiety symptoms.

Coping strategies. *The Cognitive Emotion Regulation Scale* (CERQ) [27], evaluated the presence of positive or negative coping strategies. The scale contains nine subscales: self-blame, acceptance, rumination, positive refocusing, focus on planning, positive reappraisal, putting into perspective, catastrophizing, blaming others.

Working procedure

Study participants were selected from among patients who suffered amputation in the lower limb in the period 2014-2017. They were contacted by phone and informed about the study. All those who wanted to participate signed an informed consent form.

In the first stage of the study, all participants filled in the initial assessment scales (pain, irrational cognitions, body image, depression and anxiety symptoms, coping strategies). The second stage started 1-3 days after the filling in of the questionnaires and consisted of three intervention sessions by each participant in the two groups (IVR and kinesiotherapy, respectively). The sessions were scheduled 1-2 days apart. The third stage was performed 1-3 days after the last intervention session and included post-intervention measurements for all participants.

Treatments

Kinesiotherapy intervention

The subjects were given three physiotherapy sessions of 30 minutes each, where they engaged in exercises for muscle toning in the lower amputated limb (leg and thigh);

Posture exercises, designed to release contraction and avoid vicious position of the stump, coupled with toning of the healthy lower limb muscles.

IVR intervention

Patients were given three IVR-based therapy sessions. The IVR software was developed specifically for this study, using the Unity 3D platform. The device comprised a pair of 3D virtual reality glasses for smartphones, fitted with adjustable aspherical lenses, a Bluetooth gamepad for IVR and a Lenovo K6 phone. In each session, the participants performed three repetitive tasks: placing the virtual representation of the phantom limb into a coloured, sequentially-illuminated square, hitting a virtual ball and lifting the leg to 45^0 to touch a virtual bar (**Figure 1, Figure 2, Figure 3).** First the patient was familiarized with the equipment. Hitting a virtual ball was the first task: the patient controlled the simultaneous movement of the virtual limb (by using the joystick) and the stump. The same principle was applied for the other 2 tasks. The sessions lasted 30 minutes each (**Figure 4, Figure 5**).

Statistical analysis

The entire set of collected data was statistically evaluated and the significance of each intervention was calculated. A mix Analysis of variance (mixt ANOVA) was conducted, where the within groups factor was the time (pre- and post-interventions) and the between groups factor, was the condition (IVR group and kinetotherapy group). The main effect (time*group, group, time) analysis were carried out in SPSS Version 24. Also, Pearson's linear correlations were calculated using SPSS Version 24.

Results

To test the effects of the interventions on the variables, we resorted to a mixed variance analysis, where time (pre- and post-intervention) was the *within-subjects* factor, and the group (IVR/ kinesiotherapy) was the *between-groups* factor.

Pain in the phantom limb

The results show that pain assessed with the MPQ was lower in the IVR group at post-assessment compared to the kinesiotherapy group. Statistical analysis has revealed a significant time*group interaction, $F(1, 10) = 23.965$, $p < 0.001$, $\eta^2 = 0.571$. Also, a time effect $F(1, 20) = 26.809$, $p < 0.001$, $\eta^2 = 0.598$ was recorded. There was no group effect ($p = 0.464$).

Quality of life

The quality of life measured by TAPES has shown significant post-intervention increase in patients in the IVR group compared to those in the kinesiotherapy group. A significant time*group interaction $F(1, 10) = 15.158$, $p = 0.001$, $\eta^2 = 0.457$ was recorded, coupled with a time effect $F(1, 20) = 23.684$, $p < 0.001$, $\eta^2 = 0.586$. The group effect was absent ($p = 0.707$).

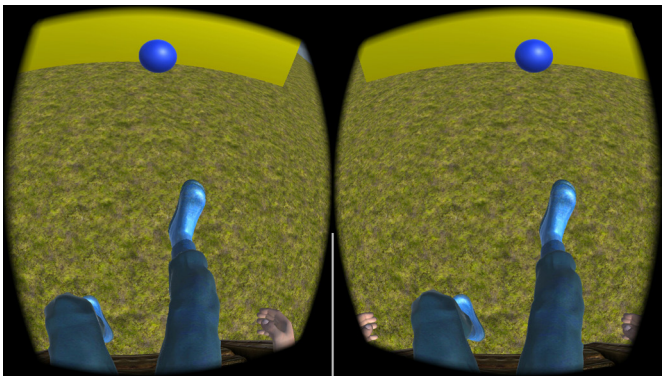

Fig. 1. First exercise - Hitting a virtual ball

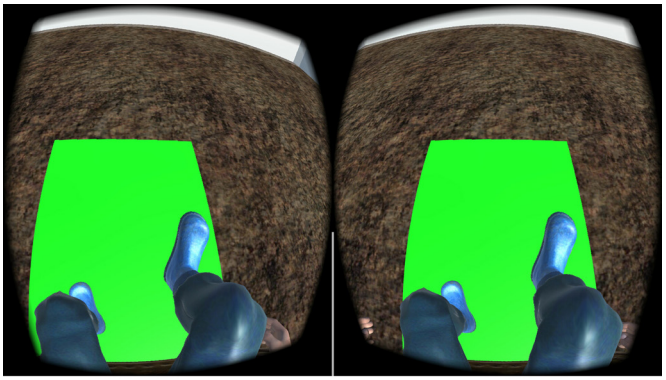

Fig. 2. Second exercise - Placing the virtual representation of the phantom limb into a colored, sequentially-illuminated square

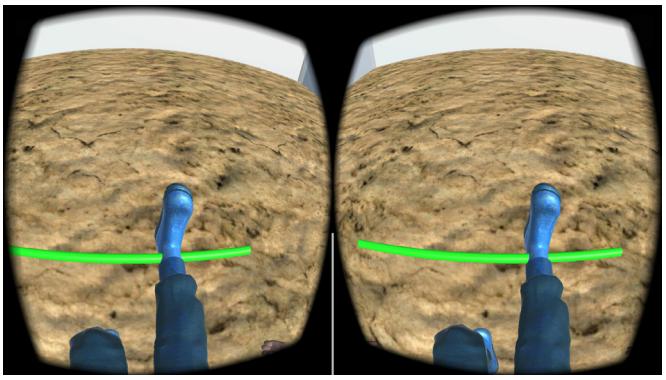

Fig. 3. Third exercise - Lifting the leg to 45° to touch a virtual bar

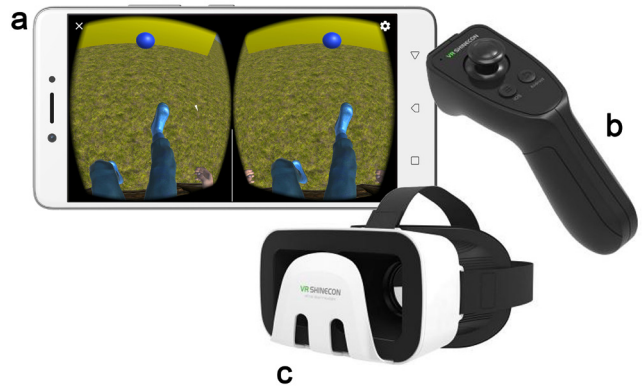

Fig. 4. IVR equipment: a. Lenovo Vibe K6; b. VR Shinecon bluetooth gamepad; c. 3D VR Shinecon glasses for mobile phone

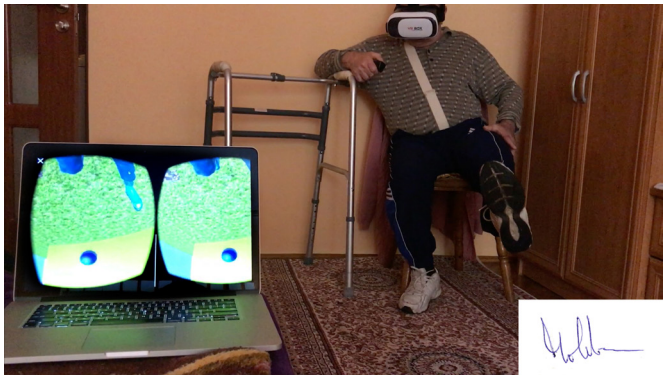

Fig. 5. Patient during IVR session

Depression and anxiety symptoms

Regarding depression symptoms, a time effect F (1, 20) = 6.560, p = 0.02, η^2= 0.267 was recorded. There was no significant time* group interaction (p = 0.404) and no group effect (p = 0.605).

As far as the anxiety symptoms are concerned, there was a time effect F (1, 20) = 9.511, p = 0.006, η^2= 0.346 recorded. No significant time*group interaction (p = 1) and no group effect (p = 0.941) were recorded.

Pain catastrophizing

The irrational cognitions related to pain catastrophizing were lower in subjects in the IVR group at post-assessment compared to those in the kinesiotherapy group. A significant time* group interaction, F (1, 10) = 12.975, p = 0.002, η^2= 0.419, was recorded. Also, a significant time effect, F (1, 20) = 18.845, p < 0.001, η^2= 0.511, was recorded. There was no group effect (p = 0.064).

Body image representation

Based on statistical analysis, no statistically significant effect occurred: time*group (p = 0.205), time (p = 0.058), group (p = 0.235).

Coping strategies

Higher scores were recorded on the positive coping strategies (positive refocusing and refocus on planning) in patients in the IVR group as compared to those in the kinesiotherapy group. However, there was an increase in the negative coping strategy (rumination) in the IVR patients compared to those in the kinesiotherapy condition.

Positive refocusing occurs when we think positive instead of thinking about our bad experiences. Studies show that positive refocusing strategy can be very beneficial to our wellbeing, while a low score on this strategy can be associated with a low level of emotional wellbeing. On this coping strategy, statistically significant results were obtained in terms of time*group interaction - (1, 10) = 23.456, p < 0.001, η^2 = 0.566, time – F (1, 20) = 17.618, p = 0.001, η^2 = 0,495 and group – F (1, 10) = 4.849, p = 0.041, η^2 = 0.212.

Refocus on planning occurs whenever we think of the steps we need to take in order to deal with a negative event or whenever we think of a plan to change a straighten up things. A high score, coupled with action, is associated with enhanced wellbeing, while a low score is a sign of emotional distress. In this study, statistically significant results were recorded on the time*group interaction, F (1, 10) = 23.529, p < 0.001, η^2 = 0.567, as well as a time effect F (1, 20) = 28.471, p < 0.001, η^2 = 0.567. There was no group effect (p = 0.143).

Ruminating is the situation when we are obstinately thinking of and are concerned about feelings and thoughts related to a negative event. A high score on this strategy is associated with emotional problems or with psychopathological symptoms. In term of rumination, statistically significant results were recorded on the time*group interaction, F = 6.661, p = 0.019, η^2 = 0.270. There were no time effects (p = 0.60) and no group effects (p = 0.772) recorded.

Pain correlations and psychological variables

No significant correlations between phantom limb pain and the various psychological variables in post-intervention measurements were identified: depression symptoms (r = 0.141), anxiety symptoms (r = 0.067), pain catastrophizing (r = -0.059). Likewise, no significant correlations were recorded between phantom limb pain and emotional and cognitive coping strategies: self-blame (r = 0.091), acceptance (r = 0.008), rumination (r = -0.132), positive refocusing (r = 0.046) (R = -0.001), refocus of planning (r = 0.077), putting into perspective (r = -0,194), catastrophizing (r = -0,008), blaming others (r = -0.256).

However, there were significant correlations recorded between anxiety symptoms and irrational pain catastrophizing thoughts (r = 0.545, p <0.005, two-tailed) and coping strategies: refocus on planning (r = -0.466, p <0.005, two-tailed) and pain catastrophizing (r = 0.784, p <0.001, two-tailed).

Discussions

A variety of treatments have been historically used to alleviate phantom limb pain, with unsatisfied efficacy. Recently, immersive virtual reality has been employed as a more

sophisticated mirror therapy.The purpose of this study was to investigate the extent to which an IVR-based intervention is more effective than an intervention based on kinesiotherapy in reducing phantom limb pain in patients with lower limb amputation. The results show that the IVR intervention is more effective, with pain alleviation in these patients recording significant levels. These results are consistent with those in some of the studies supporting the effectiveness of this method, though the major criticism is that such results come from case studies or single-subject experiments [28].

Moreover, the IVR-based intervention has also shown significant results in terms of psychological variables: increased quality of life, less irrational pain-related thoughts and positive coping strategies - positive refocusing and focus on planning. These data are very important, if we take into account the complex etiology of the pain in the phantom limb, and are underlining the positive psychological aspects that can be considered when designing multidisciplinary intervention programs.

Patients in the IVR group did not report significant decrease in their depression and anxiety symptoms, though a declining trend was seen in both groups. However, depression and anxiety symptoms in both groups recorded sub-clinical values. In addition, there were no significant concerns reported about the body image representation in the IVR patients compared to those in the kinesiotherapy group. These results are not consistent with those provided by the review conducted by Barbin et al. (2016), which suggests that an intervention based on a visual illusion of the missing limb, coupled with a virtual environment, may increase the amputee's concern about his or her body image.

Contrary to expectations, the IVR patients experienced a significant increase in the rumination coping strategy compared to those in the kinesiotherapy group. One possible explanation might be that this is a downside of the IVR therapy, which creates the illusion of an intact limb replacing the severed limb. IVR-based programs could take this aspect further into account and include psychological interventions that are specifically targeted to managing this particular kind of negative coping strategy. Again unlike expectations, depression symptoms appeared to be unrelated to the existence or intensity of pain, as it had been reported by some studies [19, 21]. On the other hand, correlations were identified between symptoms of anxiety, negative pain-catastrophizing and negative coping strategies. In this regard, future IVR-based programs should consider interventions aimed at reducing anxiety in people with phantom limb pain.

One of the main limitations of this study is the small number of the study sample (N = 20), which, though understandable for a pilot study on a new type of intervention, provides results that need to be treated with caution, being regarded as merely preliminary encouraging evidence. Future studies should validate these hypotheses on a larger and hence more representative sample. Moreover, future studies should assess the extent to which the results are replicable. Results should also be interpreted with caution, because the study did not include a passive control group of placebo or waiting list type.

Conclusions

The therapy based on immersive virtual reality (IVR) represents an efficient alternative in the management of phantom limb pain, achieving a significant reduction or even complete remission of pain. Through the positive impact IVR has upon the quality of life, coping, and irrational thinking, it increases the rehabilitation compliance and allows for an early recovery.

Across therapeutic disciplines, the evidence-base for treating phantom pain is fragile. Randomized controlled trials and systematic reviews are rare, and where they do exist, the conclusions are not encouraging. Recent progress includes the development of treatments, that directly target cortical mechanisms which have been linked to phantom pain. The initial data for IVR therapy is encouraging, but limited. The implementation of large, randomized, double-blinded, multicenter, placebo controlled trials, will be the focus of the future work.

Conflict of interest

None to declare.

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Acta Medica Marisiensis 2017;63(3):121-124 DOI: 10.1515/amma-2017-0016

RESEARCH ARTICLE

Reduced Analgesics Consumption and Pain Intensity after Injections with a New Hyaluronic Acid in Patients with Knee Osteoarthritis

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Objective: To determine the influence of a new intraarticular hyaluronic acid based hydrogel (Hymovis®) injections on the amount of analgesics consumption in patients diagnosed with primary knee OA. **Methods**: A prospective, single-center study that included 35 patients, aged 45-80 years was conducted in our orthopaedics department. Patients received two intraarticular injections of hyaluronic acid (24 mg/3 ml; 500–730 kDa; Hymovis®) at one week apart. Follow-up was scheduled at 2 and 6 months after the injections. Assessment tools included Visual Analogue Scale (VAS) and an in-house designed questionnaire regarding analgesic consumption (quantity, period and product) during the follow-up. **Results**: Compared to baseline, a significant amelioration in visual analogue scale was observed at six months' follow-up (74.2mm ± 11.7 vs. 57.3mm ± 12.1; $p < .0001$). 28% (n=10) of the patients reduced their total analgesic consumption at two months after the injections. At final follow-up, the analgesic intake was reduced by more than 50% in almost every case. **Conclusions**: Intraarticular administered injections with a novel hyaluronan-based hydrogel (Hymovis®) may reduce the amount of analgesic consumption and self-reported pain intensity in patients with knee OA.

Keywords: novel hyaluronan, knee osteoarthritis, analgesics consumption, intraarticular injections

Received 22 March 2017 / Accepted 19 May 2017

Introduction

Primary knee osteoarthritis (OA) is commonly occurring after transformations in biomechanical and biochemical properties of the articular cartilage. It is considered the most common chronic joint disorder that leads to functional deficit in the affected joint and impairment in daily quality of life. There are different conservative treatment methods in early and mild osteoarthritis, but many of them have proven to be ineffective or with small beneficial influence in relation to the normal evolution of the disease [1]. However, intraarticular injections with hyaluronic acid (HA) are considered effective in early stages of the disease, exerting positive and beneficial changes within the affected joint not only by its lubrication effect but acting as a cell, cytokine and anti-inflammatory mediator in the synovial fluid [2-6]. Numerous studies sought to asses and compare the clinical effectiveness of different injected HA products in the osteoarthritic knee, many of them providing positive results. Variables such as molecular weight, number of administrations, amount of hyaluronic acid per ml of product may influence the expected clinical results [7-11]. Several authors demonstrated that intraarticular injected low-molecular weight HA products pass easier through the synovial membrane, exerting a stimulating effect on the endogenous synthesis of high-molecular HA [9, 10, 12]. Compared to other intraarticular injected substances (e.g. corticosteroids) HA products have their analgesic effects persisting for extended duration but installed with a longer on-set [13, 14, 15]. According to guidelines from 2014 Osteoarthritis Research Society International (OARSI) all patients diagnosed with OA should be prescribed analgesics as a first line treatment [16]. Given the side-effects of common analgesics used in knee osteoarthritis (paracetamol, ibuprofen, naproxen, codein, tramadol etc.) their consumption is of great interest for clinicians [17]. Our objective sought to determine if intraarticular HA injections (Hymovis®) reduce the amount of analgesics consumption in patients diagnosed with primary knee OA.

Methods

A prospective, single-centre, clinical trial was conducted in our Department of Orthopaedics and Traumatology from Tirgu-Mures County Hospital, Romania. Local ethical committee approval was obtained. After signing the informed consent, patients diagnosed with primary knee OA received intraarticular injections of 24 mg/3 ml of HA (500–730 kDa; Hymovis®, Fidia Farmaceutici S.p.A, Italy). All patients received two injections at one-week apart. Follow-up visits were scheduled at 2 and 6 months after the injections.

