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The Editorial Board kindly informs that since 2014 *Nowiny Lekarskie* has been renamed to *Journal of Medical Science*.

The renaming was caused by using English as the language of publications and by a wide range of other organisational changes. They were necessary to follow dynamic transformations on the publishing market. The Editors also wanted to improve the factual and publishing standard of the journal. We wish to assure our readers that we will continue the good tradition of *Nowiny Lekarskie*.

You are welcome to publish your basic, medical and pharmaceutical science articles in *Journal of Medical Science*.

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ORIGINAL PAPER

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Biopharmaceutical evaluation of new semi-solid preparation with thiotriazoline and chloramphenicol

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ABSTRACT

Introduction. Periodontitis numbers among the most widespread diseases. The prevalence of the periodontal inflammation does not tend to decrease, but long-term history of the disease, the complexity in treatment significantly reduce the quality of life in patients, cause the tooth loss. An important principle for the treatment of mentioned disorder is use of drug products in the lesion location. The preference is given to semi-solid preparations with a polyvalent pharmacological action which can eliminate the causative agents and affect the different elements in the pathogenesis.

Aim. Aim of the study was to evaluate the influence of excipients, including the base-forming agents, on the thiotriazoline release from semi-solid preparation and based on the results of investigations to develop the composition of a new drug product for treatment of periodontal inflammation.

Material and methods. Thiotriazoline and chloramphenicol were selected as active pharmaceutical ingredients for the development of semi-solid preparation. Methylcellulose, carboxymethylcellulose sodium and polymethylsiloxane (base-forming agents); glycerol and propylene glycol (plasticizers); methylparaben and propylparaben (preservative agents); peppermint oil (flavouring agent) were also used in the investigations on the pharmaceutical development.

In order to select an optimal composition of the base for dental semi-solid preparation with thiotriazoline and chloramphenicol, six samples with different content were prepared. The appearance of samples was evaluated visually. Measurement of pH value was performed using pH-Meter-240 CORNING. Thiotriazoline release from the test samples was studied *in vitro* by agar diffusion method.

Results and conclusions. The results of investigations showed that excipients largely affected thiotriazoline release from the drug product. According to the results of physical, physical-chemical and biopharmaceutical investigations the following excipients were chosen for the development of dental semi-solid preparation: methylcellulose (base-forming agent), glycerol (plasticizer), methylparaben and propylparaben (preservative agents), peppermint oil (flavouring agent with antiseptic action).

Developed preparation is homogeneous mass with a thick consistency, white color and pleasant fresh smell. The drug product is characterized by stable physical-chemical and rheological properties, and has a neutral pH.

Keywords: thiotriazoline, chloramphenicol, biopharmaceutical evaluation, periodontitis.

Introduction

Periodontitis numbers among the most widespread diseases. About 75% of adults suffer from this disease [1]. The prevalence of the periodontal inflammation does not tend to decrease, but long-term history of the disease, the complexity in treatment significantly reduce the quality of life in patients, cause the tooth loss. Considering this, pharmacotherapy of periodontitis is a topical subject.

An important principle for the treatment of mentioned disorder is use of drug products in the lesion location. The preference is given to semi-solid prepara-

tions with a polyvalent pharmacological action which can eliminate the causative agents and affect the different elements in the pathogenesis.

Since commonly known drug products not always provide the desired therapeutic effect, investigations on development of composition of new combined preparation in the semi-solid dosage form were performed.

Development of the dental semi-solid preparations is justified because they are chemically more stable than liquids and they may prolong the contact time between the drug and affected tissues. Those preparations provide local and uniform release of active ingredients, creating a high drug concentration in the contact site without a significant effect on the drug level in the systemic circulation.

One of the well-known techniques which are used to enhance the therapeutic efficacy and so to shorten the treatment duration is scientifically and experimentally justified combination of several active pharmaceutical ingredients in a single dosage form [2]. Furthermore, such approach is cost-effective as it allows developing of practically new and more effective drug products based on the existing assortment of active pharmaceutical ingredients. Reduction in the number of drug products in the therapeutic scheme will undoubtedly improve the patient compliance. Taking into consideration the above mentioned aspects, thiotriazoline and chloramphenicol were selected as active pharmaceutical ingredients for development of the dental semi-solid preparation.

Thiotriazoline (morpholinium; 3-methyl-4H-[1,2,4] triazol-5-ylsulfanyl)-acetate (**Figure 1**) is a new Ukrainian agent that demonstrates antioxidant, membrane stabilizing, anti-ischemic, anti-inflammatory, immunestimulatory, antiviral activity and stimulates the tissue regeneration.

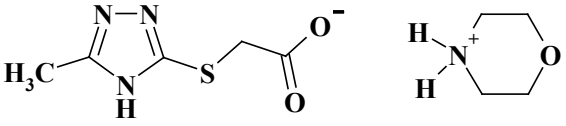

Figure 1. Chemical structure of thiotriazoline

Pharmacological effect of thiotriazoline is provided by activating the enzymatic antioxidant system and inhibition of lipid peroxidation in the ischemic tissues, normalization of the tropism of nervous system, increasing in the intensity and rate of reconstructive processes, declining the tissue inflammation, and improvement of blood microcirculation [3]. Drug products with thiotriazoline have already been used in modern medicine, particularly in cardiology, surgery, ophthalmology, hepatology, stomatology [4]. We have found that an effective concentration of thiotriazoline in drug products for topical use is 2% [5].

Since thiotriazoline does not possess the antibacterial activity, but pathogenic microorganisms are the basic etiologic factors of marginal and apical periodontal inflammations, the possibility of chloramphenicol introduction into the composition of dosage form was studied [6, 7].

Chloramphenicol (2,2-dichloro-N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl] acetamide (**Figure 2**) is a broad spectrum antibiotic with activity against gram-positive and gram-negative bacteria, rickettsia, spirochetes.

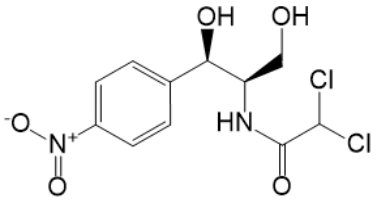

Figure 2. Chemical structure of chloramphenicol

It is the only one natural antibiotic, molecule of which contains residue of nitrobenzene that is toxic against bacteria cells. It is important to emphasize that chloramphenicol is one of the few chemotherapeutic agents that exhibit activity against anaerobic gram-negative bacteria. Minimal inhibitory concentration of chloramphenicol in dosage form for topical use is 0.5% [8].

Material and methods

Thiotriazoline (purity 99%) was generously gifted from the Department of Pharmaceutical Chemistry of Zaporizhzhya State Medical University, Ukraine, where this substance was first synthesized by prof. I.A. Mazur and coauthors.

Chloramphenicol (purity ⩾ 98%) was purchased from Sigma-Aldrich (USA) and used without further purification.

An essential stage in the development of new drug product is the right choice of excipients which serve as a carrier for active pharmaceutical ingredients. The pharmacokinetic and pharmacodynamic parameters of drug product can be controlled by selection of excipients and their concentration.

For development of the composition of dental semi-solid preparation with thiotriazoline and chloramphenі-

col the following excipients were selected: methylcellulose (A4M Pharm), carboxymethylcellulose sodium (medium viscosity) and polymethylsiloxane as base-forming agents; glycerol and propylene glycol – plasticizers; methylparaben and propylparaben – preservative agents; peppermint oil – flavouring agent. All ingredients were purchased from Ashland Inc. (USA) and Sigma-Aldrich (USA), except of polymethylsiloxane (Pharmaceutical company "Ecologoprotective firm company "KREOMA-FARM", Ukraine) and peppermint oil ("Pharmaceutical factory", State Municipal Enterprise, Ukraine).

Selected base-forming agents are hydrostable, thermostable, and allow uniform drug release in the affected areas, providing a longer therapeutic effect in comparison with liquid preparations.

The plasticizers glycerol and propylene glycol ensure the plastic, thixotropic consistency of preparation, and possess the moistening and penetrating action [9].

To prevent microbial contamination and to destroy bacteria and fungi, as well as to extend the shelf life of the drug product, preservatives methylparaben and propylparaben were introduced into the composition of semi-solid preparation [10].

Peppermint oil was chosen as a flavouring agent. It is well-known that peppermint oil is used as antiseptic, anti-inflammatory and analgesic preparation [8].

In order to select an optimal composition of the base, 6 samples with different content were prepared. The amount of thiotriazoline in all sampled was 2%, the amount of chloramphenicol – 0.5%. Composition of the test samples is described in **Table 1**.

An important characteristic of the drug products is appearance which covers such parameters as consistency, color and smell. To prevent irritating action of dental semi-solid preparation on the oral mucous membrane, the pH level of the developed product must be within the allowable range for the mouth cavity (6.3–8.0).

Therefore, all samples were controlled on the mentioned quality parameters in the moment of production and after 3 and 6 months of storage. The appearance of the samples was evaluated visually. Measurement of pH value was performed according to the requirements of State Pharmacopoeia of Ukraine using pH-Meter-240 CORNING [11].

Therapeutic effect of drug product depends mainly on the rate and extent of drug release. Thiotriazoline release from the test bases was studied *in vitro* by agar diffusion method [12]. As indicator was used Reinecke salt that in reaction with thiotriazoline produced pink color. Test samples were distributed into the wells in the agar gel. Prepared system was incubated in a thermostat TS-80-M-2 at 37°C (± 1°C) for one day, and then diameter of colored areas around each well with the drug product sample was measured using beam compass.

Result and discussion

All samples had the look of uniform gelatinous mass of a whitish color and with a distinctive smell of peppermint. Visual characteristics of the ointments with polymethylsiloxane (samples №5 and №6) were worse than ointments with methylcellulose (samples №1 and №2) and sodium carboxymethylcellulose (samples №3 and №4). The pH level of all samples was within normal range.

Introduction of propylene glycol into the composition of bases (samples №2, №4 and №6) led to the deterioration of rheological properties.

Considering the above mentioned, in order to select the most optimal base for the development of a new preparation, the biopharmaceutical evaluation was conducted of the test bases №1 and №3.

Results of the study of base influence on the rate and extent of thiotriazoline (active pharmaceutical ingredient) release are shown in **Figure 3**.

Table 1. Composition of the tested bases

Excipients	№ of the base					
	1	2	3	4	5	6
Methylcellulose	+	+	–	–	–	–
Carboxymethylcellulose sodium	–	–	+	+	–	–
Polymethylsiloxane	–	–	–	–	+	+
Glycerol	+	+	+	+	+	+
Propylen glycol	–	+	–	+	–	+
Methylparaben	+	+	+	+	+	+
Propylparaben	+	+	+	+	+	+
Peppermint oil	+	+	+	+	+	+

(+) – presence of ingredient in the base; (–) – absence of ingredient in the base

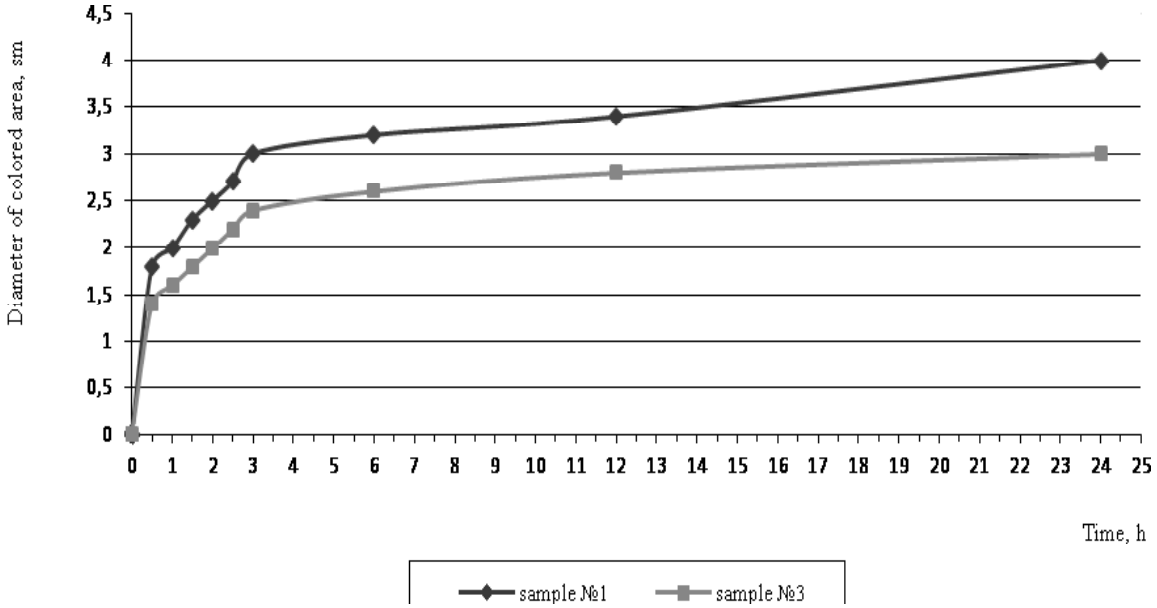

Figure 3. Influence of the nature and composition of the base on the thiotriazoline release from semi-solid preparations

As shown in **Figure 3**, thiotriazoline release from semi-solid preparations was observed within 24 h that indicated the prolonged action of test samples. But drug release from preparations was different. It was found that after an hour of the experiment the diameter of coloured area of the sample №1 was larger than the sample №3. After 90 min from the beginning of the experiment the drug release from the sample №1 was also more complete than from the sample №3 (diameter of coloured area was larger on 27%). Such a tendency was noted throughout the experiment. After 24 h the diameter of coloured area of the sample №1 was larger by 33% that indicated better thiotriazoline release from the methylcellulose based drug product.

The results of biopharmaceutical study proved that drug release from semi-solid preparations depended on the nature and amount of excipients, including the base-forming agents. An optimal base for the dental semi-solid preparation with thiotriazoline and chloramphenicol is base №1 that contains methylcellulose.

Conclusions

Combination of thiotriazoline and chloramphenicol (active pharmaceutical ingredients) in the semi-solid preparation intended for treatment of periodontitis will provide a complex therapeutic effect on the different pathogenetic links.

Performed investigations on substantiation of the composition of dental semi-solid preparation with thiotriazoline and chloramphenicol showed that excipients largely affected drug release from the drug product. According to the results of physical, physical-chemical and biopharmaceutical investigations the following excipients were chosen for the development of dental semi-solid preparation: methylcellulose (base-forming agent), glycerol (plasticizer), methylparaben and propylparaben (preservative agents), peppermint oil (flavoring agent with antiseptic action).

Developed drug product is homogeneous mass with a thick consistency, white color and pleasant fresh smell. It is characterized by stable physical-chemical and rheological properties, and has a neutral pH. The preparation adhered well to the marginal periodontium and can be easily inserted into the root canal.

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Conflict of interest statement
The authors declare that there is no conflict of interest in the authorship or publication of contribution.

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ORIGINAL PAPER

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Pharmacist's role in the system of palliative and hospice care in Ukraine and Poland

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ABSTRACT

Introduction. The efficient pharmacotherapy is an important part of palliative and hospice care, and requires a multidisciplinary approach to the patients. The pharmacist, as the member of the multidisciplinary therapeutic team, is responsible for performing pharmaceutical care, which provides safe and efficient treatment. The aim of the research was to conduct a comparative research about the status of palliative and hospice care and role of the pharmacist (clinical pharmacist) in Ukraine and Poland.
Material and methods. It was a questionnaire survey conducted in Ukraine, as well as analysis of information sources associated with this subject. The questionnaire was developed on the basis of similar study conducted in Poland. It consisted of 15 multiple-choice questions addressed to head physicians and doctors in Ukraine. The obtained results were compared to the results of the mentioned above Polish study.
Results. Eight head physicians and 22 doctors (30 questionnaires) from 13 palliative and hospice care institutions in Ukraine responded to the survey. It has been found that almost half in Ukraine (43%) believed that the pharmacist should be a mandatory member of a multidisciplinary team, because of his/her significant role in drug management in hospice.
Conclusion. A multidisciplinary approach to satisfying of patients' needs in palliative and hospice care has a great significance. Results of the study testify to the importance of including the pharmacists into the multidisciplinary team.

Keywords: palliative care, pharmacist, drug therapy, pharmaceutical care.

Introduction

Since the 80's of the 20th century, due to the biopsychosocial approach formation of a multidisciplinary approach to the provision of services was developed. This fact caused involvement as wide as possible of the range of professionals in order to solve problems of patient and his/her family. The basis of this approach is the statement, that body, mind, emotions and spirit are dynamically correlated in each person, and changes in one of these components affect changes in all others. That is why representatives from different specialties are working in multidisciplinary teams and their work promotes a comprehensive ensuring of patients' needs [1].

Because the palliative patients and their families need comprehensive support, that will maximally take into account their needs, in 1978 the World Health Organization (WHO) stated the need for multidisciplinary collaboration for successful provision of palliative and hospice care (PHC) to the patients.

The multidisciplinary team

Summary results of international studies have led to the conclusion, that the multidisciplinary team in the PHC should include four groups of employees: 1) specialists in palliative care (doctors, nurses); 2) allied health professionals (pharmacist, therapist, physiotherapist, dietician, psychologist, spiritual carers of various denominations, lawyers, social workers); 3) complementary therapists (art therapist, music therapist, massage therapist); 4) other staff (administrative staff, etc.) [7,10]. Moreover, volunteers, who support the professional staff in the care of patients, play an important role in PHC. They are members of multidisciplinary therapeutic team and perform various tasks, which help patients and their families [11].

The rational pharmacotherapy (PT) is a separate, socially important segment of PHC, which requires significant financial and organizational resources. One of the ways to optimize the efficiency of PT is the provision of pharmaceutical care to patients. It can be carried out by pharmacist in a pharmacy, which collaborates or is located directly in a hospice or in other pharmacies, when pharmaceutical care is directed to palliative patients and their family members.

The status of PHC in Ukraine

Pursuant to statistical data of the Association of Palliative and Hospice Care (APHC) over the last five years the number of deaths in Ukraine is about 800,000 people per year. According to WHO recommendations at least 500,000 end-of-life patients and nearly 2 million members of their families in Ukraine need supportive care every year [2].

Basic health care establishments, which provide palliative care in Ukraine, are hospices, palliative care departments in multi-profile hospitals and hospitals of oncological, tuberculosis and geriatric profile, and also HIV/AIDS centers. Inpatient PHC institutions provide medical care and elements of psychological and spiritual support. However, it should be noted that most people do not have access to comprehensive PHC.

The first hospices in Ukraine were established in Lviv, Korosten' and Ivano-Frankivsk in 1996–1997. In accordance with the data of APHC there are 5 inpatient hospices and 13 palliative care departments, which have 521 inpatient beds, and 7 HIV/AIDS centers (up to 50 beds) in Ukraine as of January 1, 2013. Also 3 PHC institutions (about 65 inpatient beds) are charitable, and the local health authorities are their co-founders. Bed capacity covers only about 20% of demands for inpatient hospice care (at a rate of 10 beds for palliative patients per 100 thousand population, that is 4,600 beds for Ukraine). Thus PHC is mainly administered at home by relatives or carers of patients. These data have shown a significant deficiency of specialized medical institutions for providing PHC to patients and psychological support of their families after the loss of loved ones [3, 4].

Conformably to hospice pharmacy and the role of pharmacists in the PHC system of Ukraine pharmacist's (CP) position is not included in the staffing table of establishments such as "Hospice" (Annex 50 to the Order of Ministry of Health of Ukraine "On the staff standards and typical staffing tables of health care facilities" from 23.02.2000 N 33). Only a small amount of institutions and departments of PHC in Ukraine cooperate with pharmacists (CP) (preferably in multi-profile hospitals). Therefore, pharmaceutical care is provided by pharmacists (CP) in hospital pharmacies or other pharmacies during the dispensing drugs to patients or their carers.

The status of PHC in Poland

PHC in Poland is provided in 476 various units, including 321 home hospices and 137 inpatient units (residential hospices and hospital-based palliative medicine wards) and others. There are 2232 palliative care beds, what is 58.3 per million inhabitants. Most of them were established by Catholic Church and nongovernmental organizations (associations and foundations). PHC is provided for patients with incurable diseases, like cancer, as well as for patients with heart failure, respiratory disorders, wounds and bedsores. The services are regulated by law and financed from public health care system [5, 6].

Hospice staff consists of professionals, e.g. physicians, nurses, psychologists, pharmacists, physiotherapists, priests and social workers. Nowadays, most of them are paid workers, but some perform their services as volunteers. Nevertheless, majority of volunteers do not carry out professional services and they support medical staff in patient care. Moreover, volunteers are engaged in other activates like charity and public collections. Physicians and nurses can participate in postgraduate education in palliative medicine.

According to the Polish Pharmaceutical law, pharmacists should be the members of hospice staff in the inpatient units. Residential hospices and hospital-based palliative medicine wards are obliged to establish hospital pharmacy and employ a master of pharmacy as a pharmacists team manager. It is associated with drug management and performing pharmaceutical services.

Aim

The aim of the study was to evaluate the current status of hospice pharmacy in Ukraine and to compare the role of the pharmacist in hospice and palliative care in Ukraine to that in Poland.

Methods

Questionnaire survey, analysis of literature associated with examined issues, systemic analysis was used as methods for the assessment of pharmacist's role in PHC.