Inclusion criteria were predefined as follows: (a) men and women aged 45-80 years, suffering from (b) primary idiopathic knee OA for at least 6 months, (c) radiological knee OA Kellgren-Lawrence grade II or III, (d) minimum 35 mm on the VAS, (e) daily consumption for more than 3 months of one of the following: paracetamol (up to 2g/day), ibuprofen (up to 1200mg/day) and weak opioids (tramadol, codein). Patients that met our inclusion criteria were informed about our study design and objective at the same time they signed the informed consent. Recommendations from Osteoarthritis Research Society International

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(OARSI) from 2014 suggest that knee-only osteoarthritis without comorbidities should be managed with acetaminophen (Paracetamol) or non-selective NSAIDs (i.e Ibuprofen) orally [16]. As the OARSI recommendation for weak opioids (oral) is still marked as "uncertain", they are still prescribed for the majority of patients as a second or third-step conservative treatment option when treating mild to severe knee osteoarthritis due to positive results in current systematic reviews [18]. According to the literature, these three analgesics are also among the most prescribed medications when treating knee osteoarthritis, and that is the reason for making it a major inclusion criterion [16, 19].

Exclusion criteria were as follows: (a) conditions other than primary idiopathic knee OA, (b) any type of intra-articular injections in the previous 6 months, (c) heparin or platelet anti-coagulation treatment in the last month, (d) non-steroidal anti-inflammatory drugs (NSAIDs) usage 7 days prior to injection, (e) allergy to HA injections, (f) systemic diseases that may influence the results, (g) presence of any infection or pregnancy and lactation. Primary idiopathic gonarthrosis has the highest prevalence in the etiology of this particular disease, therefore, patients that presented other etiologies that add up biases that interfere with our results were excluded from the trial ((i) post-traumatic osteoarthritis, (ii) birth defects in joint geometry that may lead to osteoarthritis, (iii) chronic corticosteroid infiltrations that lead to cartilage erosions and finally to osteoarthritis) [20].

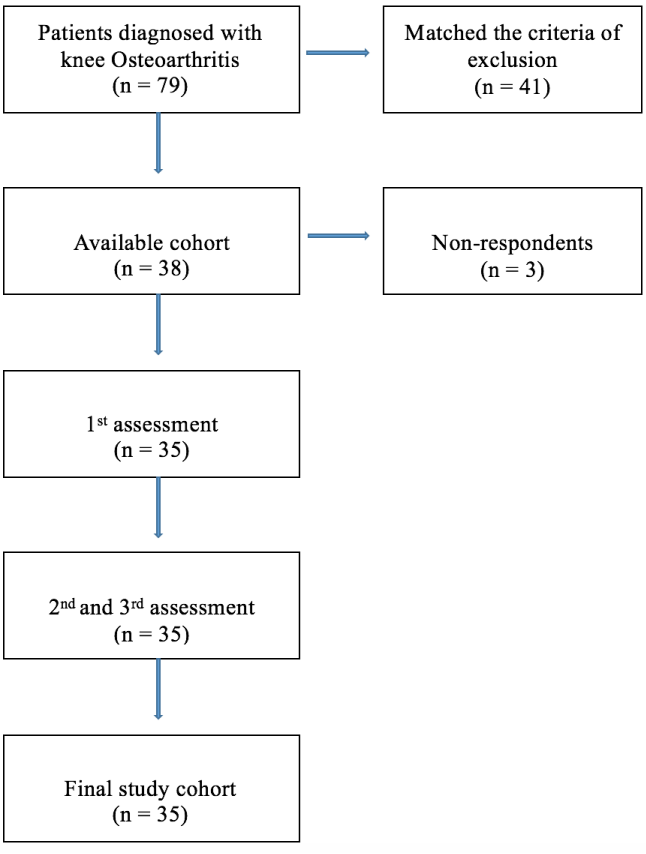

Fig. 1. Flow diagram of study cohort selection

After completing the required registration forms, thirty-five subjects were enrolled and received two injections of HA. Excluded patients and final cohort is presented in Figure 1.

An in-house made questionnaire regarding previous analgesic consumption (quantity, period and product) was completed by each patient before the first injection and at two and six months after. The changes were assessed by comparing any change in analgesic consumption since the previous visit. Visual Analogue Scale (VAS) was completed along with our in-house made questionnaire at each scheduled follow-up meeting. Every patient rated his pain by drawing a mark on a horizontal line of 100mm; afterwards the distance was measured by our study nurse. The score ranges from 0 to 100 on a 100mm scale. The scale is most commonly described by "no pain" (score 0) and "pain as worse as it could be" or "worst imaginable pain" (score 100). VAS is a consistent and validated instrument in measuring chronic pain intensity [21, 22, 23]. As 38% of Mures population consists of Hungarians [24], the questionnaires used in the study were translated into two languages: Romanian and Hungarian.

GraphPad (InStat) and EpiInfo v 7.1.4.0 (Centers for Disease Control and Prevention, Atlanta, USA) softwares were used to analyze data (chi-square test and student t-test). The level of statistical significance was set at $p<0.05$.

Results

Gender distribution and demographic data are presented in Table I. There were no correlations between smoking status and analgesics consumption reduction or VAS decline.

28% (n=10) of patients reduced their analgesic consumption compared to baseline at two months follow-up. At the final follow-up the reduction was over 50% in most cases. In six cases (17%) patients completely stopped analgesics intake. Rescue analgesic intake was reported by twelve patients.

The baseline VAS score was 74.2 ± 11.7 before the treatment. At the second follow-up the pain score decreased to 69.6 ± 9.8 with no statistically significant difference from baseline (Table II).

VAS improved significantly from 69.6 ± 9.8 at two months to 57.3 ± 12.1 at the end of the study ($p< 0.0001$). No major complications were reported during the follow-up. Minor adverse effects included arthralgia and pain at the injection site.

Table I. Demographic Data

Age (years), mean ± SD	63.2 ± 8.1
Height (cm), mean ± SD	168 ± 8.2
Weight (kg), mean ± SD	82.2 ± 16.2
Sex, male/female	10/25
Smokers	14

Table II. Analgesic consumption and VAS results

	Baseline	First follow-up (2 months)	Second follow-up (6 months)	P value*
Analgesic consumption reduction, mean %	n.s	35%	58%	-
Visual Analogue Scale, mean ± SD	74 ± 11	69 ± 9	57 ± 12	<.0001

n.s – not significant; * P value – comparison between the first and second follow-up

Discussions

Our study demonstrates that two injections of low-molecular weight HA may reduce the daily analgesic consumption in patients diagnosed with knee OA by more than 50% in a six-month period. Additionally, Hymovis® injections proved to be a safe, successful and well-tolerated treatment in mild to moderate knee OA. VAS score reduction was correlated with the reduced consumption of analgesics at the end of the study, showing that the intensity of pain is in relationship with the amount of self-administered analgesics.

The product used in our study has proven to inhibit the expression of degrading enzymes (MMP1, MMP13, ADAMTS5) and inflammatory mediators (IL6, PTGS2) [25]. In 2011, Finelli et al. published an experimental comparative study in which they compared the rheological properties of different HA products; from the variety of gel-like HA products presented in their research, Hymovis® was the only product with the capability to completely recover its viscoelastic properties after several cycles of mechanical stress [26]. Benazzo et al. studied the efficacy of Hymovis® on 49 patients with clinical and radiological confirmed knee OA. At one-year follow-up, stiffness, physical function and pain were significantly improved [27]. They concluded that patients who received two cycles of Hymovis® had reduced pain for up to twelve months post-injection. Additionally, only 26% of patients had radiological progressed OA compared to baseline after two cycles of HA injections. Conrozier et al. also questioned the amount of analgesics consumption after injecting HA of different molecular weight in knees affected by OA [28]. Their results were similar to ours; they reported a reduction in analgesics intake by more than 50% for every patient. Pain reduction was an interesting point to study in our trial. Even though pain intensity was only assessed using a validated subjective method (VAS), outcomes at six months follow-up were promising. According to VAS results, HA treatment was able to ameliorate the existing pain in knee OA. Intraarticular injected HA therapy shown to have prolonged effects in relieving pain compared to other similar treatments. This is consistent with other published studies to date [14, 29, 30].

Compared to other treatments for alleviating pain, Hymovis® proved its safety in patients with knee OA, having no adverse effects related to the product. Pain and swelling at the injection site were not considered to be product-based adverse or secondary effects.

Our study also had limitations that should be mentioned. The small sample of patients and absence of a control group may be considered limitations for the study design. Moreover, the tools used to assess reported pain might be exposed to biases in orthopaedics due to their subjectivity. Pain intensity and analgesics consumption were the only variables studied due to that fact that United States Food and Drug Administration Draft Guidance for Clinical development of Drugs, Devices and Biologic products, recommends that this should be a variable to be assessed when testing a new product in this particular type of disease [31]. Studies based on larger cohorts are required to strengthen the evidence regarding pain management in this type of treatment.

Conclusions

Two intraarticular injection cycles of the novel hyaluronan (Hymovis®) could reduce the amount of analgesics consumption and this type of treatment may be considered viable in patients with mild to moderate knee OA. Future studies should aim to assess the functional and biomechanical benefits induced by this new HA-based hydrogel.

Conflict of interest

None declared.

Acknowledgments and funding

This research was supported by the University of Medicine and Pharmacy of Tirgu Mures with financial support from S.C. Angelini Pharmaceuticals Romania S.R.L through the grant 15270/03.11.2015.

We thank Michael Hirschmann, MD, PhD for assistance with the development of our study methodology, and for his comments that greatly improved the manuscript.

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Acta Medica Marisiensis 2017;63(3):125-128 DOI: 10.1515/amma-2017-0017

RESEARCH ARTICLE

Changes in Knee Joint Space Width in Treatment with a New Hyaluronic-Based Hydrogel

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Objective: Our purpose was to assess the effect of a new hyaluronic acid-based (Hymovis®) injections on joint space width narrowing in patients diagnosed with knee osteoarthritis. Methods: A prospective clinical trial was conducted in the Department of Orthopedics and Traumatology II from the Clinical County Hospital, Tîrgu Mureş, Romania. Thirty-five patients diagnosed with idiopathic knee osteoarthritis received two intraarticular injections with hyaluronic acid-based hydrogel (24 mg of hyaluronic acid/3 ml) at one-week interval. Anteroposterior radiographs were obtained before the injections, at six and twelve months after. Minimum joint space width was measured by two senior orthopaedics surgeons at each follow up. Each radiograph was measured again by the same evaluators two weeks apart. Results: Thirty-one patients were present at the final follow-up. A minor reduction in mean weight was noticed (from 82.2 kg ± 16.2 kg to 80.9 kg ± 16.0, $p > 0.398$) without any correlation with joint space width narrowing. There were no major changes at the first follow up (6 months) regarding joint space narrowing. A reduction in joint space width was observed however at 12 months varying from 4.4 mm (SD ± 1.64, range 1.8-7.1) at the first assessment to 4.3 mm (SD ± 1.26, range 0.0-6.8) at the final follow-up but with no statistical difference (p=0.237). Conclusion: No significant modification in joint space width at the final follow-up secondarily proved that two injections of Hymovis® may slow down narrowing in the knee joint space over a one-year period.

Keywords: joint space width, hyaluronic acid, knee osteoarthritis

Received 18 April 2017 / Accepted 24 May 2017

Introduction

Knee osteoarthritis (OA) is considered one of the most common degenerative diseases with increasing prevalence in the elderly over the past decades [1]. Studies suggest that 10% of men and 13% of women aged 60 or older suffer from symptomatic knee OA [2]. A rise in OA incidence is expected due to an increase of obesity prevalence and a growing elderly population [3]. Even with the increased prevalence there are only a few therapies that may prevent cartilage degeneration. Treatment management comprises a succeeded combination of pharmacological and non-pharmacological alternatives, based on different variables such as age, physical activity, stage of the disease and associated pathology [4, 5]. One of the common therapies used in the early and mild stages of the disease are intraarticular injections with hyaluronic acid (HA) [5]. Studies revealed that HA leads to a positive and beneficial change within the affected joint by its mechanic effect (lubrication), its chondroprotective mechanism and by acting as a cytokine and anti-inflammatory mediator in the synovial fluid [6-9]. It is also considered that viscosupplementation with hyaluronic acid based products may reduce the time to total knee arthroplasty for up to 8.7 months [10]. Cartilage loss assessment and joint space width (JSW) measurements using serial standardized radiographs are important instruments in the diagnosis, treatment and progression evaluation of knee OA [11-13]. Tibiofemoral JSW measurement is recommended by Osteoarthritis Research Society (OARSI) as the main measurement tool in assessing the biological progression in OA [14]. The United States Food and Drug Administration recommends the use of joint space narrowing (JSN) as a primary evaluation tool in the progression of osteoarthritis in trials with medication that aims to reduce cartilage loss and degeneration. An increase in JSW (i.e. widening of the joints space) at a study endpoint may suggest a new or regrown cartilage and should be the aim of structural outcomes in these trials [15]. The main endpoint used in current clinical trials and large cohort studies is the minimum joint space width (mJSW) and it is considered an indirect measurement tool for cartilage thickness on both medial and lateral compartments of the tibiofemoral joint [14]. A novel biochemical innovation was capable to confer increased viscoelasticity and residence time to natural HA polymers, while maintaining its biotribological characteristics [16]. This innovative technology was implemented in a new molecule "HYADD®4", presenting an unprecedented HA-based hydrogel (Hymovis®) on the market. As it is a new product, its effects on mJSW are not yet extensively described, consequently, our aim was to assess the effect of the new HA derivative Hymovis® injections on mJSW in patients diagnosed with knee OA.

Methods

A prospective, single-center, clinical trial was conducted in a university hospital (Department of Orthopedics and Traumatology II from the Clinical County Hospital, Tîrgu Mureş, Romania). The study was approved by the local ethical committee and performed in compliance with the

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principles of Good Clinical Practice and the Declaration of Helsinki concerning medical research on humans and the country-specific regulations.

Inclusion criteria were male and female individuals aged 45-80 suffering from idiopathic knee OA for at least 6 months and diagnosed with radiological knee OA Kellgren-Lawrence grade II or III [17]. Exclusion criteria were the following: conditions other than primary idiopathic OA, any type of intra-articular injections in the previous 6 months, heparin or platelet anti-coagulation treatment in the last month, non-steroidal anti-inflammatory drugs (NSAIDs) usage 7 days prior to injection, allergy to HA injections, systemic diseases that may influence the results, presence of any infection or pregnancy and lactation. Out of 79 subjects that were screened and diagnosed with knee OA, 41 matched one or more of our exclusion criteria and were not included in the study. Due to education limitations, three individuals were unable to fill in the forms necessary for enrolment and were excluded. Patients that met our inclusion criteria were informed about our study design and objective and after signing the informed consent they received two consecutive intraarticular injections of 24 mg/3 ml of (500–730 kDa) modified HA (Hymovis®, Fidia Farmaceutici S.p.A, Italy) at one-week interval. As the anterolateral portal proved to be less painful in intra-articular administration of treatments [18], all injections were performed in the outpatient clinic under aseptic conditions in the aforementioned location. Follow-up visits were scheduled at 6 and 12 months after the injections. Conventional digital anteroposterior (AP) radiographs of the tibiofemoral joint were obtained before the infiltration and at each follow-up visit. At 48 hours after each infiltration, every patient was telephoned and questioned about local inflammatory signs, in order to assess the local tolerability and safety. A study nurse was responsible for the patients' evaluation and data collection.

Radiographs JSW analysis

Standard digital tibiofemoral, weight bearing AP digital radiographs were obtained in full joint extension at the time of enrollment in the study, at six and twelve months after the injections. Standing extended weight bearing radiographs were used in order to achieve maximum standardization and have the ability to properly grade and classify disease advancement. Moreover, in Romania, for economic reasons, the first X-ray examination is made this way in order to assess the type of prosthesis must be prepared in case of future surgery, a type that is influenced by the degree of instability. Also, according to EULAR recommendations, radiographic views are important for optimizing detection of OA features; in particular for the knee, weight-bearing and patellofemoral views are recommended [13]. Lequesne's method of measurement was used to determine the joint space narrowing (JSN) [19]. The radiographs were AP aligned and two senior orthopedic surgeons separately and electronically measured the mJSW differences using the OSIRIS image processing software (University Hospital of Geneva). Each physician repeated the radiograph measurements after two weeks. A mean result from the two measurements and assessors was calculated. As the two senior surgeons involved in radiographic evaluations were similar of age and experience and were trained in the same facility under the surveillance of the same supervisor, only inter-rater and intra-rate agreement was assessed.

Statistical Analysis

GraphPad (InStat) and EpiInfo v 7.1.4.0 (Centers for Disease Control and Prevention, Atlanta, USA) were used for the statistical analysis. mJSW changes from baseline were compared with the outcomes at 12 months post-therapy using Chi-square test and paired T test. Considering the relatively short follow-up period, a change of >0.3mm on the final radiograph was interpreted as further progression of OA.

Results

Out of the initial cohort (n=35) four individuals were missing from the final radiograph evaluation and their results could not be assessed. The final study cohort contained 31 patients. Baseline demographic and patient characteristics are presented in Table I. Usage of NSAIDs involved daily consumption of ibuprofen (up to 1200mg/day) and/or paracetamol (up to 2g/day) or rescue intake of weak opioids (tramadol or codein), paracetamol and/or ibuprofen.

There was no correlation between gender, smoking status, weight and mJSW changes. However, minor changes were reported regarding patients' characteristics at the final follow-up: a slight decrease in mean weight was observed (from 82.2 ± 16.2 to 80.9 ± 16.0, $p > 0.398$); and self-reported NSAIDs usage was reduced in 17 patients. OA related characteristics are shown in Table II.