The questionnaire developed on the basis on similar study conducted in Poland, contained 15 multiple-choice items, and has been adopted for survey in Ukraine [12]. It consisted of three parts: the first contained questions concerning information about the respondents (age, position, profession); the second was devoted to the basic characteristics of PHC institution (number of beds, funding, drugs and commodities supply, presence of pharmacist (clinical pharmacist – CP in the staff, etc.); the third was dedicated to the duties and tasks that pharmacist (CP) can perform in a hospice, possible cooperation with doctors and advantages of including pharmacist (CP) in the multidisciplinary hospice team.

Because of pharmacist's (CP) absence in staffing table of PHC establishments in Ukraine, a questionnaire was designed to survey head physicians and doctors of hospices and palliative care departments.

Data obtained from Ukraine was compared to the similar study previously conducted in Poland, which was published in Polish scientific journal on palliative medicine [12].

Results

As a result of the study, conducted in Ukraine, 8 head physicians and 22 doctors (30 questionnaires) from 13 PHC institutions responded to the survey.

It has been found, that the number of inpatient beds in almost half of the establishments in Ukraine was more than 20 beds, and almost two-fifths – with number of beds from 10 to 20 (**Figure 1**).

Suppliers of medicines for PHC establishments in Ukraine are primarily community pharmacies, pharmaceutical warehouses or wholesale pharmaceutical companies (**Figure 2**). Only 27% of respondents answered that institutions are partially supplied with medicines by pharmacies, which are located in them (usually in multi-profile hospitals). Among other sources the respondents indicated charitable organizations, local authorities and relatives of patients (presented percentages do not add up to 100%, because of the multiple-choice question).

Most respondents in their answers indicated a nurse as a person responsible for supply the hospice with drugs (**Figure 3**). Ukrainian respondents did not indicate a pharmacist as the responsible person for provision with medicines. Among other persons, responsible for supply of medicines, respondents indicated the relatives of patients.

Among the tasks that a pharmacist should perform in hospice the searching for domestic analogues and comparative assessment of their value were the most often met in answers of Ukrainian respondents (**Figure 4**). Also other functions were marked: tracking changes in legislation, appearance of new medicines; providing information about drugs; incoming control, account-

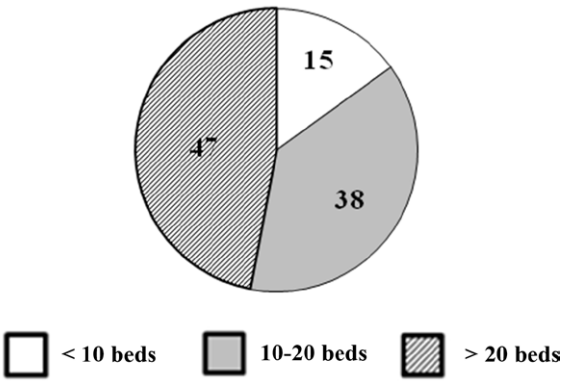

Figure 1. The number of inpatient beds in PHC institutions in Ukraine

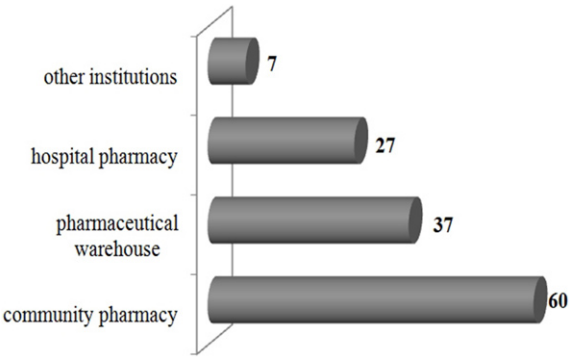

Figure 2. Organizations, which provide Ukrainian PHC institutions with drugs (%)

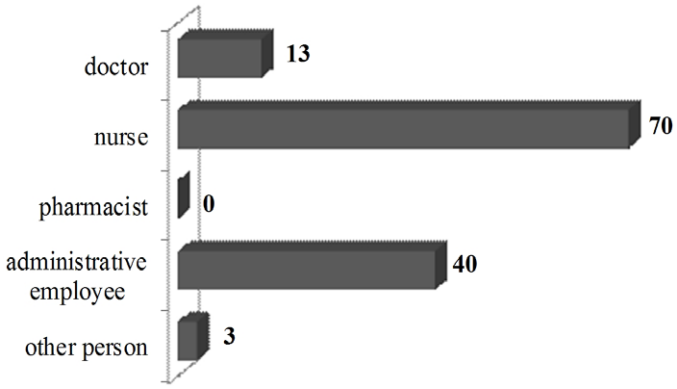

Figure 3. Persons, responsible for supply of the establishment with drugs (%)

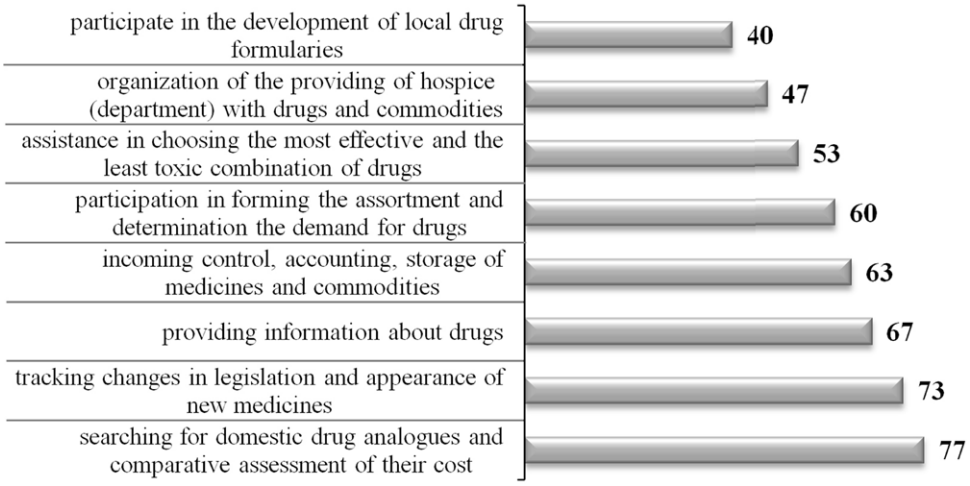

Figure 4. Tasks that a pharmacist should perform in hospice in Ukraine (% of respondents)

ing, storage of medicines and commodities; participation in forming the assortment and determination the demand for drugs.

Discussion

Comparison of surveys

The survey showed that there is no position of pharmacist in inpatient hospices and palliative care departments in Ukraine. Contrary, in Poland pharmacists are working in half of inpatient hospices. Polish hospices employ usually no more than one pharmacist [12].

In Ukraine, only one fifth of physicians (20%) indicated the cooperation with pharmacists and the others do not cooperate with them. By contrast, in Poland in more than 40% of hospices the pharmacist consults the members of therapeutic team [12].

Polish Pharmaceutical law provides two types of departments in health care institutions, in which pharmaceutical services are performed. The first one is hospital pharmacy, and the second – hospital pharmacy department. On the other hand, in home hospices patients buy medicines prescribed by hospice physicians in community pharmacies. In practice, only hospital pharmacy departments are present in residential hospices, and the most of PHC units have no specific department in which pharmaceutical services are performed [12]. Only 27% of Ukrainian respondents answered that institutions are partially supplied with medicines by pharmacies.

Three-quarters of Polish hospice directors and less than a half (43%) of Ukrainian respondents affirmatively answered to the question "Should the pharmacist be a mandatory member of a multidisciplinary team?" This difference is due to the distinctions in pharmaceutical legislation and staffing tables of PHC establishments in both countries.

In both countries not a pharmacist but a nurse is the person responsible for supply the hospice of drugs [12].

In Ukraine a pharmacist should perform the following tasks in hospice: the searching for domestic analogues, comparative assessment of their value

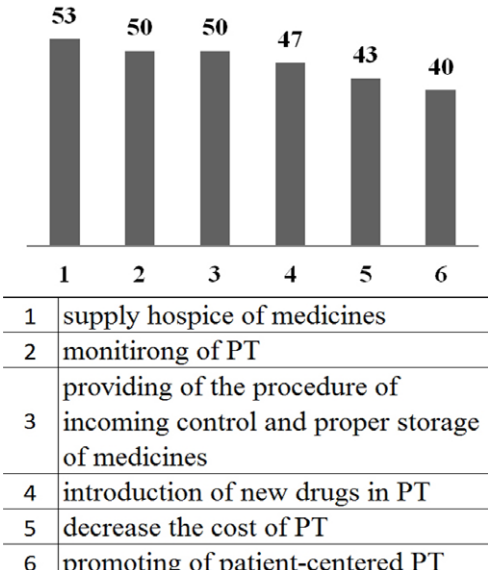

Figure 5. Advantages of presence a pharmacist in hospice in Ukraine (%)

etc. In comparison, in Poland hospice pharmacists are engaged in carrying out such activities as: giving information about drugs, their ordering and dispensing as well as providing training for hospice staff [12].

Respondents in both countries consider that the main advantages of presence a pharmacist in hospice are: improving of supply of drugs, providing the documentation of opioid and psychotropic drugs management, the procedure of incoming control, accounting, dispensing and proper storage of medicines, decreasing the cost of PT (**Figure 5**) [12].

Therefore, a multidisciplinary approach to the comprehensive satisfying of patients' needs in PHC has a great importance. Thus, the participation of pharmacists (CP) in PHC is integral and important part of it, and the results of the study testify to the importance of their inclusion in the multidisciplinary team.

Conclusion

The results of analysis of PHC status in Ukraine and in Poland suggest a lower level of availability of inpatient palliative care for the population in Ukraine than in Poland. Since a multidisciplinary approach for successful provision of PHC to the patients has a great importance, the participation and inclusion of pharmacists (CP) in multidisciplinary team is extremely relevant. As a result of questionnaire survey it has been found that pharmacists work in half of the studied hospices in Poland, and that the position of a pharmacist in Ukrainian PHC institutions is absent. In Poland there are legal and organizational constrains to including the pharmacists in PHC and for residential hospices and in hospital-based palliative medicine wards it is obligatory. Three-quarters of respondents in Poland and almost half in Ukraine believed that the pharmacist should be a mandatory member of multidisciplinary team. The advantages of presence of a pharmacist in the PHC institution, as the respondents of both countries consider, were the following: improvement of supplying the hospice with drugs, organizing incoming control, accounting, storage of medicines and decreasing the cost of pharmacotherapy.

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Isokinetic studies for detection of functional disorders in patients with unilateral shoulder impingement syndrome

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ABSTRACT

Introduction. The isokinetic assessment allows objective evaluation of muscle strength in patients considered for surgery in cases of rotator cuff injury.
Aim. The goal of this study was to define functional disorders of shoulder joint in patients with rotator cuff injury.
Material and methods. The examination was conducted in two groups, ten patients each. One group consisted of subjects with rotator cuff injury while the other was a healthy control group. Isokinetic test was performed with use of Biodex System 4 Pro Device. The following parameters were evaluated: peak torque, peak torque/body weight (peak TQ/BW), total work, average power, range of movement (ROM) and peak torque ratio of external to internal rotators (ERPT/IRPT ratio). Studies of Shoulder Pain and Disability Index (SPADI) scale supplemented clinical evaluation.
Results. Examination indicated a significant deficit of muscle strength during external rotation and ROM limitation only on symptomatic side in shoulder impingement group. Alteration between agonistic and antagonistic muscles strength for 240^0/s was found. Significant differences between involved and uninvolved shoulders during pain and disability tests were detected. There was no correlation between result of isokinetic and SPADI tests.
Conclusions. Patients after injury of rotator cuff present functional disorders that occur mainly during external rotation in isokinetic evaluation.

Keywords: shoulder impingement syndrome, rotator cuff, isokinetic dynamometry, shoulder pain.

Introduction

Rotator cuff injury combined with shoulder impingement syndrome is a common pathology of shoulder joint. Frequency of prevalence increases with age. After conducting USG tests it was observed that 13% of the population in their fifties, 20% in their sixties and 31% in their seventies suffer from this pathology [1]. Symptoms of rotator cuff disease include pain, muscles weakness, decreased range of motion, and as a result the impaired functionality of shoulder joint in daily activities [2–4].

The isokinetic assessment becomes more and more common in orthopedic practice. It allows for the objective evaluation of muscle strength in various velocities and different positions of the subject's body. The results of evaluation make possible to examine a lot of different parameters. However, most studies took into consideration only peak torque values and peak torque ratio of agonist to antagonists muscles at velocities 60^0/s and 180^0/s [4–10].

Aim

In this research we will evaluate peak torque, peak torque/body weight, total work, average power, range of motion and peak torque ratio of external to inter-

nal rotators at 4 velocities 60^0/s, 120^0/s, 180^0/s and 240^0/s to define functional disorders of shoulder joints in patients with rotator cuff injury. Moreover, we will try to correlate the above parameters with pain, disability and quality of life with the use of Shoulder Pain and Disability Index.

Material and methods

Subjects

Twenty subjects were evaluated. The first group consisted of ten patients with clinically diagnosed unilateral rotator cuff injury combined with shoulder impingement syndrome (5 women and 5 men). The average age of the patients was 59.7 ± 17.94 years, the average height was 168 ± 8.92 cm and the average weight was 72.1 ± 14.94 kg. All of them had passed positively the followings tests: painful arc, Jobe's test, Neer and Kennedy-Hawkins impingement signs. Ultrasonography confirmed the narrowing of subacromial space and total disruption of supraspinatus muscle tendon. Exclusion criteria were: bilateral rotator cuff injury, previous injuries of shoulder joint, upper limb or neck instability, degenerative spine disease, rheumatoid arthritis. The symptoms were manifested from 6 to 12 months. Most subjects had applied some forms of physical therapy for their shoulder problem. It should be mentioned that we did not have any impact on the applied therapeutic method or any insight into details collected on the subject of the type of therapy provided. The second group consisted of 10 patients without shoulder pathology (4 women and 6 men). The mean age of the healthy control group was 25.9 ± 3.81 years, the mean height was 173.8 ± 5.94 cm and the mean weight was 70.2 ± 9.19 kg. The age of the members in the control group was intentionally chosen to minimize the coexistence of any significant shoulder or cervical spine disorders typical for osteoarthritis in elderly people.

Instruments

Isokinetic testing of the shoulder rotator muscles was performed using a Biodex System 4 Pro Dynamometer. Measurements were performed in shoulder impingement group both on symptomatic and asymptomatic sides and in healthy volunteers on dominant and non-dominant sides. The procedure used in the study was a bilateral concentric shoulder internal and external rotations protocol. Before testing subjects took part in a warming-up consisting of 5 minutes of upper limb exercises. Subjects were evaluated in the seated position with the arm in the plane of scapula, which is the shoulder position at 45^0 abduction and at 30^0 forward flexion. The elbow was at 90^0 flexion and forearm was in the neutral position. Subjects' trunks were stabilized with seatbelts. The evaluation started with the external rotation. Isokinetic assessment was performed at 3 speeds: 120^0/s, 180^0/s and 240^0/s. The evaluation at each speed consisted of 5 repetitions. There was also an endurance trial, which included 10 repetitions at 240^0/s velocity. A resting period of 30 seconds was kept between each run. During the test, the subjects were encouraged by the therapist to develop maximum strength in all contractions. Peak torque, peak TQ/BW, total work, average power, ROM and ERPT/IRPT ratio were assessed.

Pain and disability factors were evaluated with the numeric version of SPADI developed by Roach at 1991. It is consisted of two subscales: pain (5 questions) and disability (8 questions). The total SPADI score was counted up by averaging scores of these two subscales. The higher score represented greater intensity of pain and disability of shoulder joint.

Statistical analysis

All numerical data were expressed as mean ± standard deviation values. Significant differences were evaluated using t-student test. Spearman's trial was used to assess correlations. The level of statistical significance was accepted at $p < 0.05$. The results were analyzed using Statistica 10 software.

Results

Only in external rotation differences between symptomatic and asymptomatic sides were found. **Table 1** shows the results of isokinetic external rotation test. In the values of peak torque from the patients' group there was found a significant discrepancy between symptomatic and asymptomatic side only at velocity 240^0/s. Similar differences were not found in the control group. When comparing deficits of peak torque between symptomatic and asymptomatic sides in patients' group and dominant and non-dominant side in the control group there was found a significant difference between deficit values at the highest tested velocity. It should be however underlined, that values of peak TQ/BW at all velocities significantly differed between symptomatic and asymptomatic sides what makes this parameter more sensitive than the peak torque alone. Moreover, no distinction between values of mentioned above parameter on dominant and non-dominant side was observed in the control group.

There were not found any differences at average power referring to symptomatic and asymptomatic sides in the patients' group, dominant and non-dominant side in the control group or comparing deficits in the patients and control groups. The mean values of total work significantly differed between asymptomatic and symptomatic sides but not in the control group. The endurance test showed significant differences in values of peak TQ/BW and total work between symptomatic and asymptomatic side only in the patients' group.

Table 2 depicts results of ROM and ERPT/IRPT ratio. There were found differences between symptomatic and asymptomatic sides in the patients' group in ROM at each velocity. There was no contrast in this parameter in the control group. Comparing ERPT/IRPT ratio there were perceptible discrepancies between

Table 1. Isokinetic test results of external rotation. Significance accepted at $p < 0.05$

	Patients from group with shoulder impingement syndrome			Control group of healthy volunteers			p
	Angular velocity 120^0/s						
	asympt	sympt	p	dom	nondom	p p	
Peak torque [Nm]	20.7 ± 7.7	16.32 ± 5.33	0.16	19.7 ± 6.9	17.8 ± 5.9	0.53	
Deficit [%]	18.05 ± 20.53			7.6 ± 15.5			0.22
Peak TQ/BW [%]	28.1 ± 5.1	22.8 ± 5.9	0.04	27.6 ± 8.05	25.2 ± 7.1	0.48	
Avg. Power [W]	20.4 ± 11.4	14.5 ± 7.6	0.19	23.2 ± 10.0	19.7 ± 9.4	0.43	
Deficit [%]	25.0 ± 29.8			15.1 ± 15.2			0.37
Total work [J]	57.7 ± 23.9	36.9 ± 18.0	0.04	62.5 ± 22.3	52.0 ± 23.0	0.32	
Deficit [%]	33.6 ± 26.7			17.8 ± 17.1			0.13
	Angular velocity 180^0/s						p
	asympt	sympt	p	dom	nondom	p p	
Peak torque [Nm]	20.9 ± 6.8	16.6 ± 4.2	0.11	17.7 ± 6.2	16.9 ± 6.2	0.78	
Deficit [%]	17.5 ± 17.2			3.6 ± 21.7			0.13
Peak TQ/BW [%]	28.7 ± 5.0	23.4 ± 5.2	0.03	24.9 ± 7.1	24.0 ± 8.4	0.8	
Avg. Power [W]	21.5 ± 12.6	14.2 ± 8.4	0.15	22.5 ± 10.7	19.7 ± 11.6	0.58	
Deficit [%]	34.1 ± 14.4			15.4 ± 27.5			0.07
Total work [J]	77.5 ± 34.8	48.5 ± 22.0	0.04	80.1 ± 34.3	67.1 ± 41.8	0.46	
Deficit [%]	35.9 ± 13.9			14.4 ± 3			0.05
	Angular velocity 240^0/s						p
	asympt	sympt	p	dom	nondom	p p	
Peak torque [Nm]	21.7 ± 7.0	16.3 ± 3.2	0.04	15.8 ± 6.0	15.7 ± 6.4	0.98	
Deficit [%]	19.9 ± 22.8			0.61 ± 14.2			0.04
Peak TQ/BW [%]	30.3 ± 7.4	23.2 ± 4.9	0.02	22.4 ± 8	22.5 ± 8.9	0.99	
Avg. Power [W]	17.1 ± 7.7	13.2 ± 6.5	0.24	18.3 ± 9.0	16.9 ± 11.8	0.77	
Deficit [%]	19.4 ± 29.1			16.9 ± 28.5			0.85
Total work [J]	65.3 ± 21.7	45.0 ± 16.2	0.03	59.0 ± 26.1	55.4 ± 35.3	0.8	
Deficit [%]	29.0 ± 20.0			15.1 ± 26.3			0.2
	Angular velocity 240^0/s (endurance)						p
	asympt	sympt	p	dom	nondom	p p	
Peak torque [Nm]	19.3 ± 5.5	16.1 ± 3.5	0.14	17.1 ± 6.05	16.9 ± 7.39	0.95	
Deficit [%]	14.2 ± 17.5			2.5 ± 20.5			0.19
Peak TQ/BW [%]	26.8 ± 5.0	22.7 ± 4.8	0.08	24.4 ± 8.1	24.3 ± 10.9	0.99	
Avg. Power [W]	18.4 ± 9.9	13.9 ± 8.1	0.29	19.4 ± 10.5	16.1 ± 11.2	0.51	
Deficit [%]	24.3 ± 19.3			26.4 ± 35.5			0.87
Total work [J]	123.4 ± 41.2	90.8 ± 38.6	0.09	123.3 ± 60.1	105.7 ± 72.0	0.56	
Deficit [%]	26.1 ± 17.3			23.0 ± 36.6			0.81

sympt – symptomatic side; asympt-symptomatic side; dom – dominant side; nondom – nondominant side
Deficit:
1 to 10% – No significant difference between extremities
11 to 25% – Rehabilitation recommended to improve muscle performance balance
> 25% – Significant Functional Impairment
(–) Negative deficit indicates that a symptomatic extremity performed better than asymptomatic one

Table 2. Isokinetic tests results: ROM and External rotation peak torque (ERPT)/Internal rotation peak torque (IRPT)

	Patients from group with shoulder impingement syndrome			Control group of healthy volunteers		
	Angular velocity 120^0/s					
	asympt	sympt	p	dom	nondom	p
ROM	89.17 ± 6.17	76.63 ± 14.09	0.02	93.26 ± 11.48	86.31 ± 8.93	0.15
ERPT/IRPT	68.68 ± 17.53	58 ± 9.27	0.13	69.12 ± 13.02	66.47 ± 16.09	0.69
	Angular velocity 180^0/s					
	asympt	sympt	p	dom	nondom	p
ROM	88.65 ± 6.17	76.42 ± 13.11	0.02	92.9 ± 11.3	86.55 ± 9.17	0.19
ERPT/IRPT	70.09 ± 14.5	60.6 ± 8.77	0.09	69.64 ± 20.23	69.72 ± 22.94	0.99
	Angular velocity 240^0/s					
	asympt	sympt	p	dom	nondom	p
ROM	87.78 ± 6.06	75.57 ± 12.93	0.01	92.01 ± 11.15	85.89 ± 9.98	0.21
ERPT/IRPT	85.02 ± 17.96	69.71 ± 9.73	0.03	73.45 ± 24.88	80.04 ± 28.23	0.59
	Angular velocity 240^0/s (endurance)					
	asympt	sympt	p	dom	nondom	p
ROM	87.96 ± 6.13	75.64 ± 13.11	0.01	92.16 ± 11.29	85 ± 9.79	0.19
ERPT/IRPT	67.57 ± 11.29	53.55 ± 8.37	0.01	70.98 ± 20.51	74.81 ± 26.94	0.72

symptomatic and asymptomatic side at velocity 240^0/s and during the endurance trial. There was not any difference in the control group.