Two patients in the left knee group progressed from OA grade 2 Kellgren and Lawrence (KL) to grade 3 at the end

Table I. Baseline demographic and patient characteristics

Characteristic	
Female, no. (%)	21 (68)
Weight, kg, mean ± SD	82.2 ± 16.2
Age, years, mean ± SD	63.2 ± 8.1
Smoker >1 year, yes, no. (%)	12 (39)
Weight bearing workplace, yes, no. (%)	16 (52)
Usage of NSAIDs*, yes, (%)	31 (100)
History of corticosteroid injections >6 mo.**, yes, no. (%)	6 (19)

*NSAIDs - nonsteroidal anti-inflammatory drugs; **mo. - months

Table II. Disease related characteristics

Characteristic	Left knee (n=17)	Right knee (n=14)
Kellgren and Lawrence grade, no. (%)		
2	8 (47)	6 (43)
3	9 (53)	8 (57)
Morning stiffness, yes, no. (%)	13 (76)	11 (79)
Medial compartment*	12 (71)	11 (79)
Lateral compartment*	5 (29)	3 (21)

* based on radiographic predominance of cartilage changes

Table III. mJSW at baseline and at each follow-up

	mJSW at baseline (mm, ± SD)	mJSW at 6 months (mm, ± SD)	mJSW at 12 months (mm, ± SD)	Average mJSW change from baseline***
Mean mJSW* KL** grade				
2	4.7 ± 1.13	4.7 ± 1.08	4.5 ± 1.19	0.23 mm
3	4.2 ± 2.15	4.2 ± 2.22	4.1 ± 1.33	0.18 mm

*minimum joint space width; ** Kellgren and Lawrence

of the trial. No significant change was noted regarding mJSW at the first follow-up (6 months). A reduction in mJSW was observed varying from 4.4 mm (SD ± 1.64, range 1.8-7.1) at the first assessment to 4.3 mm (SD ± 1.26, range 0.0-6.8) at the final follow-up (p=0.237). Inter-rater agreement was 87% for the first radiographic assessment and 89% for the second one. Intra-rater error for the two assessments was 5.8% and 5.6% respectively. Measurements for each follow up are presented in Table III.

Discussions

Based on our single-center 12 month follow-up trial results there were no major changes in mJSW in patients treated with the intraarticular hydrogel Hymovis®. These stagnant mJSW values might validate the hypothesis that hyaluronic acid based products have a diminishing effect in the progress of knee OA. In a systematic review analyzing different chodroprotective therapies, Gallagher et al. concluded that JSW changes were ameliorated only in 1 out of 3 studies that assessed HA injections compared to placebo [20]. In a case control study, 60 patients were evaluated for radiographic changes (osteophyte, JSW narrowing, joints space area, tibiofemoral angle) and knee pain after intraarticular injections with HA acid [21]. At a mean follow-up of 2.9 years, medial and lateral compartments joint space areas were significantly bigger in case patients compared to controls. However, in the same study, they concluded that mJSW was not influenced by intraarticular infiltrations with HA. In a phase III, 12-month clinical study that evaluated the same product (HYMOVIS®), Benazzo et al. found similar results to ours, with a slight reduction in JSW (from 4.6 mm at baseline to 4.2 mm at one-year follow-up. In contrast to our results, they found a significant reduction in morning stiffness ($p < 0.001$) at 3 months after viscosupplementation [22]. A frequently encountered debate in the literature addresses the molecular weight (MW) of the HA product injected. It was previously demonstrated that HA products with a MW of 500-730 kDa pass easily through the synovial membrane stimulating the endogenous synthesis of HA [23]. It is also specified that this may lead to a large quantity of high molecular HA to be secreted in the synovial liquid [24]. Also, the quality and quantity of the native HA synthesized by the human synovial fibroblasts is determined by the structure and provenance of the HA existent in the extracellular environment [25]. Rheological and viscoelastic properties have an essential function in HA products with low molecular weight. Ambrosio et al. managed to obtain a wide range of rheological behaviors on low molecular weight products, from dilute solutions to hydrogels [26]. Our study outcomes reported at one year may be considered limited by some authors, as changes in cartilage and periarticular structures may occur over longer periods in knee OA. However, a stalled advancement of cartilage destruction and disease progression may be recognizable even after shorter periods [27]. Even with the existent synergic results, analgesics and NSAIDs usage was studied as an independent variable in our study due to the fact that our therapy is considered a second-line treatment after selective and non-selective NSAIDs [5]. The lack of a control group is a limitation for the current work and sample size calculation results could not be implemented due to limited finances the relatively small sample of patients. Additionally, due to financial limitations no extra-radiographs could be added to the ones that are standardized for orthopedic assessment in our department. Upcoming trials investigating mJSW on a screening-based evaluation in larger cohorts with extended follow-up intervals might offer further evidence regarding OA radiologic progression during this type of treatment.

Conclusions

There were no major variations in joint space width at the final follow-up, secondarily proving that two injections of Hymovis® may slow down narrowing in the knee joint space in a 12-month period. However, a strong encouraging conclusion could not be drawn due to the gradual advancement of radiologic changes characteristic for the disease.

Acknowledgments and funding

This research was supported by the University of Medicine and Pharmacy of Targu Mures with financial support from S.C. Angelini Pharmaceuticals Romania S.R.L through the grant 15270/03.11.2015.

Conflict of interest

None to declare.

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Acta Medica Marisiensis 2017;63(3):129-132 DOI: 10.1515/amma-2017-0024

RESEARCH ARTICLE

Assessment of Post-Occlusive Reactive Hyperaemia in the Evaluation of Endothelial Function in Patients with Lower Extremity Artery Disease

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Background: The aim was to assess endothelial function with photoplethysmography (PPG), by post-occlusive reactive hyperaemia (PORH) combined with alprostadil challenge test in patients with peripheral artery disease (PAD). **Methods**: Forty-nine PAD patients stage II-III Fontaine (39 male, 10 female, mean age 68.45±5.86 years) and a control group of 49 healthy individuals (24 male, 25 female; mean age 25.1±3.8 for a young subgroup; 71.0±0.16 years for an elderly subgroup) were included. Ankle-brachial index (ABI) was assessed at baseline, peripheral perfusion (PP) and PORH were assessed at baseline and after the 30 minutes administration of parenteral alprostadil. **Results**: After 3 minutes of arterial occlusion, peripheral perfusion increased from 0.69±0.94 mV/V to 2.27±2.42 mV/V ($p<0.0001$). After alprostadil challenge, peripheral perfusion increased from 0.84±1.24 mV/V to 4.52±3.52 mV/V ($p<0.0001$). In controls PP was 2.4±1.7 mV/V versus 3.8±1.5 mV/V, $p<0.0001$. **Conclusion**: In patients with PAD, an increase in PORH after alprostadil challenge due to the release of nitric oxide (NO), provides information on the endothelial function and could reflect the presence of collaterals. In the healthy control group, the increase in PORH could reflect the integrity of main arterial branch. In PAD patients with an increase in PORH, conservative therapy should be preferred over surgical revascularisation.

Keywords: peripheral perfusion, post-occlusive reactive hyperaemia, alprostadil

Received 31 March 2017 / Accepted 22 June 2017

Background

The prevalence of peripheral arterial disease (PAD) of the lower limb is rising and it is still underdiagnosed [1]. Despite a decrease in ankle-brachial index (ABI), only one-half of the population with occlusive atherosclerotic vascular disease is symptomatic [2]. This compensatory mechanism might be present in case of progressive arterial occlusion, accompanied by the development of collateral blood vessels that by-pass the stenotic lessions [3]. The growth of these small arteries–called arteriogenesis is triggered by the shear stress due to arterial occlusion [4]. So far, to assess collateral circulation, several high-cost, invasive diagnostic methods have been proposed, but their use at the primary care level is still limited. Therefore, there are several studies searching for a reliable, non-invasive method to assess the presence of collateral microcirculation [5, 6, 7, 8]. In this field, the non-invasive vascular reactivity test, called post-occlusive reactive hyperaemia (PORH) gained attention. This procedure is based on the ability of the endothelium to release vasodilator agents like nitric oxide (NO) as a response to proximal arterial occlusion [9]. After occlusion, when the peripheral perfusion (PP) is restored, flow is increasing because of the reduced vascular resistance. An inexpensive method, the photoplethysmography (PPG) has been proposed to determine theese blood volume changes [10]. This optical technique allows detecting blood volume changes during a cardiac cycle, which is recorded by a sensor consisting of a light source and a photo detector, and is represented as perfusion units in mV [7].

According to the International guidelines on management of patients with PAD, administration of a prostaglandin E1 (PGE1) for 7 to 28 days, facilitate ulcer healing in patients with critical limb ischemia (CLI) [11]. Therefore the aim of the study was to assess endothelial function in patients with PAD using PPG, and to evaluate whether post-occlusive reactive hyperaemia combined with alprostadil-challenge test is a suitable method to identify patients who are candidates for conservative therapy.

Methods

A number of 98 subjects were included in our study: 49 subjects (39 male, 10 female) diagnosed with lower extremity artery disease stage II-III Fontaine, and a healthy control group of 49 subjects (24 male, 25 female); the control group was devided in 2 subgroups of young and elderly individuals. In the PAD group the mean age was 68.45±5.86 years. In the young control group, the mean age was 25.1±3.8 years while in the elderly group 71.0±0.16 years. All of them gave written informed consent and the study was approved by the Ethics Committee of the County Clinical Hospital, Tîrgu Mureş, Romania. Inclusion criteria were: diagnosis of PAD - according to the current

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guidelines of the European Society of Cardiology (ESC): ABI<0.9, multiple arterial stenotic lesions or total iliofemoral or femoral-popliteal arterial occlusions confirmed on duplex ultrasound. Patients with connective tissue diseases, lymphoedema, and venous thrombosis were excluded from the study. Intermittent claudication was evaluated according to the Edinburgh Claudication Questionnaire [12]. Ankle-brachial index and PORH test were performed at the inclusion. Smoking and caffeine cessation was required for at least two hours before testing. Patients were placed in supine position for at least ten minutes prior to measurements. During examination, room temperature was maintained at 23-24°C. Ankle-brachial index was determined with a 5-10 MHz handheld Doppler device (BiDop ES-100V3 Hadeco®). The PPG measurements were performed with Bidop ES-100V3® hand-held Doppler and transmitted to the Smart-V-Link® software. The sensor was fixed with an adhesive pad on the plantar surface of the affected leg's hallux. A pneumatic cuff was placed proximal to the pad. After baseline peripheral perfusion (PP_b) recording, blood flow was interrupted for a period of three minutes by inflation of the cuff with 20 mmHg above systolic pressure. After cuff deflation at maximum speed, post-occlusive flow was measured for further 4 minutes (1 measurement /minute). Post-occlusive reactive hyperaemia was denoted as the maximum value of the four measurements performed at one minute intervals ($PORH_b$). After obtaining the $PORH_b$ value, intravenous alprostadil (10μg/kg/min) was administrated for 30 minutes. Peripheral perfusion and post-occlusive reactive hyperemia were again determined after alprostadil challenge test (PPc, PORHc). Patients with a lack of increase of peripheral perfusion by at least 50% after administration of alprostadil, were categorized as non-responders (NR) and were referred for surgical revascularization. In the control groups, only baseline measurements were performed (ABI, PP_b, $PORH_b$), alprostadil challenge test was not performed.

Patients' characteristics were collected as raw data. Numerical data were represented as mean±SD. Means were compared using t-test for continuous variables. Correlations were studied using Pearson's correlation test for data presenting Gaussian distribution. Statistical analysis was performed with Graph Pad Instat program. Statistical significance was set at p≤0.05 with confidence interval of 95%.

Results

Among responders ABI was 0.4±0.2, and in non-responders 0.30±0.22. In young controls, ABI was 1.27 ±0.5, and in elderly controls 0.99±0.16.

In responders, the peripheral perfusion increased by 1.58 mV/V (from 0,69 mv/V to 2,27 mV/V) after occlusion (Figure 1) and after vasodilator challenge, peripheral perfusion increased by 3.68 mV/V (from 0,84 mV/V to 4,52 mV/V) (Figure 2). In responders, we found no correlation between the ABI and PORH after alprostadil challenge, p=0.83 (Figure 3).

In the non-responders group, PPb versus PORHb was: 0.6±0.85 mV/V versus 0.67±0.78 (p=0.09) and after alprostadil challenge PPc versus PORHc was: 0,68±1.18 mV/V versus 0.74±1.13 mV/V (p=0.08) (Figure 4).

In the young controls group, PPb versus PORHb was 3.7±2.25 mV/V versus 5.3±2.8 mV/V (p<0.0001), while in older controls 2.4±1.7 mV/V versus 3.8±1.5 mV/V, p<0.0001.

Discussion

In our study we evaluated endothelial function as a vasodilator response to arterial occlusion with the non-invasive finger PPG in patients with lower extremity artery disease and critical ankle-brachial index. We found that in patients with PAD, despite the low ABI, post-occlusive peripheral perfusion increased significantly and this vasodilator effect was more pronounced after alprostadil challenge. In the young control group, as well as in elderly patients without PAD, despite the preserved values of ABI, we also observed an increase in peripheral perfusion after arterial occlusion. In our experience, these findings could be explained by the fact that in PAD patients the increase in peripheral perfusion after alprostadil could reflect the endothelial function of the collateral circulation that by-pass the stenosis. In patients with critical ABI, proximal arterial obstruction and the increased shear stress that occurs, trigger a series of

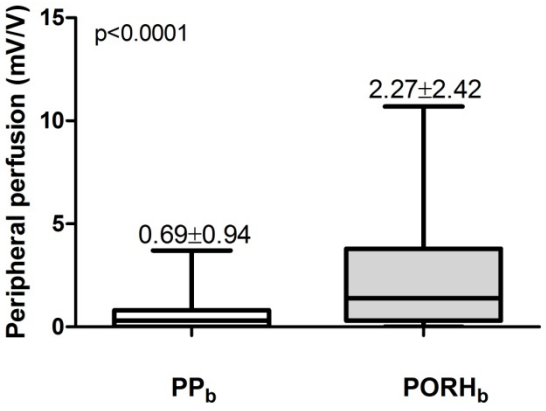

Fig. 1. Paired t-test for evaluating difference in baseline peripheral perfusion and after reactive hyperaemia in responders

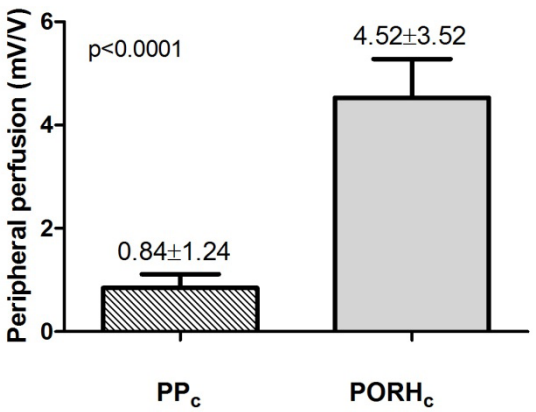

Fig. 2. Difference in peripheral perfusion and PORH after alprostadil challenge test in responders

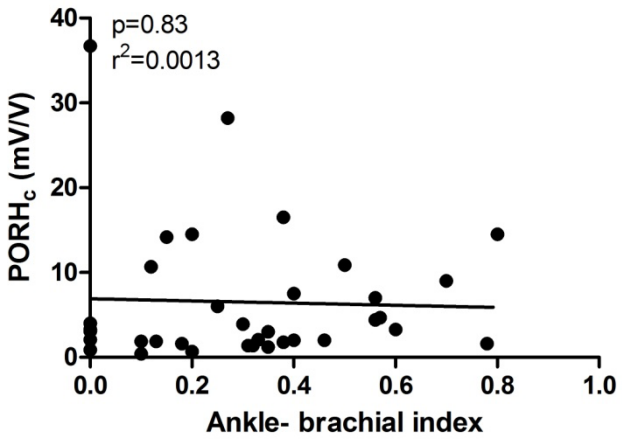

Fig. 3. Pearson's correlation between PORH after alprostadil challenge and ankle-brachial index

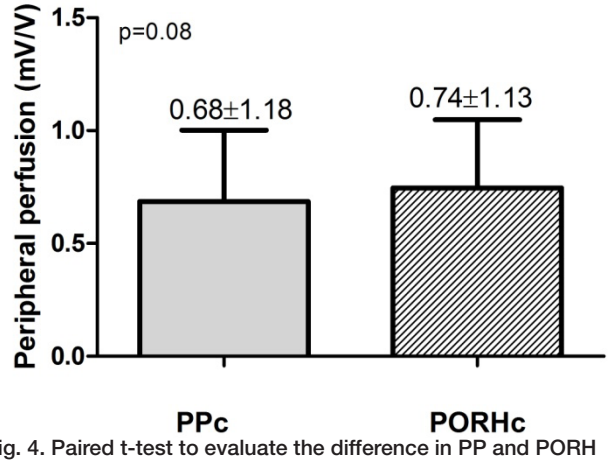

Fig. 4. Paired t-test to evaluate the difference in PP and PORH after alprostadil challenge in non-responders

inflammatory reaction that results in homing of stem cells and inflammatory cells to the "injured" tissue and the native collaterals are increasing in diameter, ensuring blood supply to the distal zones [13]. This could be the substrate for the observations that this group of patients did not develop gangrene. In our experience in non-responders with critical ABI (<0.5) and the lack of increase in PORH by at least 50% after alprostadil challenge, reflects altered endothelial function of the main arterial branch and maybe the absence of collateral vessels. Thus, for limb salvage this population should benefit of invasive high-cost investigations and surgical revascularisation. The assessment of endothelial function involves expensive equipment with a great financial burden on the healthcare system and unavailable for the primary care system. Therefore, in patients with PAD, in order to triage patients for conservative or revascularization therapy, low-cost, operator-independent screening techniques are required. Post-occlusive reactive hyperaemia assessed by finger PPG showed significant increase in peripheral perfusion due to nitric oxide release after occlusion, which could be due to the presence of collateral microcirculation that by-pass stenotic areas. This effect was potentiated after alprostadil challenge. We found no correlation between ABI and post-occlusive reactive hyperaemia, because while ankle-brachial index is a screening method to identify large vessel disease, the assessment of post-occlusive reactive hyperemia reflects the function of the endothelium and maybe the presence of small collateral circulation. Our experience is that in PAD patients with an increase in PORH after alprostadil challenge, the way towards limb salvage consists in lifestyle modification, smoking cessation, exercise therapy, as well as pharmacotherapy including cilostazol, pentoxifyllin, antiplatelet agents, lipid-lowering agents as well as parenteral administration of PGE1 for 7 to 28 days [14].

Finger PPG is a low-cost, operator-independent optical technique that enables us to detect blood volume changes in response to post-occlusive hyperemia. Although it seems to be a promising method that helps physicians in therapeutic decision making at the primary care level, there is no consensus available for this technique regarding occlusion time as well as a normal range for PORH.