Table 3 shows results of SPADI test. Statistical analysis showed significant differences between symptomatic and asymptomatic sides in results of SPADI questionnaire, but there was no correlation found between SPADI and isokinetic evaluation.

Table 3. Shoulder Pain and Disability Index

	sympt	asympt	p
Pain	0.41	0	0.004
Disability	0.21	0	0.002
Total	0.34	0	0.003

Discussion

The results of the present study demonstrated a decrease in functionality of patients with shoulders impingement syndrome in isokinetic assessment as well as in pain and disability evaluation. In the patients' group, deficits of external rotators were present while internal rotators didn't exhibit significant weaknesses. The significant differences were observed in mean values of peak TQ/BW, total work, ROM and SPADI evaluation. There were deficits in peak torque ERPT/IRPT ratio, but only at 240^0/s velocity. No significant deficits between dominant and non-dominant sides in the control group were noticed. Variations of mean peak torque values were found only at 240^0/s, when comparing both symptomatic and asymptomatic sides in the patients' and control group. It is possible that deficits appear only at higher velocities. This might be explained by morphologic changes in the muscle tissue. Irlenbush and Gansen performed a biopsy of supraspinatus muscle and the acromial part of deltoid muscle in patients with a supraspinatus syndrome and partial or complete supraspinatus rapture. This research demonstrated abnormal fibers distribution in those muscles. Their study highlighted greater changes at fast-twitch fibers (type II), which are rapidly contracting elements but undergo the quick fatigue, than slow-twitch fibers (type I), which are slowly contracting structures but they are fatigue resistant [11]. This is the explanation of the deficit observed in our study at higher velocities of testing.

Previous studies described measurements of peak torque at lower velocities: 60^0/s and 180^0/s. Erol et al compared the mean peak torque values of patients with subacromial impingement syndrome and in a control group. It should be mentioned that only velocity of 60^0/s was considered. Additionally, only the significant deficit of internal rotators compared in patients and a control group was detected. In the control group, the mean value of peak torque measured on the dominant side was significantly higher than on non-dominant [4]. Tyler et al tested isokinetically the peak torque at 60^0/s and 180^0/s velocities. They compared patients with subacromial impingement syndrome with control group, but didn't find any major deficits of peak torque in these velocities [5]. Also, the position of shoulder and range of motion may have impacted the results. We were testing shoulders in a scapular plane and the painless range of motion. Scapular plane position guaranteed a comfortable, physiological position, which ensured the optimum result of peak torque measure-

ments. It didn't cause any symptoms of impingement, as it didn't exert the pressure on tissues. Moreover, the peak torque was measured at the middle of range of motion in isokinetic tests but the impingement occurs only when the shoulder is at the end of range. So, both position and range of motion considered in this study, didn't cause symptoms of impingement. Tyler et al tested rotator cuff power isokinetically in two different positions, and with a handheld dynamometer (HHD) as an isometric strength trial. Testing positions were scapular plane and shoulder abduction to 90^0 in a frontal plane. They didn't find any significant deficits in isokinetic tests, but testing in shoulder abduction positions demonstrated higher deficits of peak torque. Using HHD testing they found deficits both at scapular plane and shoulder abductions positions. They indicated that testing using HHD may be more sensitive than isokinetic testing alone, because HHD evaluates strength at the end of a range. The authors believe that strength may be normal at the middle range, but deficits may appear at the end of range, when there can appear the symptoms of impingement [5]. Dupuis et al didn't find any significant differences between results of scapular plane testing and shoulder abduction testing. However, they tested only healthy people who didn't show symptoms of shoulder impigement. No pain appeared while testing this range of motion, which could affect final results [6]. We didn't find previous studies describing isokinetic testing at 240^0/s velocities. In our study deficits of peak torque values have already appeared only at 240^0/s. The findings of our research provide the evidence that peak torque should be tested only at such a velocity.

We detected significant differences between mean values of ERPT/IRPT ratio only at 240^0/s. The same phenomenon was detected during the endurance trial. It should be underlined that this was calculated for the peak torque values. That is why a relation between results of peak torque and ERPT/IRPT ratio may occur. Erol et al compared results of mean values ERPT/IRPT ascertained in patients with results obtained in a control group. Results were similar. This might indicate that both external rotators and internal rotators were impaired [4]. Mattielo-Rosa et al compared results of ERPT/IRPT measurements in patients and a control group at 60^0/s and 180^0/s velocities. They found significant differences of this ratio only at higher velocity [10]. Edouard et al indicated that peak torque is more reliable than ERPT/IRPT, because it has an impact on the two values of this ratio thus, a distortion of each of them may change the result [7].

Peak TQ/BW seems to be more reliable indicator than peak torque, because it takes the anthropometric attributes of a subject into consideration. In our study, symptomatic upper limb gained significant higher result than asymptomatic one at 120^0/s, 180^0/s and 240^0/s. Unfortunately, there are only few studies describing changes of this parameter. Wassinger et al tested peak TQ/BW at 60^0/s and observed significant weakness of symptomatic side in comparison with asymptomatic one [8]. In our study a value of peak TQ/BW was lowered in symptomatic extremity even at 60^0/s and 180^0/s velocities. We believe that this is better factor to evaluate dysfunction of a rotator cuff muscle in comparison with peak torque. Mean values of both total work and ROM demonstrated dysfunction of shoulder joint. No mention of these factors was found in a previous literature. Mean values of ROM are often evaluated during shoulder joint functionality trial, but none of them tested this factor during isokinetic condition. Deficits of total work mean values appeared even at lower velocities than 240^0/s. It seems to be a reliable factor through which an evaluation of functions of shoulder joints can be performed.

We also found significant differences between mean values of SPADI test referring to symptomatic and asymptomatic sides. Additionally, there were no correlations between the result of isokinetic assessment and SPADI test. As it has been already mentioned, during the biodex test patients didn't feel any pain and that is why there was no correlation between those two tests. Erol et al provided the same explanation of lack of relation between results of these two trials. In addition, they think that low velocities may have an impact on the results of this test [4]. Wessinger et al evaluated influence of pain on muscle strength in isokinetic assessment. The authors compared mean peak torque values with mean value of VAS results before and after analgesic injection in the subacromial space. Their study showed that the level of pain affected the result of strength evaluation. Their patients demonstrated weakness both in external and internal rotations. After a decrease of pain, mean strength values of symptomatic side came closer to the results detected on the asymptomatic side. Mean value of peak TQ/BW during external rotation increased, but it was still lower than strength found on asymptomatic side. Pain had a large impact on a decrease of strength in patients with rotator cuff disease, particularly with partial thickness tearing and inflammation [8]. Forthomme et al considered the impact of pain levels on the mean peak torque value. They observed that after analgesic injection only

external rotators were still weakened. It might indicate that external rotators were more affected than internal ones [9].

Psychological factors may have an impact on the lack of correlation between results of SPADI test and isokinetic assessment. It has to be highlighted that the pain is a subjective sensation and patient's personality may affect the result of test. Hill et al evaluated the impact of range of motion in shoulder joint on result of SPADI test. They also didn't find any correlation [12]. There are many studies which acknowledge the reliability of SPADI questionnaire [13–15].

Conclusions

The results of this study showed a significant decrease of external rotators function expressed in mean values of peak torque, peak TQ/BW and total work. Additionally, the abnormal relationships between agonistic and antagonistic muscles strength and decrease of ROM were discovered in patients representing the shoulder impingement syndrome. Decrease of shoulder joint functionality detected in isokinetic evaluation was confirmed in results of SPADI test. Moreover, the results indicate that higher velocities of isokinetic testing are more reliable for evaluation of shoulder joint.

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Idiopathic hirsutism – medical and psychological aspects

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ABSTRACT

Introduction. Hirsutism is a condition which results in excess of male pattern hair growth - the androgen dependent hair in females. Excessive hair growth usually starts around puberty. The aim of this study was to show the significance of hormonal diagnosis in female adolescent hirsute patients.
Material and methods. The material consisted of medical documentation of 128 female adolescent patients aged 13–22 years who came for a consultation because of excessive male pattern hair growth (second degree of hirsutism according to the Ferriman-Gallwey scale) which they considered unacceptable. The history was indicative of considerable discomfort, decreased mood in subjects and low self-esteem (especially regarding patient's femininity).
Results. Results of the analysis were confronted with the results of a group of an equal number of participants.
Conclusions. Hormonal tests in female patients with hirsutism were proven to constitute a major diagnostic element allowing for correct diagnosis of the cause of hirsutism, planning further tests and treatment methods. Considering its causes and effects, hirsutism in adolescent patients is a serious concern for developmental gynecology. This disorder is a endocrinological or gynecological problem, which influences patient's well-being.

Keywords: hair, gynecology, sexuology, girl, women.

Introduction

Hirsutism is a condition which results in of excess male pattern hair growth – the androgen dependent hair in females. Excessive hair growth usually starts around puberty [1–5].

Aim

The aim of this study was to show the significance of hormonal diagnosis in female adolescent hirsute patients.

Material and methods

The material consisted of medical documentation of 128 female adolescent patients aged 13–22 who came for a consultation because of excess male pattern hair growth (second degree of hirsutism according to the Ferriman-Gallwey scale) which they considered unacceptable. The history was indicative of considerable discomfort, decrease of mood in subjects and them experiencing feminity inferiority complex.