Conclusions

The fact that in PAD patients with low ankle-brachial index, associated with stenotic lessions, as well as in the healthy control group, PORH increased significantly, allows us to conclude that the post-occlusive reactive hyperemia test reflects the endothelial function. In our opinon, an increase in PORH after alprostadil challenge in PAD patients despite a low ABI could reflect the endothelial function of the collateral vessels, while in healthy subjects the increase in PORH could reflect the endothelial function of the main arterial branch. Although, in PAD patients a comprehensive investigation has to be done, for a screening method at the primary care level, PORH test combined with alprostadil challenge test, could offer an inexpensive screening method in order to help physicians in therapeutic decision making. In our experience, in patients with lower extremity artery disease, and an increase of PORH after vasodilator challenge by at least 50%, conservative therapy with alprostadil, cilostazol, lipid-lowering agents, antiplatelet agents as well as lifestyle modification are able to delay surgical revascularisation. The long-term follow-up of these patients is of major importance, and if conservative therapy fails, surgical approach should be performed.

Conflict of interest

Authors declare no conflict of interest.

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Acta Medica Marisiensis 2017;63(3):133-135 DOI: 10.1515/amma-2017-0029

RESEARCH ARTICLE

A Standardized Dissection Protocol to Generate Aortic Valvular Scaffolds from Porcine Hearts

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Objective: To describe a particular harvesting procedure for isolating intact porcine aortic heart valve roots as potential sources for biologic scaffolds. **Methods**: Fresh porcine hearts were brought to the Tissue Engineering and Regenerative Medicine Laboratory at the University of Medicine and Pharmacy in Targu Mures. The aortic roots were extracted from the porcine hearts by anatomical dissection. For this purpose, we used a basic surgical instrument kit. This initial phase was the first step in obtaining acellular extracellular matrix as a biologic scaffold material. **Results**: Aortic roots were isolated with preservation of the ascending aorta as well as the intact aortic sinus and coronaries together with the adjacent myocardial tissue and anterior leaflet of the mitral valve. This approach allowed for safe mounting of roots into mounting rings for perfusion decellularization. **Conclusions**: The described procedure is a feasible protocol for obtaining intact biological valvular scaffolds from porcine hearts. Reduced requirements regarding tools and personnel underline the easiness of aortic root harvesting using this particular procedure.

Keywords: scaffold, valve, extraction, decellularization

Received 4 March 2017 / Accepted 26 July 2017

Introduction

Aortic valve pathology is an area of interest due to the severity of symptoms, the impact on quality of life and it is usually fatal if neglected [1]. Valvular substitutes, represented by mechanical or biological valves proved their importance by increasing life expectancy and providing patients a better quality of life [2]. Although history reveals a constant improvement of mechanical valves design and sourcing and reduced immunogenicity of the biological ones, an entire panel of risks remains associated with each of them. Mechanical valves require lifelong anticoagulation therapy, which associates specific therapy risks, i.e. patient compliance and under and over dosage of anticoagulant drugs [3]. Biologic replacements are known to last only 10 to 15 years until degeneration and calcification occur [4]. These shortcomings highlight the need to develop a brand new valvular substitute that comes closer to a healthy heart valve, appropriately adapted to the physiological parameters and requirements.

Regenerative medicine, a new medical research field, aims to produce the ideal valve prosthesis, a living and functional heart valve using scaffolds, stem cells and bioengineering [5,6]. Scaffolds are biocompatible and biodegradable tridimensional structures that provide mechanical support to stem cells and adequate stimuli for appropriate cell function [7,8]. Numerous papers report on decellularization of aortic valves, but rarely give account on details regarding optimal dissection protocols to create intact roots for tissue engineering [9-12].

In this paper, we propose and describe a standard method for the dissection of intact porcine aortic root used for further processing as biologic scaffolds.

Methods

Procurement of porcine hearts

This work is part of a grant which has the approval of the Ethics Committee of the University of Medicine and Pharmacy Targu Mures.

Fresh porcine hearts provided by a local abattoir immediately after harvesting were brought to the Tissue Engineering and Regenerative Medicine Laboratory, University of Medicine and Pharmacy Targu Mures.

Preparation for aortic root harvesting

As a first step, all hearts underwent screening and selection. Only those with undamaged ascending aorta and aortic arch were kept. At the beginning of the procedure, the entire valvular complex was carefully analyzed to identify potential congenital malformations of the valve and degenerative or mechanical lesions. Then, the porcine hearts were placed in ice water and the aortic valve roots were harvested using basic surgical instrument: two curved blunt dissecting scissors, two anatomical blunt forceps, one of each being a delicate one, suited for fragile tissues, one regular No. 10 scalpel blade.

Identification of main anatomical components

A “Y” incision was performed in the pericardium by using a pair of scissors, revealing the heart. The aortic root,

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continuing the left ventricle outflow tract, provides the supporting structure for the leaflets of the aortic valve, and forms the bridge between the left ventricle and the ascending aorta. Therefore, in order to isolate an intact aortic root, it's necessary to identify the left ventricle and the aorta. Then, the proximal segment of the aorta is isolated from the pulmonary trunk using a forceps and a dissection scissor (Figure 1a), ending with a transversal section of the pulmonary artery. Then a transversal cut is applied to remove all tissues above the aortic arch. Caution should be taken at the base of the root to avoid the coronary arteries, as the right emerges from the right sinus of Valsalva, surrounds the right heart and ends frequently as the posterior interventricular artery. The left coronary, emerging from the left sinus of Valsalva, passes an area between the pulmonary artery trunk and the left atrium; it then splits into two branches: left anterior descending artery and left circumflex artery.

The dissection that follows their identification is performed using delicate scissors and forceps. The procedure is completed with a transversal section of the coronary vessels 1 cm distally from their ostia and ligation at the origin (Figure 1b).

Aortic root removal and myocardial processing

The height of the root extends externally from the sinotubular junction to the ventricular-arterial junction and internally from the sinotubular junction to the basal ring defined by the plane passing through the cusps base [13]. To make sure that every root component is maintained intact after the decellularization fixation method, the dissection should be extend beyond these limits. The aorta was further dissected just below the origin of the brachiocephalic trunk. One third of the aortic root base is formed by the anterior mitral leaflet and the other two thirds of the ventricular endocardium and myocardium, 3 cm below the basal ring. Depending on the root mounting type, the thinning of the endocardial tissue might be necessary.

The under- leaflet component is formed by the myocardial muscle, belonging to the muscular part of the interventricular septum, anterior and posterior sides of the left ventricle and anterior mitral leaflet. Myocardial tissue is gently trimmed, avoiding fenestration, with a No. 10 scalpel blade, so that the thickness of the myocardium will be the same as for the mitral cusp. This step is essential in order to balance the forces applied during mounting.

Discussions

The porcine aortic valves are prepared in this manner in order to be placed in a perfusion decellularization system. This preparation protocol represents the first step of the procedure in order to obtain completely decellularized porcine derived aortic valve scaffolds using a transvalvular pressure gradient. The technique offers a large variety of options which can be adapted for any protocol that follows the porcine heart valve isolation, needing only a basic surgical instrument kit, the No. 10 scalpel blade could be replaced with any other model or size. This method requires myocardium thinning which should be performed with great care because the excessive thinning of the myocardium can lead to valve integrity damage (Figure 1c), during the decellularization process. This concept was designed as a solution to earlier report on decellularization failure [14]. Variations of the presented protocol could be applied regarding the ligation of the coronary arteries. It could be replaced with a surgical suture with the downside of damaging the coronary ostia, restricting their use in surgical procedures that require coronary arteries reimplantation, such as the Bentall procedure [15] and varied modified procedures[16-18]. The literature presents various methods and protocols used in order to obtain decellularized aortic valves, a search on PubMed by the key words "aortic" "decellularization" returned 302 results, but minimal and limited information are found in these papers regarding the procedure of aortic valve extraction. To prove this statement, a search using the terms "scaffold", "porcine", "aortic", "decellularization", "surgical", "extraction", returned only one result.

Conclusions

A standardized protocol for aortic root harvesting allows reproducible generation of aortic roots for tissue engineering, also offering high availability, low costs and easy procurement procedures. It also fulfills the need of having a rigorous step by step described protocol. The simplicity and advantage of this particular surgical technique

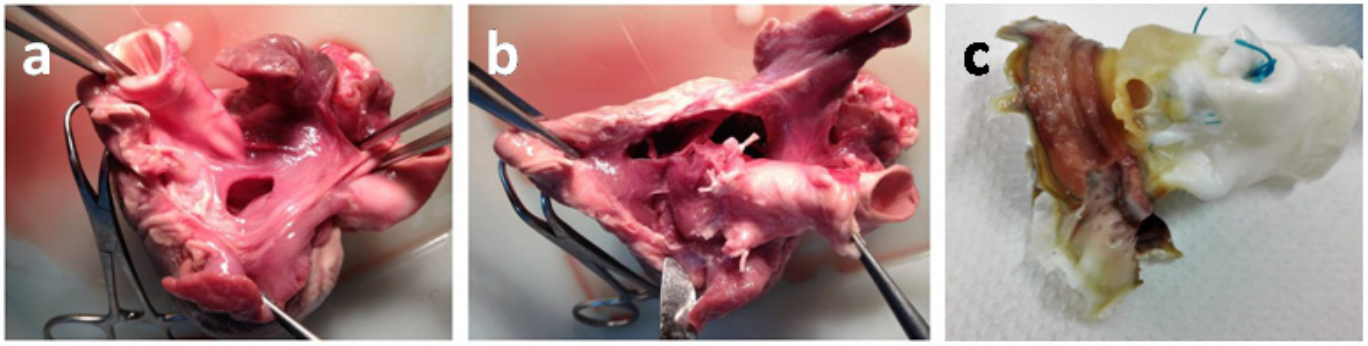

Fig. 1. General aortic root aspects: a. Anatomic position of great vessels; b. Ligated coronary arteries; c. Myocardial rupture due to over thinning- three days before finishing the protocol

are also underlined by the fact that there is a minimal requirement of tools and does not require highly trained personnel.

Acknowledgement

This work was supported by a grant from the Competitiveness Operational Programme 2014-2020, Tissue engineering technologies for cardiac valve regeneration, valve-regen, id P_37_673, Mysmis code: 103431, contract 50/05.09.2016 .

Conflict of interest

The authors have declared that there is no conflict of interest.

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DE GRUYTER OPEN

Acta Medica Marisiensis 2017;63(3):136-139 DOI: 10.1515/amma-2017-0015

RESEARCH ARTICLE

Clinical Outcomes after Regenerative Periodontal Therapy with Emdogain

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Objective: Regeneration is defined as a reconstruction of a lost part of the body in such a way that the structure and function of the lost tissue are completely restored. The aim of this study is to compare the clinical outcomes of intrabony defects treatment using regenerative periodontal therapy with enamel matrix proteins (Emdogain, EMD) with a control group. **Methods**: Ten patients with chronic periodontitis were included in this randomized, controlled clinical study. Two groups received conservative periodontal therapy. In the test group, different teeth received regenerative treatment with EMD. In the control group teeth received solely conservative periodontal therapy. Pocket depth probing (PD) and bone reduction (based on X rays) were registered at baseline and after eight months in both groups. In the control group **Results**: Both groups showed a significant reduction of PD. The teeth treated with EMD showed a significant attachment gain. Within the test group, the radiographic examination of the teeth treated with EMD showed no significant change, whereas the teeth in the control group showed significant bone reduction. **Conclusions:** Intrabony defects in teeth treaded with EMD exhibit a substantially higher gain in clinical attachment and defect filling. The use of EMD in dental practice can prevent further bone loss.

Keywords: chronic periodontitis, regeneration, emdogain, intrabony defects

Received 28 February 2017 / Accepted 27 April 2017

Introduction

Periodontal diseases have become the most frequent ailment of the human body, affecting it irrespective of age, sex or geographical area. The factors incriminated in this condition are local associated with microbes or general factors (cardiovascular, haematological or haepatical conditions, diabetes, endocrine or immune dysfunctions, nutrition deficiencies, nervous system related diseases).

As such, periodontitis represents an infectious disease resulting in progressive 1oss of attachment and bone and ending in dental loss. Conservative therapy of periodontal disease aims at halting the progression of the disease by reducing the pockets, increasing the soft tissue gain and stopping the bone loss. Conservative therapy includes covering such issues as scaling and root planning and results in periodontal repair. Generally, the procedure leads to healing without having to restore the tooth attachment apparatus thus dramatically improving the quality of life in the patient [1,2].

However, restoration of a fraction of the original tissue can be achieved only by regenerative periodontal therapy where regeneration is defined as a"reconstruction of a lost or injured part of the body in such way that the structure and function of the injured tissue are completely restored". However, regenerative periodontal therapy can only restore a fraction of the original tissue. In many clinical situations, where regenerative techniques have been used, significant probing depth reduction in clinical attachment are gained, yet residual defects may still remain [3,4,5].

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More than ten years have passed since Emdogain was introduced as an adjunctive to periodontal surgery. Emdogain was developed to promote regeneration of the periodontal tissue by mimicking the normal development of these tissues [1].

The purpose of the present study is to compare the clinical outcome of intrabony defects treatment using regenerative periodontal treatment using regenerative periodontal therapy with enamel matrix proteins (Emdogain, EMD) with a control group.

Material and Methods

Subjects. In this clinical study, ten patients (four females, six males) aged between 25-55, with chronic periodontitis were included. The subjects were selected from a private practice. The patients were distributed in 2 groups according to the following inclusion criteria: presence of intrabony defect with probing depth (PD) ≥ 6mm, no smoking, no sistemic diseases, good oral hygiene.

In the test group, different teeth received regenerative treatment with EMD after scaling and root planing. The control group received only conservative periodontal therapy.

Conservative periodontal therapy. After recording the patients' periodontal condition, conservative periodontal therapy was performed in both groups. This conservative periodontal treatment consisted of hygiene instructions, full mouth scaling and root planing.

Surgical treatment. The regenerative therapy using EMD used the papilla preservation technique (as described by Cortellini): on the buccal aspect of the damaged teeth, a vertical incision is performed and the site is conditioned

with 24% EDTA for 2 minutes to remove smear layer. Then the site is carefully rinsed with sterile saline, and EMD is applied with a syringe starting at the most apical level. The mucoperiosteal flaps are replaced and sutured carefully in order to obtain primary closure and wound stability. Finally, patients are instructed concerning post-surgery maintenance care. Informed consent was obtained from each patient.

Supportive periodontal therapy. Patients were seen weekly postsurgery for professional tooth cleaning. After that, the patients were recalled monthly for maintenance, oral hygiene control, and reinstruction in oral hygiene.

Clinical parameters. The following parameters were recorded at baseline and after 8 months: bone reduction based on x-rays, probing depth (PD) and bleeding on probing. Tooth mobility was recorded using Miller's index. Plaque index (O'Leary et aI.1972) was used to evaluate the presence of plaque. Gingival index(Loe and Silness, 1963) was used to evaluate gingival inflammation[6,7].

Statistical analysis. The statistical analysis was carried out using t-test and chi-square. The level of significance was set at $p<0.05$.

Results

The groups consisted of ten patients (four females, six males). The average age in the test group was 41.347±10.891, the mean age in the control group was 43.965±11.008. The average observation period was 7.26±0.97 months (test group) and 7.42±0.35 months (control group).

In the test group, 106 teeth were treated with scaling and root planning; later 38 thereof with EMD. In the control group 129 teeth were treated with scaling and root planing. Table 1 shows the distribution of the teeth received regenerative treatment with EMD after scaling and root planing (Table I).

In both groups a significant reduction in PD was found: in the test group 1.6mm and in the control group 0.9mm ($p=0.000$) (Fig.1 a, b). The difference between the two groups was significant ($p<0.0001$). The teeth treated with EMD showed a significant attachment gain with a mean of 1.84±0.2mm ($p<0.001$). Figure 1 shows the changes of PD in test group at baseline (C) and after 8 months (D) in comparison with control group (A, at baseline and B, after 8 months).

The difference between groups was significant ($p<0.0001$). The teeth treated with EMD showed a significant attachment gain with a mean of 1.84±0.2mm ($p<0.001$) (Fig. 2, Fig. 3).

A statistically significant increase in mean PD was observed at 8 months in test group ($p<0.0001$). Mean PD reduction in the recorded sites at 8 months was 5.1±0.5mm (Fig. 2). The reduction was maintained during the 1 year observation period, with no significantly change. No significant correlation was found the baseline PD between the control group and the test group (Fig. 3).

In both group gingival aspects improve after regenerative periodontal therapy in comparison with baseline .

Table I. Distribution of Emdogain treated teeth

Teeth	Upper teeth	Lower teeth
Frontal teeth	7	8
Premolars	4	7
Molars	6	6

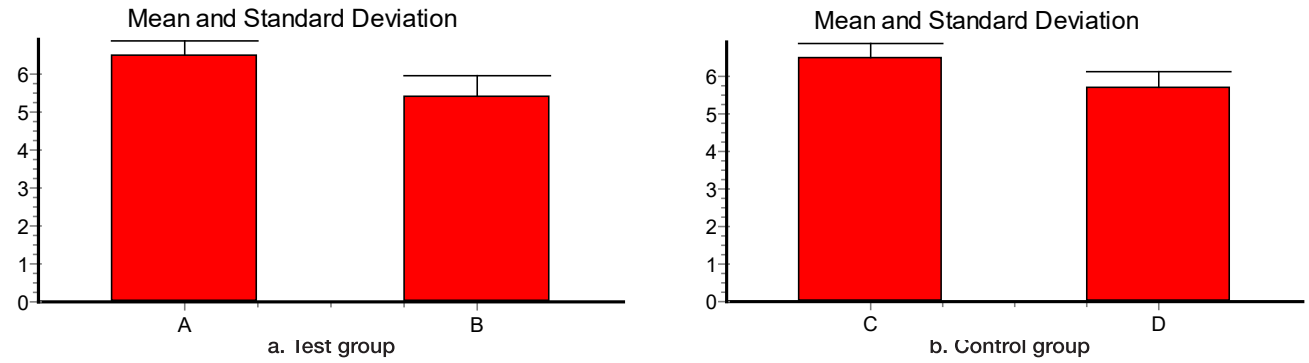

Fig. 1. Changes of PD in test group (a) and control group (b) at baseline (A,respectively C) and after 8 months (B, respectively D).

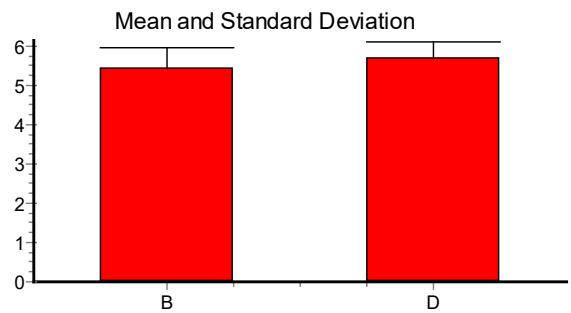

Fig. 2. Changes of PD in test group (B) and control (D) after 8 months

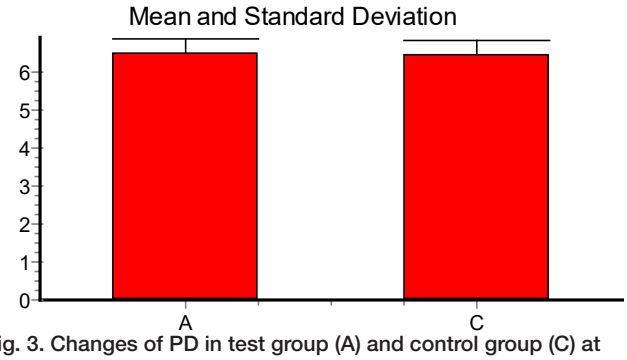

Fig. 3. Changes of PD in test group (A) and control group (C) at baseline

There was no significant change in tooth mobility after 8 months. Minimal significant changes were evidenced radiographically in the test group over the observation period, while the control group showed no changes whatsoever.

Discussion

The present study carried on regenerative periodontal therapy with Emdogain. The results showed significant PD reduction and CAL gains. Wound healing following EMD application appeared to be favourable. EMD may influence soft tissue healing, in addition to its capability of promoting periodontal regeneration. The results reported in our study are consistent with those published by other authors.

Several studies have been published concerning degree of clinical success, possibilities for combining Emdogain with other agents, or means to promote periodontal regeneration, as well as cellular effects and mechanism of action [8]. The introduction of Emdogain as an adjunct to periodontal surgery therapy has stimulated a great number of research projects concerning its effects and efficacy.

The majority of these publications show that Emdogain is able to significantly regenerate cementum, periodontal ligament and alveolar bone when it is used to treat deep intrabony defects, as was originally indicated [9].

Our findings that the regenerative periodontal therapy with Emdogain may results in higher significantly improvement regarding CAL and PD when compared to the baseline are in agreement with the results of other studies. In the first controlled clinical trial, Heijl was ccompared the efficiency of EMD treatment used to support periodontal flap surgery to the efficiency of surgery alone in treating intrabony defects. The parameters followed were radiographic bone level and clinical attachment level. Follow-up examination after three years showed that mean radiographic bone gain in the EMD -treated sites had increased from 2.2mm to 2.6mm. The bone level at the control sites was more or less unchanged after three years.

The results showed clinically relevant difference especially since almost half of the patients were smokers [10].

In a controlled clinical study, it was demonstrated that treatment with EMD was superior to open flap debridement (OFD) at 12 months postsurgery [11,12]. In addition, it was demonstrated that the percentage defect fill after adjusting for crestal bone resorption was more than three times greater for EMD than for OFD alone [13,14].

In a multicentric study, Tonetti et al. reported a mean CAL gain of 3.1mm at one year [14]. Saito et al. evaluated the long-term clinical outcomes of treatment with EMD in a private practice setting [15]. The mean CAL gain at six months was 3.6mm which was significantly greater.

In our study we reported higher CAL gain after regenerative periodontal therapy with Emdogain. These clinical results are also suported by other study. Sculean et al. reported the formation of new attachment at six months following EMD treatment of advanced intrabony lesions. Their results showed bone regeneration after formation of new attachment was not always followed by bone regeneration, although the newly formed cementum was predominantly of a cellular character [15]. Ozcelik et all emphasized that patients' perceptions on the postsurgery period were significantly better in the groups with non-surgery and surgery with EMD group as compared to the surgery group [14,15]. In our study the clinical outcomes after regenerative periodontal therapy with Emdogain are significant and we reported a significant attachement gain in the group treated with Emdogain.

However, Zetterstrom et al. and Hagenaars et al. reported no differences in pacients' perceptions and post-surgical healing between surgeries with EMD and flap operations[16-18]. Saito et al. in a study regarding the treatment of periodontal defects with enamel matrix derivative, showed after three to six months that periodontal surgery with EMD results in a clinically relevant reduction in probing depth and a gain in clinical attachment [19-22].

Our results are in agreement with those of others authors. Further research need to be performed,due to the reduced number of patients included.

Conclusions

Treatment of intrabony defects with EMD may lead to substantially higher gains in clinical attachment and defect filling. The use of EMD in dental practice can prevent further bone loss. However, these results need to be confirmed on a larger scale in multicenter controlled clinical trials.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

This paper was published under the frame of internal research grant University of Medicine and Pharmacy Tirgu Mures, nr. 1262/2015 financed by SC ANDSER SRL.

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Acta Medica Marisiensis 2017;63(3):140-146 DOI: 10.1515/amma-2017-0012

RESEARCH ARTICLE

Evaluation of Early Postoperative Cognitive Dysfunction Incidence and Involved Neurocognitive Functions in Patients with Cardiac and Noncardiac Surgery Under General Anesthesia

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Objective: To analyse postoperative cognitive dysfunction's (POCD) incidence and cognitive areas involved, in patients with cardiac and general surgery. **Material and Methods**: Prospective observational study on 130 patients undergoing general or heart surgery on cardiopulmonary bypass, under general anesthesia. Two groups, 65 members each. Group A had a heart surgery and group B a noncardiac surgery. The same type of anesthetic drugs were used. All patients completed the Montreal Cognitive Assesment (MoCA) questionnaire: preoperative, 24 hours after stopping any medicine acting on central nervous system and 7 days postoperative. We compared the MoCA scores obtained on different cognitive domains in this moments for each group of patients, for neurocognitive functions: visuo-spatial executive, naming, attention, verbal fluency, abstraction, recall, orientation, final score. We compared the scores between the two groups at 24 hours and 7 days postoperatively for the same domains. **Results**: POCD was found at 24 hours testing in both groups. At 7 days postoperatively POCD was not found in any of the groups. There was no statistically significant difference in total final score between two groups at 24 hours nor at 7 days postoperative testing. There are significant differences between the two groups, with lower score in cardiac group in 5 of 7 fields at 24 hours testing, with the persistence of difference in 2 of 7 fields at 7 days. **Conclusions**: Overall POCD was present at 24 hours but was not found at 7 days testing for none of the groups. POCD is present in some neurocognitive domains and this depends on surgery type.

Keywords: POCD, neurocognitive domain, cardiovascular surgery, noncardiac surgery, general ansthesia

Received 21 January 2017 / Accepted 24 April 2017

Introduction

The postoperative neurocognitive dysfunction definition emerged about mid-1990s when it began to be described as a decline in cognitive performance. This postoperative decline which has to come out at least in two or three tests of a battery especially created for this aim [1].

Nowadays postoperative cognitive dysfunction is a well known perioperative syndrome, accounting for about 15% in patients older than 60 years old, when a proven decrease in cognitive function appears. This postoperative reduction is proven by objective testing and is due to anesthesia and surgery, without being able to precisely know the exact cause of it [2].

The following risk factors for POCD were identified: age, the extent of surgery, duration of anesthesia, postoperative complications, education level, neuropsychiatric disorders and medication and already existing cognitive impairment [1]. From all above, patient age impact and involvement on POCD occurrence was studied in large groups of adult patients who completed neuropsychological tests and questionnaires. These were applied at various time moments reported to surgery: before and after surgery, at hospital discharge and 3 months postoperatively. Classifying the subjects according to their age as young age: range 18-39 years old, middle-aged: 40-59 years old and over 60 years old, it was observed that POCD could be detected in 36.6% in young people, 30.4% in middle aged to 41.4% in old aged subjects [3].

Defining postoperative cognitive dysfunction: first of all it must not be confused with postoperative delirium which is a transient disorder with fluctuant conscious state and which occurs very nearly to postoperative period. While this entity is a transient change, POCD is a persistent change in cognitive performance which can be objectively demonstrated by comparing pre and postoperative acquired scores in certain type of tests [4].

This change is common at the time of discharge from hospital in adults of all ages having undergone a major surgery, with a significant and greater risk for long-term problems in population above 60 years old [3].

Objective

The main objective of our study was to analyse postoperative neurocognitive dysfunction development and incidence in patients with cardiac and general, noncardiac surgery.

Second objectives were to determine cognitive areas involved in POCD pathology, by applying pre- and postoperative evaluation questionnaires, and also to determine the role of anesthesia and surgery type POCD etiology.

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Materials and methods

We performed a prospective observational study in the following two clinics: Clinic of Intensive Care Unit in Tirgu Mures Emergency County Hospital and Tirgu Mures Emergency Institute for Cardiovascular Diseases and Transplantation. The study was conducted from 1st of June 2013 to 1st of April 2016 on a sample of 130 patients undergoing general or heart surgery.

The study included patients undergoing general surgical noncardiac interventions, under general anesthesia and cardiac surgery on cardiopulmonary bypass under general anesthesia.They all completed the MoCA (Montreal Cognitive Assesment) questionnaires for assessment of postoperative cognitive dysfunction.

We did not included in our study patients under 18 years old, patients who suffered a gynecological, orthopedic or thoracic surgery (other than the cardiac one), nor patients whom cardiac interventions were proceeded without extracorporeal pump, patients who had been reoperated. We also excluded ones who were sedated for more than 24 hours postoperatively, those with visual disturbances and those who developed postoperative delirium or psychosis. We also excluded patients who deceased.

The study was possible following approval of the ethics committees of University of Medicine and Pharmacy of Tirgu Mures (approval dated on 5/30/2016), of Emercengy County Hospital of Tirgu Mures (approval dated on 23.09.2015) and of Tirgu Mures Emergency Institute for Cardiovascular Diseases and Transplantation (dated on 09/10/2016) and after we obtained the patient 's signed informed consent.

Subjects were divided into two groups with 65 members each. Group A had a heart surgery under general anesthesia and patients in group B undergone a noncardiac surgery also under general anesthesia.

Patient management

The multimodal balanced anesthetic technique was applied. All patients were anesthetized with the same type of anesthetic drugs from the following classes: the class of sedatives and hypnotics, benzodiazepines (same type of benzodiazepine were used) and propofol. Analgesia has been provided with the same opioids and same volatile was used. Neuromuscular blocade has been accomplished with non-depolarizing neuromuscular blocking. Postoperative sedation was provided with propofol while for postoperative analgesia same NSAIDs class and opioids were used.

All patients completed three times, after they have been explained and signed the informed consent form, the "Montreal Cognitive Assessment" (MoCA) in the following moments: preoperative, 24 hours after stopping any sedative or hypnotic drugs or other medicine acting on conscious state and 7 days postoperative.

The MoCA test is a free assessment tool designed for quickly screening for mild cognitive impairment. It evaluates the following skills: alternative coupling, visual-constructive skills, language related processes and the ability to repeat certain concepts, verbal fluency, memory recall, attention, abstraction skills. This skill are related to correspondent areas of cognition like executive function, language area, attention and concentration field, memory, orientation, visual field, conceptual thinking and orientation.

Score interpretation: a final score of 26 points and more is considered normal. A score between 22-26 points suggests a mild cognitive dysfunction, while a score below 22 points, around the average of 16 may indicate a severe cognitive dysfunction, phenomenom of Alzheimer dementia type [5-9].

Data recording

The follow-up sheet included the next parameters: sex, BMI, the highest level of education attained, previous medical history, preoperative medication, the diagnostic, surgery type, time of surgical intervention, time of anesthesia (for those with noncardiac surgery), time of extracorporeal circulation, the ASA anesthetic risk, allergies, hospitalization in intensive care unit period, necessity of transfusion of blood products, inotropic support required, postoperative arrhythmias with their management, duration of postoperative mechanical ventilation, postoperative hemodynamic, neuropsychological , respiratory, renal, hepatic, or gastrointestinal complications, postoperative bleeding, postoperative infectious complications or other perioperative incidents.

The next column in the follow-up sheet was the scoring for MoCA questionnaire before surgery, 24 hours after any sedative-hypnotic infusion had stopped and 7 days after surgery for the following sections: visuo-spatial-executive field, naming, attention, language, abstraction capacity, delay recall, orientation and final score.

Data anlayse

Data were analysed using statistical processing programs GraphPad Prism 7.0 and Epilnfo. The established alpha value was $\alpha = 0.05$ and with confidence interval of 95%.

The normality of the data series was established using D'Agostino test. For comparing the data sets the Man Whitney test for nonparametric data series was used; while student t-test for unpaired data was used in assessment of Gaussian distributions. The data series were compared using paired t-test for paired data, if normal distribution, and Wilcoxon test for non-Gaussian distributions. The differences between proportions was tested using Chi-square test. The correlations between variables were investigated by calculating the Pearson coefficient, Spearman r, depending on the normality of the given series of data, of course.

The description of the study

We compared the preoperative MoCa score versus 24 hours postoperative ones, preoperative versus 7 days postoperative and 24 hours postoperative versus 7 days postoperative as it follows, for all the cognitive domains, for each group

of patients (group A-cardiac surgical patients and group B-noncardiac surgical patients).

After that we compared the scores for cognitive domains among patients undergoing noncardiac surgery and those with heart surgery as it follows: MoCA scoring at *24 hours after surgery* in noncardiac patients versus cardiac patients, MoCA scoring at *7 day postoperatively* for the same as above neurocognitive functions in noncardiac patients versus cardiac patients.

Results

Comparisons results for patients in group A-with cardiovascular surgery (see table I)

MoCA score for the visuo-spatial executive neurocognitive domain

When we compared scores for test battery of MoCA questionnaire for patient in group A in visuo-spatial executive functions, we noticed that 24 hours postoperative score was lower than the preoperative ones. The difference was statistically significant, with a p value < 0.0001. Differences between scores obtained preoperative and 7 days postoperative scoring and that between postoperative scores at 24 hours and 7 day were not statistically significant with a confidence interval of 95%.

MoCA scoring for naming cathegory

When we compared the scoring for "naming" obtained preoperatively with those scored 24 hours and then 7 days postoperatively no statistically significant difference was noticed. Neither when comparing 24 hours with 7 days after the surgery. Surgery and anesthesia did not influence the capacity of naming and this neurocognitive domain.

MoCA scoring for "attention"

In case of cardiovascular patients, 24 hours postoperative score for attention was significantly lower than the preoperative one, p was equal with 0.0037. It was not statistically significant lower than that scored 7 day postoperative. When 24 hours score was compared with that at 7 days the difference was not significant different.

MoCA scoring for language field

When we compared the scores for neurocognitive domain of language, we noticed that 24 hours postoperatively scoring was significantly lower than the preoperative one and than those scored at 7 days after surgery, when p was equal with 0.0038. When we compared preoperative moment with postoperative moment 7 days after surgery, the difference was not statistically different.

MoCA scoring for abstract skills

For this neurocognitive part, the score difference was statistically significant only when comparing postoperative test at 24 hours versus those at 7 days; p was equal to 0.0452.

Scores for orientation

When we compared the scores for "orientation" domain those scored 24 hours after surgery were significant lower than those scored before surgery and 7 days after, when p value was 0.0018 but with no statistically significant difference before surgery and 7 days after.

"Recall" neurocognitive field scores

In this field score analyse, the difference between 24 hours and 7 days after surgical procedure was statistically significant with a p of 0.0043 for a confidence interval of 95% with lower score at 24 hours moment. The medium score improved 7 days later after surgery with a value of 3.541 versus 24 hours after surgery, of 2.541.

Total MoCA score

Overall postoperative score differ in a statistically significant manner, being lower at 24 hours postoperative than the preoperative ones. These scores tend to improve 7 days after surgery, being significant higher than those at 24 hours after surgery but not being significant different than the preoperative ones.

Comparisons results for patients in group B-with noncardiac surgery (see table II)

MoCA score for the visuo-spatial executive neurocognitive domain

Scores achieved in tests for visuo-spatial-executive neurocognitive field for patients in group B, those with noncardiac surgery, 24 hours after surgical procedure were significant lower than those preoperative ones. The difference kept up in scoring 24 hours versus 7 days postoperative, but with higher (better) scores at 7 days postoperative, with a p of 0.0114. There was no statistically significant difference between preoperative and 7 days after intervention achieved scores for a confidence interval of 95%.

MoCA score for "naming" neurocognitive domain

For this category the only statistically significant differences between the scores achieved were those in preoperative versus 24 hours postoperative, when p=0.0051.

MoCA score for "attention"

For this category the only significant difference was between the postoperative scores, when that one scored at 24 hours after surgical intervention was lower than that one score after 7 days, with p=0.0025, for the same 95% confidence interval.

MoCA scoring for "language" neurocognitive domain

When we compared the scores achieved by the patients in group B, for the neurocognitive field "language" we noticed that 24 hours postoperative scores were significant lower than the preoperative ones and p had a value of 0.0004. 24 hours scores was significant lower that 7

Table I. MoCA results for group A (cardiac heart surgery patients)

Neurocognitive function	Visuo-spatial executive functions	Naming	Attention	Language	Abstraction	Orientation	Recall	Total
Preoperative score	3.80 ± 1.19	2.79±0.54	4.66±1.27	1.73 ± 0.97	1.65 ± 0.58	5.89 ± 0.37	3.16±1.53	24.26 ± 4.11
24 h postoperative score	2.80 ± 1.41	2.82±0.53	3.96±1.54	1.26 ± 1.12	1.57 ± 1.59	5.52 ± 1.08	2.54±1.73	21.21 ± 4.99
7 days postoperative score	3.34 ± 1.45	2.83±0.52	4.70±1.44	1.86 ± 1.05	1.82 ± 0.38	5.91 ± 0.27	3.54±1.39	24.64 ± 4.23
p value < 0.05 Preop vs postop 24 h	Yes	No	Yes	Yes	No	Yes	No	Yes
p value < 0.05 Preop vs postop 7 days	No	No	No	No	No	No	No	No
p value < 0.05 Postop 24 h vs postop 7 days	No	No	No	Yes	Yes	Yes	Yes	Yes

*Results are given as medium ± SD *p value < 0.05 is considered statistically significant

days (postoperative) scores also, when p was equal to 0.05. Medium scores for preoperative test moment and that at 7 days postoperative was equal and it was of 1.738 with standard deviation of 0.973 and 1.122 and confidence interval of 95%.

MoCA scoring for "abstraction" neurocognitive domain

For this category the only statistically significant difference was between preoperative and postoperative 24 hours tests when patient scored lower test value. p=0.0026 for this comparison.

MoCA scoring for "recall" neurocognitive domain

Here the significant difference was between postoperative scores when that at 24 hours was significant lower than that at 7 days postoperative with p value of 0.0219.

MoCA scoring for orientation skills

When testing orientation skills there were no statistically significant differences between pre and postoperative scores.