The degree of hirsutism was assessed with the use of Ferriman-Gallwey scale which rates hair growth from 0 to 4 points in twelve locations. Male pattern hair growth is diagnosed if one scores more than eight points on the scale:

- 8–16 points – moderate hirsutism,
- 16–36 points – severe hirsutism.

The statistical analysis by way of Chi-square test and Fisher-Freeman-Halton test has demonstrated p = 0.0213.

Results

The results are presented in the **Table 1**.

Discussion

Clinical observations show that hirsurism, which is the masculine type hair growth in females influence patient's self-esteem, their feelings related to gender identity or may even adversely affect their sexual relations [17].

Hirsutism is a hair growth problem in women characterized by unwanted and excessive hair production [6–11].

The analysis has shown the importance of diagnostic aspect of hormonal testing in clinical cases of hirsutism [12–14]. As we mentioned in the introduction, hormonal testing in hirsutism is used to confirm clinical observation of hyperandrogenism and to find the source of excessive androgen production [15–18]. Here, the most important examinations include evaluation of testosterone serum concentration (as testosterone is the main circulating androgen) [19–22].

The sources of circulating testosterone in females include the following:

- ovarian origin (probably ovarian stroma) – 5–20%,
- adrenal origin – 0–30%,
- the result of peripheral transformation – 50–70%.

Medical test results do not confirm virilizing tumors or congenital hydroxylation defects.

Patients whose serum LH concentrations are respectively higher than FSH concentrations are hirsute as a result of polycystic ovarian syndrome confirmed by ultrasound scan of ovaries.

Polycystic ovary syndrome (PCOS) affects 5–10% of the population of women, and the spectrum of its symptoms such as obesity, hirsutism, skin problems, and finally fertility problems has a huge negative impact on the individual's psychological and interpersonal functioning [21, 22].

According to literature, PCOS can be found in 10% of women in reproductive age. Despite 70 years of research on PCOS, its etiopathogenesis remains unexplained.

The relation between patient's lifestyle and the course and effects of her disease, which has been confirmed in literature, gives reasons to continue research aimed at starting preventive actions as early as possible. From a patient's point of view clinical symptoms accompanying PCOS may adversely influence her mental health and sexuality. These women often suffer from low self-esteem and have a negative image of their femininity. Ekback et al. (2009) interviewed women who suffered from hirsutism due to polycystic ovarian syndrome. The study has confirmed that women with excessive male pattern hair growth, the participants of the study had a negative image of their bodies which they detested and experienced as a prison.

Additionally, elevated testosterone levels may be a risk factor in depressive episodes, which in turn have impact on quality of patient's family life and her effectiveness at work.

Table 1. The tests used include Chi-square test for independence or Fisher-Freeman-Halton test

Hormone concentrations	Experimental group Mean ± SD	Control Mean ± SD	p-value
FSH	5.253 ± 2.25	5.110 ± 1.94	p = 0.301
LH	9.517 ± 2.25	9.930 ± 1.94	p = 0.00000
E2	84.180 ± 65.29	57.812 ± 38.72	p = 0.00000
PRL	22.867 ± 20.25	14.687 ± 7.02	p = 0.000000
Total testosterone	0.719 ± 0.26	0.491 ± 0.29	p = 0.00001
DHEA-S	10.224 ± 5.04	6.651 ± 3.02	p = 0.0001
SHBG	41.97 ± 20,94	66.572 ± 0,042	p = 0.042

No correlation was found

Table 2. Plasma total testosterone concentrations

Above 3.4 nmol/l > 1 ng/ml		Below 3.4 nmol/l < 1 ng/ml
Virilizing adrenal or ovarian tumors Total testosterone concentration > 6.8 nmol/l => 2 ng/dl Extremely elevated androstenedione, DHEA or DHEA-S levels Localizing examinations of the tumor	Congenital hydroxylation defects Androstenedione, DHEA, DHEA-S and total testosterone concentrations slightly elevated (50–100% higher) Extremely elevated progesterone and 17-hydroxyprogesterone levels in response to ACTH (10 times as much)	PCOS Enlarged ovaries LH/FSH elevated Elevated PRL Lowered TBG Elevated free T

Patients whose hormone concentrations remain within the normal reference ranges were diagnosed with idiopathic hirsutism (these girls were found to have a correct ovary structure – which is not the subject of this paper)

Hirsutism is caused by three main reasons:

- nonandrogenic factors are the ones that are not related to disproportionate androgen activity,
- result of androgen excess,
- idiopathic hirsutism.

Since androgens are the main hormones that affect hair production and development in humans, the most common cause of hirsutism is traced to androgen malfunction. Idiopathic hirsutism is the second most common cause of hirsutism after polycystic ovarian syndrome (PCOS), which is associated with androgen dysfunction. Idiopathic hirsutism accounts for approximately five to 17 percent of hirsute cases.

Idiopathic hirsutism can be defined as excessive terminal hair production in a male-like pattern in androgen-receptive body parts of patients who show no signs of endocrine or androgen disorders. This kind of hirsutism occurs in the presence of regular ovulation and normal androgen levels.

In hirsute patients, one of the crucial tasks is to conduct a through examination in order to distinguish idiopathic hirsutism from other forms of hirsutism. Though a lot more research needs to be done about its pathophysiology, patients with this kind of hirsutism have a probable excessive peripheral 5a-reductase action in skin and hair follicle, other variations in androgen metabolism or greater sensitivity of the androgen receptor.

To understand any kind of hirsutism it is first necessary to know a bit about human hair biology [11].

Human hair follicles first form in a human fetus. The number of human hair follicles that grow, which is 3–5 million (20% of which is in the scalp), is genetically predetermined.

To understand hirsutism better, one needs to know the three human hair types and their life cycle:

- lanugo hair: This soft, downy hair is first formed in the fetal stage in the mother's womb and is lost in late gestation or early postpartum stage,
- vellus hair: It is non-pigmented, soft and short and occurs in the seemingly hairless body regions,
- terminal hair: This is pigmented, dense, coarse and longer than vellus hair and composes the eyebrows, eyelashes, scalp hair, the pubic hair and axillary hair, etc.

The permanent conversion and development of vellus to terminal hair is a normal physiological process triggered by androgens, testosterone and dihydrotestosterone (DHT) in body areas that are androgen sensitive. This cycle normally begins at puberty, continues through adult life and gradually lessens with age and reproductive capacity in both sexes.

The normal life cycle of hair is made up of three alternating stages:

- anagen stage: Growth,
- catagen phase: Involution,
- telogen phase: Rest.

Primary symptoms of idiopathic hirsutism are excessive terminal hair growth in androgen-sensitive body areas. However, menses (i.e. ovulation) and circulating androgen levels remain unaffected. Moreover, studies about this kind of hirsutism found altered functioning of androgen receptors and an altered androgen metabolism. It was found that in the peripheral blood lymphocytes of certain patients with idiopathic hirsutism, the longer of the two androgen receptor alleles was "preferentially methylated" (and hence dysfunctional). Hence, experts suggest that it is probable that genetic modifications of the androgen receptor function and possibly 5a-reductase functions can alter the manifestation of hirsutism. Around 40 per cent of eumenorrheic hirsute women show signs of anovulation and hence they are diagnosed for polycystic ovary syndrome (PCOS) and not idiopathic hirsutism [11].

On the basis of analysis of literature and research results it should be concluded that, most importantly, hormone concentrations in certain types of hirsutism are the following:

- idiopathic type – hormone levels usually remain within the normal reference ranges,
- ovarian type – elevated free testosterone levels (LH/FSH concentration ratio in PCOS > 2.5),
- adrenal type – elevated DHEA-S concentrations and congenital adrenal hyperplasia, (CAH) – elevated 17-alpha-hydroxy-progesterone concentrations,
- mixed type – elevated DHEA-S and free testosterone concentrations.

Although it has not been a topic of this paper, one must bear in mind that differential diagnostics by way of the so-called dynamic tests are used except for physical examination, medical interview and basic hormonal testing [6, 7].

According to literature, patients with elevated DHEA-S and free testosterone concentrations undergo a Dexamethasone Supression Test while ACTH stimulation test must be used to confirm the diagnosis of congenital adrenal hyperplasia. In treatment of hirsutism best results are achieved by psychotherapy connected

with cosmetic procedure and pharmacological therapy. Except for rare tumor cases, the role of surgery remains insignificant [8–10].

As it was mentioned in the introduction, psychological aspect of hirsutism is of particular importance especially in case of young patients because their psychosexual development takes place simultaneously with somatic development [12, 13].

The problem of hirsutism should be explained to adolescent girls. Although hairiness does not play an important role in physiological processes, characteristic differences in its seating arrangements in both genders are relevant for one's normal psychosexual and social functioning. Patients need consultation about their apprehensions and fears connected with the problem of hirsutism (or other hyperandrogenization characteristics). A doctor should assess patient's perception of the severity of the problem. The participants of the study had a 2nd degree of hirsutism. Despite the fact that it was not the highest degree of hirsutism, it was the most important problem for them at the moment of consultation [14–16].

Most investigators agree that even low degree hirsutism may adversely influence self-confidence and can cause self-consciousness which in turn leads to one's withdrawal from social life and further difficulties in social life. Keegan et al. (2003) interviewed 53 women to assess psychological consequences of perceived hirsutism. Authors of the study suggested that it was not the excessive hair growth in itself but idealized cultural norms for hair growth which caused problems with sexual identity and feelings of stigma. Our results support Keegan's conclusions. All participants of our study did not accept and were deeply frustrated because of hirsutism. As it was mentioned before, it was the main reason why they sought gynecological consultation. They were observed to be highly motivated to start difficult, sometimes painful testing and treatment. In contrast to some data in literature, which claim that the patients are not concerned with their fertility, patients in the experimental group showed their apprehension and fears connected with that problem [17, 18]. It was confirmed that from clinical point of view it was very important to talk to them about it [19–22].

Hirsutism, associated with social and psychological difficulties including anxiety, social avoidance and a confusion of gender identity and although it raises important gender issues is not only endocrinological, gynecological problem, but a psychological-sexuological too [23, 24].

Conclusions

Hormonal tests in female patients with hirsutism were proven to constitute a major diagnostic element allowing for correct conclusions about the cause of hirsutism, planning further tests and treatment methods.

Considering its causes and effects, hirsutism in adolescent patients is a serious concern for developmental gynecology.

Girls and women with excess hair growth need to be evaluated by a healthcare provider, especially if the hair develops or worsens rapidly, or if her relatives have comparatively less hair growth. In the vast majority of cases, hirsutism is not caused by a serious medical condition; however, the cause of hirsutism should be determined, and underlying conditions may need to be treated [21].

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Assessment of heparanase and heparin-binding growth and angiogenesis factors in the uterine cavity fluid in women with impaired reproduction

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ABSTRACT

Introduction. Numerous reports lead to conclusion that either the absence or insufficient amounts of heparanase and heparin binding growth factors on the luminal surface of the epithelium in the endometrium may be associated with impaired reproduction. The aim of this study was to assess the suitability of the fluid from the uterus to predict reproductive disorders.