Total final MoCA scoring

Total score achieved by the patients with noncardiac surgical interventions in MoCA questionnaire were significant lower when they completed the tests 24 hours after stopping any SNC active medication after surgery, than the preoperative ones. These maintained low and with significant difference versus 7 days postoperative tests, p=0.0001. Although postoperative scoring lowers after surgery statistically significant, they tend to increase after 7 days, such as this difference is no more visible in terms of statistical significance compared with that before surgical procedure.

Result of comparison of MoCA 24 hours postoperative scores between group A and B (see table III)

Although in majority of cases (except "naming", "language"and "abstraction" categories) 24 hours postoperative scores were lower for group A, that of cardiac surgical patients, the statistically significant differences between group scores was found for the following neurocognitive fields: visuo-spatial executive domain, naming capacity, in case of abstraction skills, recall ability and orientation, where p value was < 0.05 for a confidence interval of 95%.

Comparison results of MoCA 7 days postoperative scores, between group A and B (see table IV)

The difference between groups maintained at 7 days postoperative also; with lower score for group A than group B in the same neurocognitive fields as for 24 hours after surgical interventions. Still the only two domains where this difference maintained statistically significant at this point after operation were visuo-spatial-executive functions and abstraction skills. There were not significant differences in total scores between two groups.

As it can be observed in the figures below (fig. 1 and fig.2), the final score was comprised, for most patients somewhere between 22 and 28 points. It showed an important decrease, higher that 2 points, sometimes as low as 20 points and below this number at 24 hours postoperatively, with a subsequent increasing at third testing, the one applied 7 days after surgery. The interesting fact is that the

Table II. MoCA results for group B (noncardiac surgery patients)

Neurocognitive function	Visuo-spatial executive functions	Naming	Attention	Language	Abstraction	Orientation	Recall	Total
Preoperative score	3.80 ± 1.19	2.79 ±0.54	4.66 ±1.27	1.73 ±0.97	1.65 ± 0.58	5.89 ± 0.37	3.16 ±1.53	24.26 ± 4.11
24 h postoperative score	3.30 ± 1.35	2.53 ±0.68	4.12 ±1.52	1.13 ±0.98	1.33 ± 0.66	5.89 ± 0.43	3.15 ±1.31	22.05 ± 4.52
7 days postoperative score	3.95 ± 1.12	2.76 ±0.49	4.95 ±1.26	1.73 ± 1.12	1.55 ± 0.63	5.954 ± 0.211	3.75 ±1.11	25.14 ± 3.93
P value < 0.05 Preop vs postop 24 h	Yes	Yes	No	Yes	Yes	No	No	Yes
P value < 0.05 Preop vs postop 7 days	No	No	No	No	No	No	No	No
P value < 0.05 Postop 24 h vs postop 7 days	Yes	No	Yes	Yes	No	No	Yes	Yes

*Results are given as medium ± S.D. *p value < 0.05 is considered statistically significant.

Table III. MoCA results at 24 hours testing for group A and B.

Neurocognitive function	Visuo-spatial executive functions	Naming	Attention	Language	Abstraction	Orientation	Recall	Total
Group A	2.80±1.41	2.82±0.53	3.96±1.54	1.26±1.12	1.57±1.33	5.52±1.08	2.54±1.73	21.21±4.99
Group B	3.30±1.35	2.53±0.68	4.12±1.52	1.13±0.89	0.59±0.66	5.89±0.43	3.15±1.31	22.05±4.52
P value	0.04	0.004	0.58	0.60	0.03	0.008	0.04	0.37

*Results are given as medium ± S.D. *p value < 0.05 is considered statistically significant

Table IV. MoCA results at 7 days testing for group A and B.

Neurocognitive function	Visuo-spatial executive functions	Naming	Attention	Language	Abstraction	Orientation	Recall	Total
Group A	3.34±1.45	2.83±0.52	4.70±1.44	1.86±1.05	1.82±0.38	5.91±0.27	3.54±1.39	24.64±4.23
Group B	3.95±1.12	2.76±0.49	4.95±1.26	1.73±1.12	1.55±0.63	5.95±0.21	3.75±1.11	25.14±3.93
P value	0.01	0.21	0.29	0.54	0.01	0.41	0.54	0.46

*Results are given as medium ± S.D. *p value < 0.05 is considered statistically significant

average of this increase reached the preoperative scoring or even slightly above it.

In summary, the results obtained by our patient and our study were as it follows:

In case of patients of *group A*, cardiac surgical patients overall 24 hours postoperative score was lower than the preoperative one, being statistically significant lower for *visuo-spatial-executive functions* (p<0.0001), in *attention* testing score (p=0.0037), for *language* neurocognitive domain (p=0.0038), in case of *orientation* skills (p=0.0018) and for *total scoring* also (p<0.0001).

Still this postoperative decreasing of scores doesn't persist at 7 days postoperative, when score tend to have a closer value than the preoperative one. When comparing preoperative scores with those at 7 days postoperative there are no statistically significant differences.

Scoring at 24 hours after surgery is also lower than that at 7 days after surgical intervention, with significant difference for the following neurocognitive domains: *language* (p=0.0038), *abstraction* skills (p=0.0452), *orientation* capacity (p=0.0018), *recall* (p=0.0043), and *total* final score (p<0.0001).

Patients in *group B*, those with noncardiac surgery also had an lower overall 24 hours postoperative score than that scored at the preoperative testing, with statistical significance for the next neurocognitive fields: *visuo-spatial execcutive* (p=0.0114), *naming* capacity (p=0.0051), *language* test (p=0.0004), *abstraction* skills (p=0.0026) and *total final* score (p=0.0001). The score improved 7 days after surgery like in group A, but with no statistically significant difference compared with the preoperative one. The increased was statistically significant when compared to the scores marked 24 hours after the procedure for the next fields: *visuo-spatial executive* functions (p=0.0114), *attention* (p=0.0025), *language* (p=0.0004), *recall* (p=0.0219) and for *final score* (p=0.0001).

24 hours postoperative score was significant lower *for cardiac* patients for following tested neurocognitive functions: *visuo-spatial executive* (p=0.0492), *recall* (p=0.0473) and *orientation* (p=0.0083) and statistically significant lower *for grop B, noncardaic* patients for the following fields: *naming* (p=0.0041) and *abstraction* (p=0.0387).

The difference maintained statistically significant at 7 days postoperative for *visuo-spatial executive* function, when group A had lower scores than group B (p=0.0143) and *abstraction* skills testing, when group B had lower scores than group A (p=0.0121).

Discussions

We chose the MoCA questionnaire, a validated 30 points test for screening even of mild cognitive impairment, with a sensivity of 90% because the older used MMSE (Mini Mental State Examination) tool is a test with low sensivity and because the majority of the adult subjects of any age score

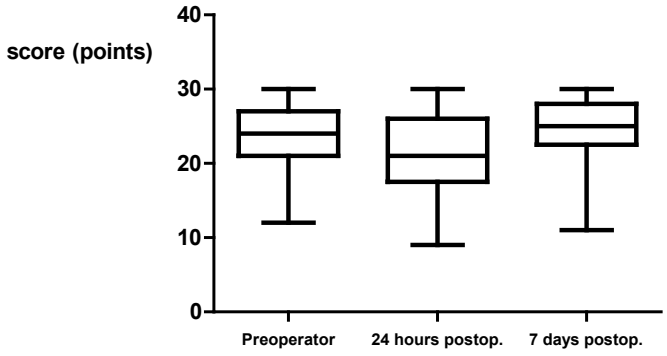

Fig. 1. Group A (cardiovascular surgery patients) results

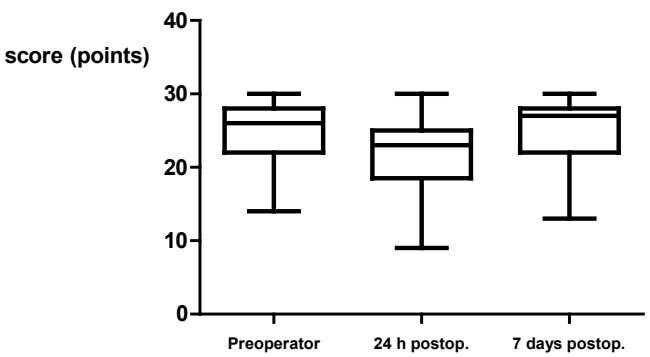

Fig. 2. Group B (noncardiac surgery) results

very close to the maximum score and thus the minor degrees of cognitive decline are missed by applying MMSE [5, 10].

Unlike other studies [11], we did not take a cutoff/ threshold score to diagnose the emergence of POCD, since there is still no established agreement on how low the score it should be. As opinions about this vary along a cutoff point of 1, 1,5, or 2 SD (standard deviations) and is still hard to classify patients who scored lower one domain but scored more in others, we made statistical comparisons for each domain and for final score between moment before operation and after this.

Since there is no general consensus regarding the timing of assessment for POCD, and the measurements had been done beginning 1 day as long to 5 years postoperatively we chose to assess the patients at 24 hours after stopping any medication with effect on nervous system and at 7 days postoperatively. As we found no statistically significant differences at 7 days, we did not continued the investigation at 3 month after the surgical intervention. Another main reason is that in three month many external, familial and other factors have impact on patient state and may affect cognition [12].

Although the time for completing the questionnaire it was supposed to be in average 15 minutes, our subjects took in average 40 minutes to complete [13].

We also noticed an improvement in postoperative test performance in some domains which can be a learning or practice phenomenom which was also studied and described in other cited studies [14, 15].

For cardiac operation group of patients, the results of our study are different from the citated studies, with 7 days postoperative outcome showing no significant difference from the preoperative scoring with medium scores slightly higher than the preoperative ones for the majority of domains (see table 1) [16, 17].

We also found no statistically significant difference for noncardiac surgical group of patients between preoperative scoring and 7 days postoperative which is a different result by that shown in another study that reviewed several big databases and which found some evidence of POCD in the early period after major noncardiac surgery especially in older people [18].

A possible explanation for this difference between our study and the cited ones, could be the fact that in our research both group A and group B had a lower preoperative total score in MoCA testing than the established cutoff point for normal scoring of 26 points [5, 19] with a *medium of 24.26 (4.111 S.D.).* The cutoff point of <25, <24, <23 in MoCA scores [20] must be taken under the reserve that cutoff points must be established appropriate to culturally context and maybe that the bonus point given to those having under 12 years of schooling is just not enough to homogenize the subjects capacity of completing the questionnaire [21].

In what concerns the postoperative results for group B, noncardiac surgical patients these are not different from other studies and where was no evidence of long-term effect of cognitive decline [22].

When we take a look at domain scores we see no statisticaly significant difference for none of fields at 7 days postoperatively, neither for cardiac group, nor for noncardiac group. For noncardiac group the most affected cognitive fields at 24 hours are visuo-spatial executive, naming, language and abstraction, while for cardiac group visuo-spatial executive, attention, language and orientation were most affected. The comparison between the cardiac and noncardiac group showed that at 24 hours testing, group A had significant lower scores than group B for the following domains: visuo-spatial-executive, naming, abstraction, recall and orientation, while at 7 days testing the difference remains significant only for two fields like: visuo-spatial-executive and abstraction capacity.

Since we did not find any POCD at 7 days postoperative evaluation, and since after patient is discharged many familial and external factors interfere with his/her cognition we did not consider a must, in following the patients at three months after surgery. This decision was also sustained by the low patients' compliance.

Conclusions

POCD was found at 24 four hours after surgery both in cardiac and noncardiac group. The difference was statistically significant between scores before surgical procedure versus those at 24 hours after. At 7 days postoperatively POCD was not found in any of the groups. The difference between preoperative score and that at 7 days postoperative was not statistically significant. There was no statistically significant difference in total final score between two groups at 24 hours testing nor at 7 days postoperative testing. So we can conclude that type of surgery and anesthesia did not influence the POCD emergence. Still there are some cognitive domains where POCD is present at 7 days postoperatively and this depend on type of surgery. There are statistically significant differences between the two groups, with lower score in cardiac group in 5 of 7 fields at 24 hours testing, with the persistence of difference in 2 of 7 fields.

Final conclusion of our study is the fact that we could identify different types of POCD detectable at 24 hours postoperative. Early memory and learning deficits and psychomotor slowing were reversible and disappeared in the first week in the majority of patients.

Conflict of interest

None declared.

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Acta Medica Marisiensis 2017;63(3):147-151 DOI: 10.1515/amma-2017-0025

RESEARCH ARTICLE

Ultrasound Guidance Versus Peripheral Neurostimulation for Brachial Plexus Block Anesthesia with Axillary Approach and Multiple Injection Technique

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Introduction: There are several approaches for brachial plexus anesthesia: supraclavicular, infraclavicular, interscalenic and axillary. Out of these, the axillary approach is considered to be the safest because of the low risk of lesioning the adjacent structures, low risk of phrenic nerve blockade or of producing an iatrogenic pneumothorax. The block can be performed by one single injection at the site, by two injections or by several injection, among each nerve of the plexus. Ultrasound was introduced in regional anesthesia since 1978, being used initially as an auxiliary method to peripheral neurostimulator. **Objectives**: The evaluation of ultrasound efficiency as an auxiliary method for brachial plexus block performance, in terms of success rate, vascular punctures. The influence of obesity on performing time, total duration of the block, and success rate of brachial plexus block. **Material and method**: Prospective, randomized study which enrolled adult patients, scheduled for surgical emergency or elective surgical intervention on upper limb with brachial plexus block by axillary approach, using either the peripheral nerve stimulation or the ultrasound guidance. **Results**: We enrolled 160 patients, grouped in two sets- the ultrasound group= 82 patients (US) the neurostimulation group = 78 patients (NS). Vascular punctures were statistically significant different p= 0, 04. The success rate was not influenced by the obesity. **Conclusions**: Ultrasound guidance makes axillary brachial plexus block safer, we can recommend ultrasound guidance as routine for axillary brachial plexus block. The obese patient can beneficiate by both methods of brachial plexus blockage.

Keywords: brachial plexus block, ultrasound, neurostimulation, obesity, vascular punctures

Received 4 June 2017 / Accepted 18 July 2017

Introduction

Anesthesia and analgesia methods for hand surgery consists of general and regional procedures. Regional anesthesia is getting ahead for hand surgery. This is due to the known advantages of regional anesthesia compared to general anesthesia. Among these advantages, we point out the awake state of the patient, the fact that it does not imply major modifications on the major body systems and organs: lungs, heart, and vascular system. Postoperatively, this mode of anesthesia offers up to 8 hours analgesia [1].

There are several approaches for brachial plexus anesthesia: supraclavicular, infraclavicular, interscalenic and axillary. Out of these, the axillary approach is considered to be the safest because of the low risk of producing lesions to the adjacent structures, low risk of phrenic nerve blockade, and of producing an iatrogenic pneumothorax. Although superior among the other approaches of brachial plexus, even with this procedure, the risk of inadvertent intravascular injection of the local anesthetics persists [2].

For performing the axillary brachial block by axillary approach, a few technique possibilities exist. The block can be performed by single injection at the site, by two injections or by several injections, for each nerve of the plexus. The last one is considered to be the safest method, ensuring a high success rate. A study of Casati et al. showed a failure rate of 3% when using ultrasound and multiple injection technique, for this regional procedure [3].

At the beginning, for brachial plexus blockade the techniques, which implied paresthesia, or the trans-arterial technique. When the peripheral neurostimulator was introduced in 1912 by Perthes, the nerves could be properly and precise localized, most of the disadvantages of the above-mentioned methods being eliminated. This became the preferred method for nerve localization, until the ultrasound was introduced as an auxiliary method to brachial plexus block [4].

The possibility of direct visualization of the anatomic structures, the vessels and the nerves as well as the needles and the anesthetic spreading during the technique, increased the blocks success rate to up to 99% [5, 6].

Ultrasound was introduced in regional anesthesia in 1978, being used initially as an auxiliary method to peripheral neurostimulation. However, accidents such as vascular punctures and intravascular anesthetic administration continued to be present, this being the main reason that made needle visualization as well as the anesthetic spreading, to be a must in regional techniques. The industry developed needles with special properties, such as a different angle in faceting, which helps for a better reflection of the ultrasounds. These proprieties increased their visibility under ultrasound. Also, the ultrasound machines were improved to better localize the nervous structures. [7]

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Objectives

The evaluation of ultrasound efficiency as an auxiliary method for brachial plexus block, in terms of success rate, vascular punctures.

The influence of obesity on performing time, total duration of the block, and success rate of brachial plexus block.

Material and method

The prospective, randomized study was conducted in the Emergency Clinical County Hospital Tîrgu-Mureș. The study was approved by the Ethics Committee of the Emergency Clinical County Hospital, (No.2987/2013) and by the Ethics Committee of the University of Medicine and Pharmacy of Tîrgu Mureș (No.40/2013).

The patients were recruited by signing an informed consent form before performing the technique. The study enrolled adult patients, scheduled for emergency surgery or elective surgery on upper limb. The anesthetic procedure was the brachial plexus block with axillary approach, by multiple injections technique, using either the peripheral nerve stimulation or the ultrasound guidance. The inclusion of the patients was randomized, one patient had the brachial plexus block performed with the help of the peripheral neurostimulation, and the next one had the brachial plexus block performed with ultrasound guidance.

The materials used for anesthesia were the following:

- Peripheral nerve stimulator –B Braun (BBraun, Germany) - Stimuplex HNS 12
- Ultrasound -G&E Logique (General Electric, Chicago, Il, USA) - 9mm linear probe
- Needles 50mm STIMUPLEX Ultra Insulated Needles with 30° Bevel B Braun (BBraun, Germany)
- Anesthetic substances were Ropivacaine-10mg/ml-(Fresenius Kabi Pharma, Portugalia) and Lidocaine 1%, (Infomed Fluids, Bucharest, Romania), ana partes aequales 0.5% concentration, the anesthetic volume administered was between 8-10ml/ nervous structure.

We anesthetized the following nerves: musculocutaneous, median, ulnar and radial.

After informing and obtaining the written consent, the patient was positioned in supine position, with the arm of the injured limb abducted at 90 degree.

After inserting a peripheral venous cannula and standard monitoring the vital signs (noninvasive blood pressure, EKG, peripheral oxygen saturation) the exact spot of the puncture was established by identifying the axillary artery, in the axilla. Before the start of the anesthetic procedure, an amount of 1-2 ml of local anesthetic was administered subcutaneously for subcutaneous brachial nerve anesthesia.

The anesthetic procedure was performed either by peripheral neurostimulation or by ultrasound guidance.

By peripheral neurostimulation

A 50 mm stimulating needle was initially introduced anterior and superior to the axillary artery for musculocutaneous and median nerve localization, then the needle was oriented inferiorly to the artery for the ulnar nerve and posteriorly and inferior for radial nerve localization. The amplitude of the electric stimulus was initially set at 1,2mA, being reduced as we approached the nerve. The anesthetic administration was made at an amplitude lower than 0.5mA, but higher than 0.2mA.

By ultrasound guidance

The axillary artery was located by direct visualization along with the nerves, which are located around the artery. For anesthetic injection, 50 mm needles with increased ultrasound visibility were used.

The anesthetic injection method was that of multiple injections, around the each nerve.

A complete motor and sensitive brachial block was considered a successful anesthesia.

The processed data were:

1. The performing time of the anesthesia – measured from the moment of the puncture until the moment of needle extraction.
2. Vascular incidents – accidental vascular punctures encountered during the anesthetic procedure
3. Body mass index and success rate, vascular punctures.

The data were processed with the program GraphPad Prism 7.0. A value of 0.05 alpha (α=0.05) was established, and a confidence interval of 95%. The normality of the series was established by D'Agostino test. For series comparison, the following statistic tests were used: Man Whitney for non-parametric data and t student test for unpaired data, when a normal distribution was present. The difference between proportions was tested by Chi test. In order to analyze the variables, we deemed necessary to use logistic regression models.

Results

We enrolled 160 patients, two groups. The ultrasound group comprised of 82 (US group) patients and the neurostimulation group had 78 patients (NS group).

When the groups were compared as to performing time, no significant statistic difference was noted between the two groups, p= 0.74. The mean performing time in the NS group was 12.27 ± 6.96 minutes, very similar with the US group, where the mean performing time was of 12.26± 6.21 (Figure 1).

For the accidental vascular punctures the US group was advantaged by a significantly lower rate (p= 0.04) (Figure 2).

Obesity was defined as a value of BMI over 25. We enrolled a total of 88 (55.7%) patients with an BMI> 25 out of 158, for two patients from the US group the necessary data to calculate BMI were not obtained. Figure 3 shows the number of each category of patients, in terms of BMI, from the two studied groups.

There were no statistically significant differences regarding the number of the obese patients or the mean body mass index between the studied groups.

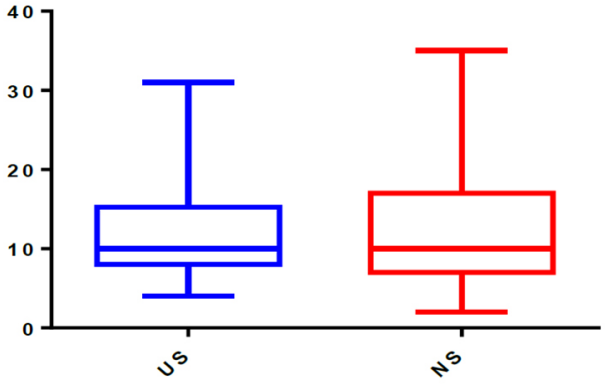

Fig. 1. The performing time of the brachial plexus block by the studied methods.

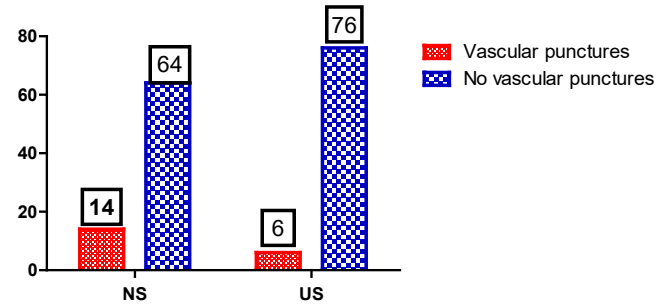

Fig. 2. Vascular punctures in the US group compared with NS group

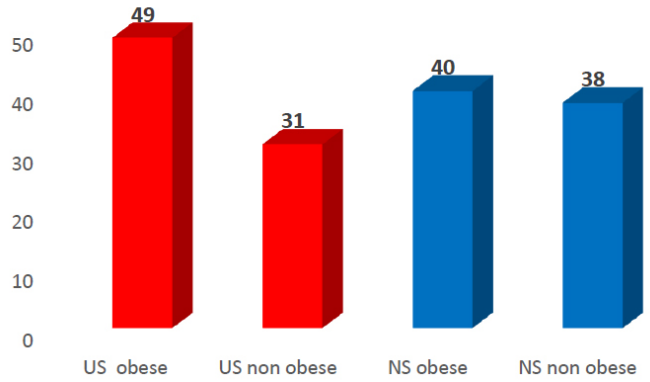

Fig. 3. Obese patients in the two groups.

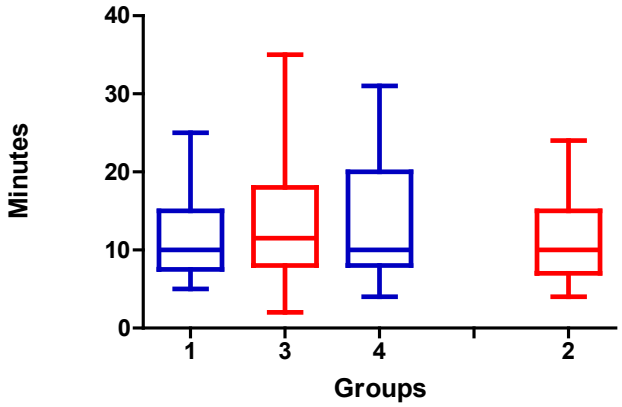

Fig. 4. Performing time in the studied groups 1- Obese patients US; 2- Obese patients NS; 3- Non obese patients NS; 4- Non obese patients NS

The success rate of the block was not influenced by the obesity. Table I represents Pearson correlation results when the groups were compared in terms of success rate and obesity.

Table I. Relationship between BMI and the success rate of the block

p- values Pearson correlation			Partial Block non obese - NS	Partial Block non obese US
Partial Block obese US		0.21	0.61	0.69
Partial Block obese NS	0.21		0.61	0.96
Partial Block non obese – NS	0.61	0.61		0.96
Partial Block non obese US	0.69	0.96	0.96	

Table II. Correlation between vascular punctures and the obese vs non obese patients from the two groups.

p- values Pearson correlation	Vascular punctures- obese NS	Vascular punctures obese US	Vascular punctures non obese NS	Vascular punctures non obese US
Vascular punctures- obese NS		0.43	0.09	0.35
Vascular punctures obese US	0.43		0.14	0.37
Vascular punctures non obese NS	0.09	0.14		0.33
Vascular punctures non obese US	0.35	0.37	0.33	

Obesity did not influence significantly the number of vascular punctures. There was no significant statistic difference between the subgroups; p values of the statistical correlation are listed in table II.

The performing time of the procedure when the obese were compared with non-obese in the two formed subgroups, showed a positive significant correlation between the times needed to perform the procedure in non-obese patients from the US group and the obese patients from the US group. Table III presents the detailed correlations.

The time needed to perform the procedure for the different groups is presented in figure 4. Table IV shows the exact time needed to block the brachial plexus in every studied group.

Discussions

Brachial plexus anesthesia for hand surgery is increasingly used either to complete general anesthesia or as a single technique. The classical approach for nerve localization is peripheral neurostimulation, but in the last two and a half decades, ultrasound guidance is increasingly used as an auxiliary method in regional anesthesia. This is because trough peripheral neurostimulation the direct visualization of nervous and vascular structures is not possible. This aspect offers a higher safety degree during the ultrasound guided procedure. Some may still be reluctant to use the ultrasound because the need of supplementary knowledge.

Table III. The correlation between the groups in the light of obesity and performing time

p- values	Performing time obese US	Performing time obese NS	Performing time non obese NS	Performing time non obese US
Performing time obese US		0.19	0.04	0.42
Performing time obese NS	0.19		0.10	0.24
Performing time non obese NS	0.04	0.10		0.18
Performing time non obese US	0.42	0.24	0.18	

Table IV. Performing time for the studied groups

	Performing time obese US (minutes)	Performing time obese NS (minutes)	Performing time non obese NS (minutes)	Performing time non obese US (minutes)
Minim	5.000	4.000	2.000	4.000
Maxim	25.00	24.00	35.00	31.00
Mean	11.33	11.23	13.43	13.39
Standard deviation	5.051	5.881	7.722	7.260

The execution time is not affected by the method used to perform the block, the values obtained in this respect for the two groups of patients being very close and with no significant difference. Moreover, the literature shows similar results; a study conducted in 2012 obtained similar results when using the ultrasound method for axillary brachial plexus block – 12.2 minutes [8] and another study, again in 2012, presented a performing time of 15.7 minutes [9].

The obtained values in this study, for the performing time, were 12.27 minutes in the US group, value very close to the value reported in the above-mentioned studies, but with a higher specificity due to the larger number of patients that the study enrolled. The obtained value on performing the ultrasound guided brachial plexus block was also close to that obtained when the oldest approach was used, peripheral neurostimulation, this showing that ultrasound guidance is not delaying the procedure.

This study points out the significant advantage that ultrasound guidance brings to the safety of this procedure. In the US group, the number of vascular punctures was significantly lower compared to the NS group. The advantages of the ultrasound guided regional anesthesia is well recognized by specialists around the world, this study reinforcing the statements that the ultrasound guidance reduces significantly the number of vascular punctures [10]. This is what we also obtained, the ultrasound guidance significantly reduced ($p<0.05$) the number of vascular punctures, making the procedure safest, thus diminishing the risk of intravascular injection of the local anesthetics.

The obese patients are a group of patients with supplementary risks for anesthetic procedures, because the anatomical marks are altered and sometimes it is not that easy to palpate the axillary artery, which is the landmark for the brachial plexus block with axillary approach. When we studied the enrolled patients in this respect, we found no correlations between BMI and the number of vascular punctures. The same results were reported in literature, the conclusion being that the obesity does not influence significantly this regional anesthetic procedure, regardless of the method used to perform the brachial plexus bock. [11].

The literature advises the use of ultrasound guidance for patients with a body mass index above 30 because on those patients, the anatomical marks are harder to locate, but a meta-analysis conducted on the studies which evaluated this aspect, did not graded this recommendation to be representative [12,13]. This study is limited by the fact that it considered only this approach of the brachial plexus block, and that it was only conducted on the axillary block.

Conclusion

Ultrasound guidance makes axillary brachial plexus block safer in terms of incidence of vascular puncture. Therefore, we recommend ultrasound guidance as routine for axillary brachial plexus block.

The obese patient can benefit by both methods of brachial plexus blockage, ultrasound or neurostimulator guided, without any significant risks compared to non-obese patients.

Acknowledgement

This study is part of the PhD thesis entitled: Comparative pharmacological studies on the method of sodium channel inhibition by local anesthetics, anticonvulsants and antiaritmics and the use local anesthetic admixtures in brachial plexus block with axillary approach.

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Acta Medica Marisiensis 2017;63(3):152-154 DOI: 10.1515/amma-2017-0027

CASE REPORT

Transthoracic 3D Echocardiographic Imaging of Type A Aortic Dissection – Case Presentation

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In type A aortic dissection (AoD) an early and accurate diagnosis is essential to improve survival, by applying urgent surgical repair. 3D transthoracic echocardiography (3D-TTE), an advanced noninvasive imaging technique, could offer a comprehensive evaluation of the ascending aorta and aortic arch in this regard. Both modalities of real-time 3D imaging – live 3D and full-volume aquisition – proved to be useful in evaluating the localization and extent of AoD. Our case illustrates the utility of 3D-TTE in the complex assessment AoD. By providing the proper anatomical dataset, 3D-TTE could facilitate considerably the diagnosis of type A AoD.

Keywords: type A aortic dissection, 3D transthoracic echocardiography

Received 7 June 2017 / Accepted 20 July 2017

Introduction

Recently, one of the most substantial developments in the field of echocardiography was the introduction of three-dimensional (3D) imaging.

The advantages and clinical applications of this technique are multiple: (1) significant improvement in the quantitative evaluation of the cardiac chambers, including volumes and left ventricular ejection fraction, (2) better visualization of cardiac structures, like valves or congenital anomalies, (3) real-time control and guiding of percutaneous cardiac interventions (e.g., atrial septal defect closure), (4) evaluation of heart disease in the preoperative, intraoperative and postoperative setting, allowing immediate feedback on the effectiveness of surgical interventions. [1]

In our days, 3D echocardiography – due to the development of both hardware and software engineering – became a feasible, real-time examination method in the daily practice, using both the transthoracic and transesophageal approach.

The two main methods of examination in 3D echocardiography are live 3D mode and full volume acquisition with offline postprocessing.

During the live 3D examination a narrow pyramidal volume is exposed in real-time. This type of imaging has multiple advantages: (1) lack of stitching artefacts, (2) "true" real-time examination (of great value during interventions), (3) good temporal resolution, and (4) visualisation of different cardiac structures by steering the volume electronically. [1, 2]

Our case represents an excellent example of the utility of transthoracic 3D echocardiography in the evaluation of a critical cardiac condition, a type A aortic dissection.

Case presentation

KI, a 61 year old male, was referred to our department from a territorial hospital with the diagnosis of heart failure due to significant aortic regurgitation. At the admission, the patient complained of dyspnea with orthopnea, severe fatigue and a non-exertional, non-anginal chest pain/discomfort. After a detailed history taking, we found that the first acute and severe chest pain appeared three months before, while brushing his teeth, and his clinical condition progressively worsened determining admissions to the territorial hospital. Physical examination revealed a severely distressed patient with pale and perspirated teguments, bilateral, basal crackles, and a 4/VI proto-mesodiastolic murmur at the Erb point accompanied by an ejection murmur, 5/VI, in the aortic area.

Routine 2D transthoracic echocardiographic examination (Fig. 1) revealed a severely dilated ascending aorta (72mm), a severe aortic regurgitation and a great suspicion of aortic dissection, due to the existence of a linear structure in the aortic lumen, resembling with a dissection membrane.

The examination was continued with transthoracic 3D imaging, which confirmed the diagnosis of type A aortic dissection (Fig. 2, 3, 4) causing severe aortic regurgitation. The visualisation of the lesion was possible toward the thoracic descending aorta using the parasternal and suprasternal windows.

The patient was referred to the cardiovascular surgery clinic, but declined intervention, at hospital discharge being hemodynamically stable without signs of decompensation.

After 4 weeks he returned to our clinic with signs of severe cardiac decompensation. After stabilization he undervent cardiac surgery, but has died due to postoperative complications.

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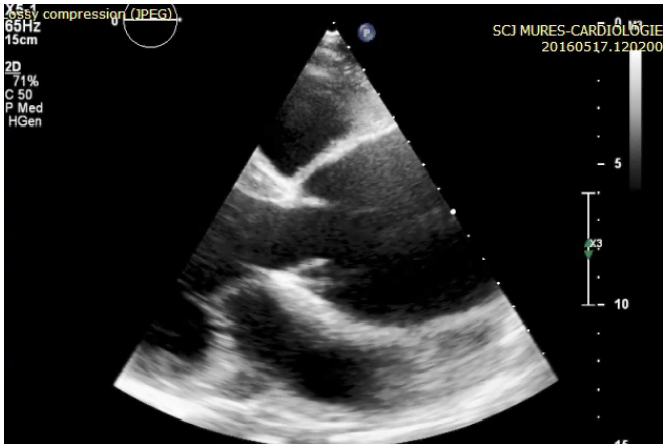

Fig. 1. 2D transthoracic echocardiography - parasternal long axis view of the ascending aorta: severe dilatation

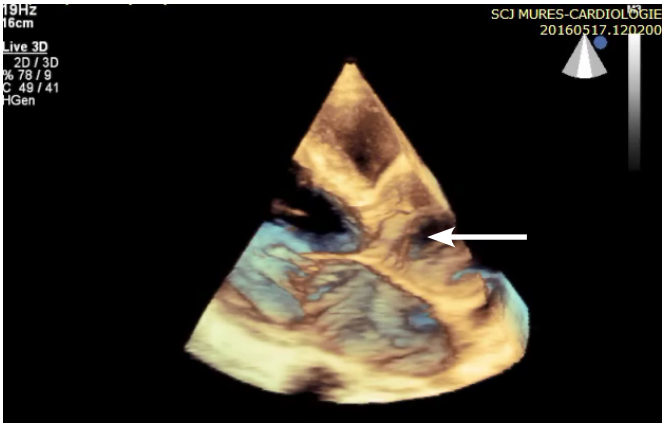

Fig. 2. 3D transthoracic echocardiography (live 3D) – parasternal long axis view: visualisation of the dissection membrane in the aortic lumen (arrow)

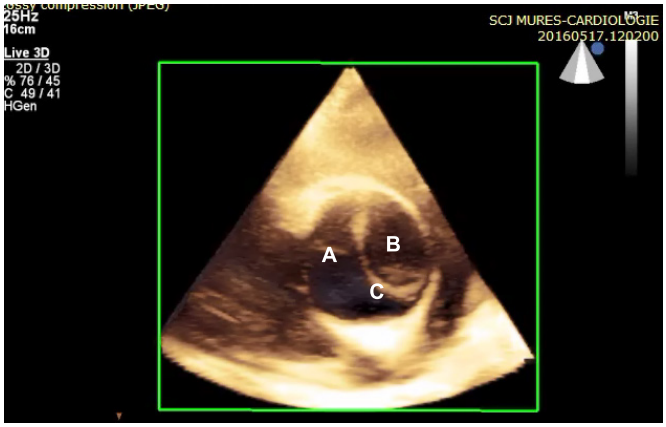

Fig. 3. 3D transthoracic echocardiography (live 3D) – parasternal short axis view (angulated superiorly): visualisation of the false (A) and true (B) lumen and the dissection membrane (C)

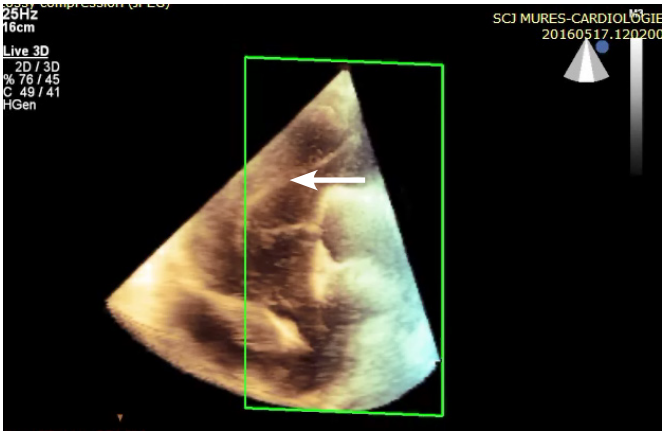

Fig.4. 3D transthoracic echocardiography (live 3D) – suprasternal long axis view of the aortic arch: visualisation of the dissection membrane (arrow)

Discussion

Aortic dissection (AoD) is life threatening condition which occurs when blood enters into the media due to an intimal tear. Its incidence is estimated at 6:100.000 person per year, being higher in men than in women and increasing with age. The main risk factor associated with AoD is hypertension, which is present in almost 70% of patients. [3]

Timely diagnosis is important since the natural history of AoD, if left untreated, includes a mortality as high as 2% per hour during the first two days, thus, urgent surgical repair repair could be lifesaver.