Material and methods. The group consisted of 32 women with 2 or more consecutive unexplained miscarriages, and 33 idiopathic infertility patients; the control group comprised 22 women with normal reproductive potential. Concentration of the studied factors was assayed by ELISA in uterine fluid.

Results. The uterine flushings from women with two or more consecutive miscarriages showed significantly lower concentrations of HPA1 ($p < 0.001$) compared to the control group and infertile patients. In contrast, we didn't observe statistically significant differences of concentration of HB-EGF, VEGF, FGF2 in the studied groups. Statistically significant correlations were obtained between the levels of HPA1 and growth factors in all groups $p < 0.05$. The ROC curve was used to test the diagnostic value of HPA1. With a cut-off point of 8.56 U/L for HPA1 levels, we achieved 58.6% sensitivity and 84.6% specificity in the detection of women with recurrent miscarriage compared to fertile controls and infertile women combined. The area under curve (AUC) value was 0.751.

Conclusions. The procedure for determining the concentrations of HPA1, HB-EGF, VEGF, FGF2 by ELISA in fluids derived from the uterine cavity is insufficient to predict either success of reproduction or reproductive disorders.

Keywords: heparanase, HPA1, heparin binding growth factors, HB-EGF, VEGF, FBF2, recurrent miscarriage, infertility, uterine fluid.

Introduction

Implantation of the blastocyst and invasion of trophoblasts are connected with changes of the extracellular matrix (ECM). Components of the ECM structure and enzymes that enable its digestion are responsible for this process. The extracellular matrix not only maintains the tissue structure, but is also responsible for its remodeling, angiogenesis and establishment of links between the trophoblast and decidua [1].

One of the main components of polysaccharide-protein coat (glycocalyx) is heparan sulfate (HS). Its presence has been demonstrated in the endometrium, the decidua and trophoblast invasion area [2]. Heparan sulfate acts as an integrating molecule, not only bonding tissue and cells, but also acting as a binding site and releasing angiogenic growth factors, including heparin-binding EGF-like growth factor (HB-EGF), vascular endothelial growth factor (VEGF), the fibroblast growth factor family (FGF's), and bone morphogenetic proteins (BMP's). Binding of these factors with the HS as a coreceptor not only increases their concentration, but also modulates their effects on target cells [3, 4]. On the other hand, ECM stimulates both angiogenesis by the trophoblast and increased release of the factor-HS complex [5].

Heparanase (HPA1) – endo-β-D-glucuronidase is responsible for the HS degradation process, cell migration and release of heparan sulfate-binding agents. It cleaves the sugar chain at specific sites, generating short oligosaccharides consisting of 10–20 sugar moieties [6]. Reduction of heparanase expression *in vitro* by nonenzymatic methods affects angiogenesis and impairs hemostasis by reducing the expression of VEGF and tissue factor (TF) [7]. It is not known whether an analogous situation occurs in the endometrium.

There are relatively few reports about the expression and role of heparanase in processes related to endometrial receptivity and embedding. In mammals, including primates and human, HPA1 expression has been found in the endometrium and placenta, and a mouse model has demonstrated its central role in the process of embedding [6–12].

In studies of mouse blastocyst implantation, it has been shown that the synthesis of HB-EGF is closely related to the implantation site. In the mouse endometrium, production of HB-EGF occurs in the form of a protein associated with endometrial epithelium. This factor binds to the HS present on the surface of the blastocyst. *In vitro* studies have also shown that endometrial HB-EGF acts as a factor for blastocyst growth by binding to receptors for epidermal growth factors (EGF) – HER1, HER4 [13, 14]. It should be noted, however, that the direct comparison of test results of implantation in animal models to humans may lead to erroneous conclusions. This is due to the specificity of biochemical implantation and the physiology of pregnancy, especially in mammalian species.

Also, other well-known growth and angiogenesis factors, e.g. FGF2 and VEGF, represent HS binding domains. So far, numerous studies have demonstrated an important role of both factors in the process of implantation and pregnancy development, both in humans and in other mammals [15–18].

There is a general agreement that the synthesis of endometrial HB-EGF, FGF and VEGF is controlled by sex hormones. The expression of these factors has been repeatedly confirmed not only in the stroma, but also in the glandular and luminal epithelium of the endometrium. The proteins are localized in the uterus secretory phase [16, 19, 20]. These reports lead to conclusion that either the absence or insufficient amounts of growth factors on the luminal surface of the epithelium of the endometrium may be associated with impaired reproduction in humans.

A common mechanism for controlling the expression of growth factors, as well as our previous observations, led us to conclusion that a positive correlation is also noticeable with respect to the concentrations of the HPA1, HB-EGF, FGF2 and VEGF proteins in the uterus. Therefore, the aim of this study was to assess the suitability of the fluid from the uterus to predict reproductive disorders. We studied the uterine cavity fluid protein concentrations of HPA1, HB-EGF, FGF2 and VEGF, which are potential markers of endometrial receptivity, and explored the correlations between HPA1 and HB-EGF, and FGF2 and VEGF in both groups of women with impaired reproduction, and a group of healthy women, i.e. the control group.

Material and methods

Patients and controls

Uterine flushings have been collected from the women hospitalized in the Division of Reproduction, Department of Obstetrics and Gynecology, University of Medical Sciences in Poznań. The study included 87 reproductive-age women. The group consisted of 32 women with 2 or more consecutive unexplained miscarriages, and 33 idiopathic infertility patients; the control group comprised 22 women with normal reproductive potential. The women in the miscarriage group had had at least two consecutive unexplained miscarriages in the first trimester of pregnancy. This group included 20 women with diagnosed recurrent miscarriage (e.g. 3 consecutive miscarriages). The mean duration of infertility in idiopathic infertility patients was 3 years (range: 1–5 years). The control group consisted of women that had at least one child, regular menses, and were without anatomical or functional lesions within the endometrium. The study protocol was approved by the Karol Marcinkowski Medical University bioethical committee, and the patients signed an informed consent form. No patients in either the study or control group had taken any hormonal preparations for at least three months prior to the study. The exclusion criteria were: current use of hormonal contraception, and any serious diseases. The age of women, number of miscarriages and parity are presented in **Table 1**.

Uterine fluid collection

Seven to nine days after ultrasound confirmation of ovulation (the putative implantation window), women underwent uterine flushing followed by endometrial biopsy with a Pipelle. Endometrial tissue samples were sent to histopathology for hematoxylin and eosin staining and assessment according to Noyes and Hertig criteria. The procedure involved placing a sterile catheter in the os of the uterine cervix, which was connected to

Table 1. Clinical characterization of the study groups

	n	Age		Number of miscarriages		Infertility duration (years)		Parity	
		Median	Span	Median	Span	Median	Span	Median	Span
Miscarriage group	32	32	23–41	3	2–5				
Two consecutive, unexplained miscarriages	12	33	27–41	2	2		NA		0
Clinically diagnosed recurrent miscarriage	20	32	23–40	3	3–5				
Idiopathic infertility	33	33	25–40	NA*		3	1–5		0
Control group	22	39	19–43	NA			NA	1	1–3

** not applicable*

a 20 ml syringe filled with 3.5 ml of sterile isotonic solution of sodium chloride (0.9% NaCl). The fluid was slowly infused into the uterine cavity and then gently aspirated in a repetitive fashion creating a turbulent flow (to achieve homogenous distribution of soluble factors). Next, the fluid was drawn into the syringe, which was transported to the laboratory, centrifuged and the supernatant was frozen at –20°C for further examination.

Protein assessment

Total protein concentration was estimated with a Pierce™BCA Protein Assay Kit (Thermo Scientific, USA). For assessment of the VEGF, FGF2 was assayed by immunoenzymatic tests (enzyme-linked immunosorbent assay; ELISA) which are commercially available (R&D Systems, Minneapolis, USA). HPA1 was determined using an ELISA kit, USCN (Wuhan, China). For the determination of HB-EGF the Sigma Aldrich ELISA kit was applied (St Louis, USA). All assays were performed according to the manufacturers' instructions. For plate reading, a Dynex Technologies MRX reader was used, (Chantilly, USA). The results were normalized to total protein concentration. All analyses were performed in the Tissue Culture Laboratory in Gynecological and Obstetric Clinical Hospital in Poznan, Poland.

Statistical analysis

For statistical analysis, SigmaStat3.5 software was used. The analysis of the results was based on the Kruskal-Wallis One Way Analysis of Variance on Ranks. Correlation of the studied proteins in the uterine flushing was performed using the Spearman rank sum test. P value less than 0.05 was considered to be statistically significant.

To test whether the obtained statistically significant values of the studied factors could be used for diagnostic purposes, we used Receiver Operating Characteristic (ROC) curves (Statistica v.10). The ROC curve displays diagnostic accuracy expressed in terms of the true-positive rate against the false-positive rate for different cut-off points [21].

Results

The uterine flushings from women with two or more consecutive miscarriages showed significantly lower concentrations of HPA1 ($p < 0.001$) compared to the control group and infertile patients. Concentrations of VEGF, FGF2 and HB-EGF did not differ between the studied groups and healthy controls. A detailed comparison of the concentrations of the studied factors is summarized in **Table 2**.

Using the Spearman rank test, we analyzed the relationships between the concentrations of HPA1 and growth factors in the uterine flushing. Statistically significant correlations were obtained between the levels of HPA1 and HB-EGF ($p = 0.015$) and HPA1 and VEGF ($p = 0.04$) in the control group. In the group of women

Table 2. Concentrations of the studied proteins in uterine fluid

Group	Control group		Miscarriage group		Idiopathic infertility		p
	Mean (SD)	Median [Span]	Mean (SD)	Median [Span]	Mean (SD)	Median [Span]	
HPA1 [U/L]	13.45 (± 5.63)	13.21 [2.14–22.3]	9.24 (± 5.15)	8.06 [2.86–23.47]	15.97 (± 8.54)	12.85 [6.4–38.82]	< 0.001*
HB-EGF [pg/ml]	168.88 (± 106.27)	141.36 [38.73–420.6]	111.27 (± 48.63)	104.5 [39.85–264.14]	128.79 (± 93.44)	110.75 [0–408.71]	ns**
VEGF [pg/ml]	110.87 (± 170.78)	72.93 [12.07–850.0]	109.75 (± 206.48)	35.48 [0–1006.0]	167.58 (± 206.54)	66.52 [0–731.61]	ns
FGF2 [pg/ml]	126.03 (± 104.94)	100.72 [14.62–517.08]	104.52 (± 110.8)	82.37 [0–490.28]	254.63 (± 323.43)	124.17 [0–1174.71]	ns

** Kruskal-Wallis One Way Analysis of Variance on Ranks with All Pairwise Multiple Comparison Procedures (Dunn's Method)*
*** Not significant*

with two or more consecutive miscarriages, we obtained correlations between HPA1 and VEGF (p = 0.009). In the infertile women group, the concentration of those HPA1 positive correlated with HB-EGF (p = 0.043), but we obtained negative correlations between the concentrations of HB-EGF and VEGF (p = 0.011). Detailed results of the assessment of the correlations in the uterine flushing are summarized in **Table 3**.