The ascending aorta (aortic root) is routinely evaluated during every standard echocardiographic examination, measuring the annulus and the sinusal, sino-tubular and tubular diameters. [4, 5]

More precise diagnosis of AoD is based on computer tomography (angio CT) and two-dimensional transesophageal echocardiography (2D-TEE). CT is a feasible method (availability, instantaneous results), but cannot be performed at the bedside, also needing administration of a contrast agent. Echocardiography is the preferred method of examination in the unstable patient who is not transportable. Despite its incontestable usefulness, two-dimensional transthoracic echocardiography (2D-TTE) has its own limitations regarding the visualisation of the whole aorta. Thus, in patients with adequate acoustic windows, real-time three-dimensional transthoracic echocardiography (3D-TTE) could provide all the relevant anatomical and functional data which make possible an accurate diagnosis of AoD, not requiring esophageal intubation or administration of intravascular contrast agent.

In our case, live 3D-TTE increased the accuracy of the diagnosis, since the dissection membrane was clearly seen on the 3D images. 3D-TTE has proven to be useful when AoD is suspected on 2DTTE, having an additional advantage, that makes possible the visualization of propagation into the branching vessels. [6-11]

Perspectively, in the diagnosis of aortic dissection, 3D-TTE is going to be more feasible, mainly because of the visualisation and measurements from the en face views, which provide more accurate and reproducible data in comparison with two-dimensional echocardiography. [1]

Conclusions

In our case, 3D transthoracic echocardiographic examination proved to be an accurate choice for diagnosing type A

aortic dissection, conferring all the relevant anatomical and functional data needed for planning surgical repair.

Ethical approval

Approval of the Ethical Committee of Clinical County Hospital Mures (no. 3865/01.03.2016) was obtained for data processing and publication.

Conflict of interest

None to declare.

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Acta Medica Marisiensis 2017;63(3):155-158 DOI: 10.1515/amma-2017-0028

CASE REPORT

Heterozygous Deletion in Exons 4-5 of SHOX Gene in a Patient Diagnosed as Idiopathic Short Stature

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Introduction: Isolated Short Stature Homeobox (SHOX) gene haploinsufficiency can be found in 2-15% of individuals diagnosed with idiopathic short stature determining different skeletal phenotypes. **Case presentation**: We present the history of an 11-year-old female patient diagnosed with idiopathic short stature. Clinically, she was moderately disproportionate, with cubitus valgus and palatum ogivale. Her breast development was in Tanner stage 1 at the time of diagnosis. The endocrine diagnostic tests did not reveal any abnormalities except a slightly elevated thyroid stimulating hormone. We have also assessed the bone radiological findings. Multiplex Ligation-dependent Probe Amplification technique used for the identification of SHOX gene haploinsufficiency showed a heterozygous deletion spanning exons 4-5 of SHOX gene. **Conclusions**: This case is determined by deletions in exons 4-5 of SHOX gene and indicates the necessity of screening for SHOX deletions in patients diagnosed with idiopathic short stature, especially in children having increased sitting height-to-height ratio or decreased extremities-to-trunk ratio.

Keywords: SHOX gene, idiopathic short stature

Received 29 May 2017 / Accepted 24 July 2017

Introduction

The short stature (SS), found in about 3% of children worldwide, can occur due to a variety of causes and is diagnosed when the proband's height is below – 2SD (standard deviation) within the mean age, gender and ethnic group of a certain population, or their height is below the third percentile curve. Within a family, short stature means < -2SD of parental height.

Idiopathic short stature (ISS, Online Mendelian Inheritance in Man (OMIM) #300582) is characterised by the above-mentioned parameters, without any apparent clinical signs of systemic, endocrine, nutritional and chromosomal abnormalities. Clinical diagnosis is based on the negative findings of standard diagnostic procedures. Nowadays more than 200 genes are known to be involved in the aetiology of syndromes characterised by short stature [1, 2]. *SHOX* gene haploinsufficiency is one of the most common monogenic causes of SS which have been described in 2-15% of ISS cases, in 50-90% of patients with Leri-Weill dyschondrosteosis (LWD; OMIM #127300) and 40% of patients presenting Langer mesomelic dysplasia (LMD) [3,4]. The *SHOX* gene is located in the pseudoautosomal region 1 (PAR1) of the short arm of the X and Y chromosomes (Xp22.33, Yp11.32) [5, 6]. PAR1 genes do not undergo X inactivation, and two copies of the *SHOX* gene are expressed, one of each of the sex chromosomes [5]. The *SHOX* gene encodes a transcription factor expressed during human embryogenesis in the pharyngeal arches and developing limbs. It plays a fundamental role in the chondrocyte function of the growth plate, as a regulator of cellular proliferation and differentiation [6, 7]. Isolated SHOX deficiency leads to a variety of different skeletal phenotypes and clinical conditions [3]. The Multiplex Ligation-dependent Probe Amplification (MLPA) is the first recommended molecular genetic method for the detection of deletions in the *SHOX*-gene and can be used to detect PAR1 deletions and duplications including SHOX and downstream enhancer region [8].

Case presentation

This study was approved by the local Ethical Committee, the informed consent was signed by the parents, and they approved to publish the medical data of the child.

The subject of our study was an 11-year-old girl from Odorheiu-Secuiesc, who was born full term with a birth weight of 4,000 g and 57 cm length. Thereafter, compared to other children of her age, she was of short stature. At the age of nine, she was 124.5 cm tall (-2.35 SD) and she weighed 40 kg. Clinically, she was moderately disproportionate, having increased sitting height-to-height (SH/H) ratio, with cubitus valgus and palatum ogivale. At the first measurement according to the Tanner scale, her breast development stage was B2/3. Presently (two years later, *Figure 1.*) she weighs 45 kg, presenting obesity grade 1 (according to the World Health Organisation criteria, year 2007), genu valgum and Tanner stage B3. She also presents cubitus valgus, palatum ogivale (high – arched palate) and micrognathia.

Binder and co-workers have developed a method based on axiological measurement which facilitates a more accurate selection of idiopathic short stature patients to whom the *SHOX* gene examination is recommended. The authors recommend calculating the sum of leg length and arm

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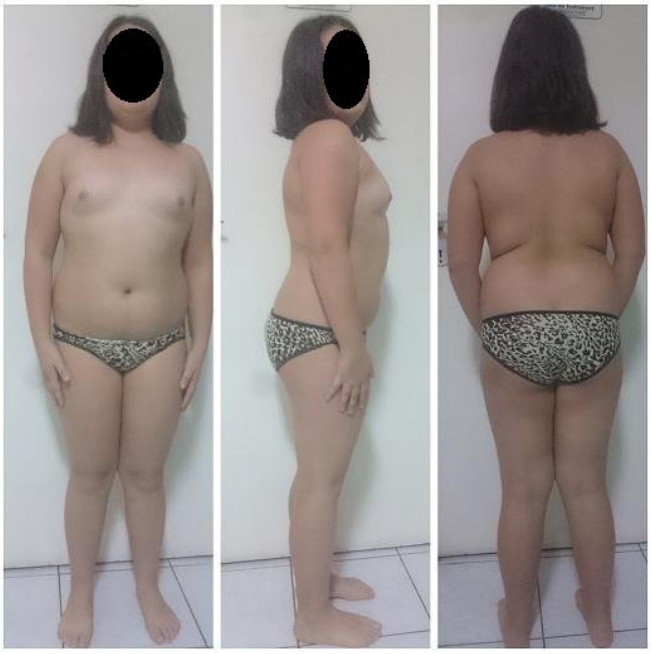

Fig. 1. The SHOX haploinsufficiency ISS patient from Romania

span, divided by sitting height. Thus the results can be of almost 100% negative predictive value, i.e. the higher this value is (>2.5), the lower the probability of SHOX gene deletion [9].

According to the phenotype scoring system proposed by Rappold and his co-workers for the evaluation of SHOX deficiency, there are eight criteria based on the data of 1608 short individuals including 68 persons with SHOX deficiency that should be taken into consideration [10]. Arm span to height (A/H) and SH/H ratios were classified as abnormal if less than 0.965 and greater than 0.555, respectively. In our case, the total score was 8. The patient presented an increased sitting height/height ratio, obesity and cubitus valgus *(Table 1).*

The common endocrinological diagnostic tests did not reveal any abnormalities which might cause growth retardation, except a slightly elevated TSH = 14.55 μIU/mL (0.25-5.00) *(Table 2).* Thyroid ultrasound described normal volume and structure, and the anti-thyroid peroxidase antibodies were between normal range excluding the presence of autoimmune thyroid disease. The high TSH level indicated a subclinical hypothyroidism that could be related to the elevated BMI. Bone radiological findings excluded the presence of Leri-Weill dyschondrosteosis. Turner syndrome (even a mosaicism) could be excluded by the fact, that in our case only the 4 and 5 exons of the SHOX gene were deleted, and not the whole gene. Similarly, in the positive control Turner syndrome patient only one SHOX gene was present, so the intensity measured with MLPA was only half of the normal controls.

To determine the possible alterations of the *SHOX gene*, genomic DNA was extracted from the leucocytes by the method recommended by QIAgen, and the Multiplex Ligation-dependent Probe Amplification (MLPA) technique was performed at the Endocrine Genetics Laboratory of the Semmelweis University, using the SALSA P018 MLPA kit (MRC-Holland, Amsterdam, The Netherlands), according to the manufacturer's protocol. Molecular genetic testing revealed a heterozygous deletion of exons 4-5 of SHOX gene, using the MLPA technique *(Figure 2).*

Short stature was present in the family history of the patient (brother, mother and father), but the genetic testing of the SHOX gene failed to show SHOX gene deletion in the family members, suggesting that this alteration occurred de novo in our patient.

The treatment plan consists of a hypocaloric diet with low fat, low carbohydrates, and protein rich foods. The patient was advised to avoid a sedentary lifestyle and do physical exercise on a daily basis, at least 30 – 60 min. Systemic treatment for subclinical hypothyroidism with small doses of L-thyroxine was also necessary. Growth hormone therapy has been approved by FDA (Food and Drug Administration) in The United States, and now it is generally accepted in the clinical treatment of these cases [11].

Discussions

Isolated SHOX haploinsufficiency is one important monogenic cause of short stature. The deletion of *SHOX gene* is characterized by feminine domination, which can be explained by the fact that the deletion of the short arm of the X chromosome is more frequent than the rupture of the short arm of the Y chromosome [12].

As far as we know, we are the first to report a case of ISS in a young patient from Romania diagnosed with the MLPA technique for SHOX haploinsufficiency. Our case

Table I. The total diagnostic score of our patient, based on axiological measurements and clinical characteristics [10].

	Criterion	Score
Arm span/height ratio	<0.965	0
Sitting height/height ratio	>0.555	2
BMI	>50 percentile	4
Cubitus valgus	present	2
Short forearm	present	0
Bent forearm	present	0
Muscle hypertrophy	present	0
Ulna elbow dislocation	present	0
Total score		8

Table II. Results of endocrine diagnostic tests.

	Patient's values	Reference range
Growth hormone at baseline (ng/mL)	2.99	0.05 – 17.3 ng/mL
Insulin-like growth factor type 1 (ng/mL)	386.9	76 – 549 ng/mL
Plasma cortisol at baseline, at 8:00 AM (nmol/L)	578.8	118 – 618 nmol/L
Anti-thyroid peroxidase antibodies (IU/mL)	< 10	< 35 IU/mL
Thyroid stimulating hormone (μIU/mL)	14.55	0.25-5 μIU/mL
Free Thyroxine (ng/dL)	1.01	0.98-1.63 ng/dL
Glucose (mg/dL)	98	60-100 mg/dL
Alcaline phosphatase (U/L)	208	51-332 U/L

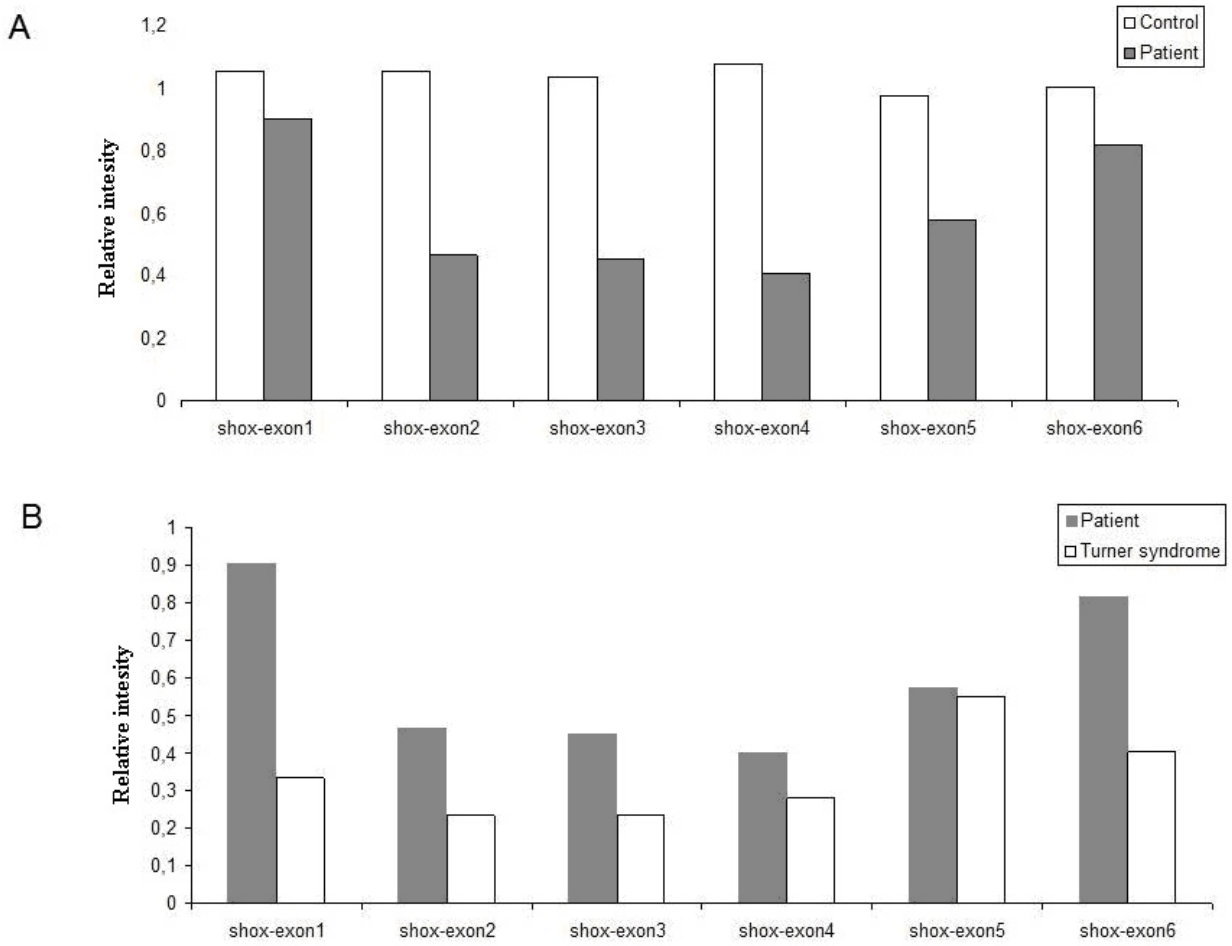

Fig. 2. Multiple ligation dependent probe amplification (MLPA) for detection of SHOX gene deletion. Panel A. Relative expression of exons of SHOX gene in our patient compared to the control (note: the relative expression of exons 2-5 in our patient was between 45-58 % compared to a healthy female, the cut-off value for diagnosis was 0.6 of the normal value). Panel B: Relative expression of exons of SHOX gene in our patient compared to a patient with cytogenetically confirmed Turner syndrome (expression of exons 4 - 5 was similar between these two patients, while the expression of exons 2 - 3 was approximately twice greater in our patient compared to those found in patient with Turner syndrome).

has a particular importance because, the deletion of exons 4-5 of the gene was identified in a patient with ISS, without any radiological signs, highlighting the importance of genetic testing even in patients who present mild clinical symptoms, or suffer from idiopathic short stature.

Based on an accurate genetic diagnosis, the physician can give proper genetic counselling for the patient and his/her family, informing them about the evolution of the disease and the potential therapeutic plans.

Treatment with GH of children with SHOX deficiency and young females with Turner syndrome was equally efficient in a two-year prospective open-label parallel study [11]. This kind of treatment is effective in ameliorating the growth deficiency and skeletal anomalies found in children with SHOX deficiency [2].

SHOX deficiency disorders are inherited in a pseudoautosomal dominant manner. The offsprings of an individual with SHOX deficiency disorder has a 50% risk to have a pathogenic SHOX variant. If both parents present SHOX deficiency, the child undergoes a 50% hazard of developing a SHOX deficiency disorder, 25% risk of developing Langer mesomelic dysplasia, and 25% chance of being healthy [13].

In a previous Romanian study Miclea et al. analysed 79 patients presenting short stature with FISH technique using probes for *SHOX* and centromeric regions. They found one (2.3%) case with *SHOX* deletion in a patient with short stature and normal karyotype [14].

The MLPA is currently the first recommended molecular method for the detection of SHOX gene deletions with a 70-75% success rate. It is a simple method that permits the concomitant analysis of a wide number of samples and can be applied for the detection of PAR1 deletions and duplications including *SHOX* and the downstream enhancer region. It also permits the evaluation of the deletion extension. MLPA is less expensive, less time consuming and more sensitive than other techniques (e.g. microsatellite analysis, or FISH technique) [15].

Conclusions

We are reporting the first case of SHOX haploinsufficiency ISS patient from Romania, specifically a deletion in exons 4-5 of the gene, detected by Multiplex Ligation dependent Probe Amplification (MLPA) technique. This case indicates the necessity of screening for SHOX deletions in patients with idiopathic short stature, especially in chil-

dren presenting increased sitting height-to-height ratio or decreased extremities-to-trunk ratio.

Conflict of interest

None to declare.

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