As mentioned above, only the HPA1 concentration in the uterine fluid obtained from women with recurrent miscarriages was significantly lower than in women from the control group and infertile women. To test the diagnostic value of HPA1 measurements for the detection of states of recurrent miscarriages, we utilized ROC curves. With a cut-off point of 8.56 U/L for HPA1 levels, we achieved 58.6% sensitivity and 84.6% specificity in the detection of women with recurrent miscarriage compared to fertile controls and infertile women combined. The area under curve (AUC) value was 0.751.

Table 3. Correlations between the levels of the heparanase and heparane-related proteins in the uterine flushing

	Control group			Miscarriage group			Idiopathic infertility		
	VEGF	HB-EGF	FGF2	VEGF	HB-EGF	FGF2	VEGF	HB-EGF	FGF2
HPA1									
Correlation coefficient	0.44	0.512	-0.231	0.478	0.101	0.359	0.227	0.378	0.292
p	0.0402	0.0149	ns*	0.009	ns	ns	ns	0.0429	ns
VEGF									
Correlation coefficient		-0.0096	-0.163		0.0875	0.349		-0.445	0.236
p		ns	ns		ns	ns		0.011	ns
HB-EGF									
Correlation coefficient			0.115			-0.00384			-0.17
p			ns			ns			ns

** Not significant*

Discussion

Fluid from the uterine cavity contains cytokines, growth factors responsible for receptivity and implantation. It has been demonstrated that fluid obtained from healthy women during the implantation window stimulated the *in vitro* growth of blastocysts and endometrial epithelial cell adhesion to fibronectin and collagen IV, extracellular matrix components, while fluid derived in the same way within the follicular fluid phase of the menstrual cycle did not show such properties [22]. Aspiration of fluid from the uterine cavity represents a minimally invasive form of testing, during which there is no damage to the endometrium. This method allows for quantitative and qualitative assessment of soluble and deposited factors on the surface of the endometrium. Some researchers question the usefulness of diagnostic fluid from the uterus. Hamilton et al. showed that the fluid obtained by aspiration of saline into the uterus contains varying amounts of protein. He also demonstrated that without the assistance of ultrasound it is not possible to assess the extent to which introduced salt solution fills the cavity of the uterus. Moreover, the results may be unreliable if it comes to aspiration of blood or mucus [23]. However, numerous studies have indicated the usefulness of fluid from the uterine cavity in the diagnosis of reproductive disorders [24–27] and the monitoring of therapy [28].

The growth factors we studied are secreted on the surface of the endometrium and associated with the glycocalyx, where they are released with the participation of HPA1 [4]. Their impact on the processes associated with endometrial receptivity and decidualization and implantation has been repeatedly confirmed in mammals including humans. The primary mechanism of the action of heparanase is to change the structure of the glycocalyx by removing the HS. This process facilitates the mutual contact of the embryo and the decidua. It has also been reported that in the process of implantation heparanase molecules can perform adhesive functions and can even act as a transcription factor [29]. During the implantation window, HPR1 can assist embryo implantation, and during pregnancy HPR1 acts as an angiogenesis-stimulating factor, especially as a catalyst for ECM changes. One of the mechanisms that control the activity of heparanase may be a change in pH. It has been revealed that the maximum catalytic activity of HPR1 is achieved at pH 5.0, while the pH of the sexual cycle of the uterine cavity is between 6.6 and 7.6. Such a pH promotes the adhesive properties of heparanase, even in the form of proenzyme, which is catalytically inactive [30, 31]. *In vitro*, the presence of heparanase in the external environment stimulates the embryo implantation process [32].

Our results of the determinations in the uterine cavity fluid showed that in women with two or more miscarriages HPA1 concentrations are lower than both in the control group and in the group of infertile women. This observation suggests that in humans endometrial heparanase is not only a factor responsible for implantation, but above all for the maintenance and development of the pregnancy. We have also observed that in fluid from the uterine cavity in the control group heparanase concentrations correlated positively with the concentrations of HB-EGF and / or VEGF. This observation is consistent with previous observations that indicate that the expression of both HPA1 and HB-EGF and VEGF are under a common expression mechanism controlled by sex hormones, and the severity of the degree of their expression correlates with changes in the cycle of the sexual endometrium [33].

It has been proven that the maximum level of expression of HB-EGF in human endometrial epithelium occurs at implantation, and the secretion of HB-EGF protein has been confirmed in fluid from the uterine cavity [19, 34]. Lessey et al. demonstrated that the HB-EGF in an autocrine manner stimulates the production of endometrial epithelium integrins, LIF and HOXA10 [35]. In turn, Stavreus-Evers et al. reported a close relationship between the synthesis of HB-EGF in the epithelium of the endometrium and the maximum of pinopod development at implantation. Moreover, HB-EGF located on the surface of pinopods lends itself as a good marker for the implantation window [36]. In our previous studies using the WB method, endometrial biopsies have shown a lower level of expression of HB-EGF proteins in women with at least two unexplained miscarriages compared to women with normal reproductive potential. At present, although HB-EGF concentrations in fluid from the uterine cavity are at their highest values in the group of women without reproductive failures, these levels did not differ with respect to the HB-EGF concentrations in the groups of women with impaired reproduction.

The fluid from the uterine cavity has also been evaluated for the VEGF concentration. In an animal model, Zhang et al. demonstrated VEGF expression in the epithelium of the endometrium and during implantation, pointing to an important role for this agent in the process of implantation and decidualization [37]. Based on a large selection of studies, it may be seen that many, especially older publications present divergent views as to the expression of VEGF in the sexual cycle [18, 38]. Despite this, the authors seem to agree on the role of VEGF in the early stages of pregnancy. In new research, Lash et al. showed that, in women with recurrent miscarriage, levels of VEGF were significantly lower in the glandular and luminal epithelium and in the vessels of the endometrium in relation to control groups [20]. Based on their results, Seo et al. proposed the use of VEGF as a predictor for pregnancy success in In Vitro Fertilization Treatment (IVF) [39]. In turn, Hannan et al. reported a lower concentration of VEGF in fluid from the uterine cavity in women with infertility and showed that VEGF is a key component of the fluid from the uterus in implantation and that it is responsible for the adhesion of epithelial cells of the endometrium and the blastocyst [22]. Although in our study the median value was the lowest in the group of patients with two or more abortions, similar to the case of HB-EGF, we did not obtain any statistically significant differences. We did not observe any difference in the concentrations of VEGF in the group of infertile women and the control group. In the group of infertile women, we obtained an inverse correlation between the concentrations of HB-EGF and VGFA. It cannot therefore be ruled out that one of the causes of infertility may be affected by the relative proportions between factors potentially conducive to implantation.

It has been shown that expression of VEGF and FGF is also stimulated by hCG [16]. Zimmermann et al. showed that the endometrium is capable of synthesis of hCG [41, 42]. FGF in the epithelial cells of the endometrium is synthesized during the entire sexual cycle. The maximum of its expression falls in the second phase of the cycle and remains at a high level in the first trimester of pregnancy. On the basis of ERK 1/2 acting as a MAP kinase [42], FGF receptors increase the binding of epithelial cells to fibronectin and collagen IV with ECM components of blastocyst trophoectoderm [43, 44]. The studied uterine cavity fluid FGF concentrations did not differ between the two groups.

In our previous studies, we observed lower levels of HPA1 expression in endometrial biopsies and lower levels of HB-EGF protein in women with recurrent miscarriage. The molecular studies of the endometrial sections showed correlations between the expression of HPA1 and HB-EGF at both the mRNA and protein levels [45]. In contrast, the present study on the uterine cavity fluid from women with reproductive disorders showed that among the four factors only HPA1 concentration exhibited a significantly lower concentration in women with two or more abortions. During the study, the level of HPA1 also correlated with HB-EGF and / or VEGF. Based on the analysis of the ROC curves, we can conclude that the determination of the concentration of HPA1 fluid from the uterine cavity does not meet

the criteria required to be considered a good predictor for this procedure. It should also be noted that the investigated factors are only part of the biochemical mechanisms responsible for the receptivity of the endometrium. Therefore, we do not have information on the level of receptors for the growth factors studied, nor do we have expression profile information on the components of the embryo.

Conclusions

The aim of this study was to search for predictors of endometrial receptivity in material non-invasively acquired in the non-conceptional cycle, thus enabling the possible conception. The procedure for determining the concentrations of HPA1, HB-EGF, VEGF, FBF2 by ELISA in fluids derived from the uterine cavity is insufficient to predict either success or reproductive disorders.

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Conflict of interest statement
The authors declare that there is no conflict of interest in the authorship or publication of contribution.

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On the presence of aluminium in human endometrial tissue and potential factors that may influence it – a pilot study

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ABSTRACT

Introduction. Aluminium (Al), the most ubiquitous metal in the earth crust, has been shown to reveal a potential metalloestrogenic action. Despite an increasing interest in Al exposure in human, there is essentially no information on its status in the reproduction system.
Aim. The present work investigated the content of Al in female endometrial tissue (n = 25) and its association with endometrial thickness and histological image, female age, place of living, history of cigarette smoking and diet.
Material and methods. The endometrial samples (n = 25) were obtained during routine procedures. The Al content was determined using microwave induced nitrogen plasma atomic emission spectrometer. The relationships between metal level and histological image, endometrial thickness, female age, place of living, cigarette smoking and diet were investigated.
Results. The Al was detected in every analysed sample. Its concentrations varied from 0.9–16.0 μg/kg dry tissue. The lowest Al level was found in atrophic endometrium. The metal content in polyposis, hyperplasia and unaltered tissue was comparable. The study failed to find significant association with the metal content and endometrial thickness, female age, place of living, smoking habits and diet.
Conclusion. Human endometrial tissue can contain detectable levels of Al. It, in turn, indicates that endometrium may play a role in systematic accumulation of absorbed Al but also that it may represent an unique route of periodic discharge of this element. Further studies are necessary to elucidate which factors are responsible for the presence of Al in endometrium and what are the possible consequences of its increased content in this tissue.

Keywords: aluminium, endometrium, lesions, bioaccumulation.

Introduction

Despite a relatively wide interest in metallic elements and their interactions with human body, still little is known as to their status in female reproductive system and potential factors that can influence it. In general, metals constitute an important but broad chemical group of highly essential elements that can support reproduction (e.g. Zn) but also that these reveal certain toxicities (e.g. Cd, Pb), and have been associated with increased risk of infertility [1, 2], pregnancy loss [3], endometrial [4] and uterine cancers as well as endometriosis [5]. Mostly, assessments of metal exposures in humans employ the analytical investigations of blood, hair or urine [6–8]. As long as these samples are generally easy to collect and process, and can reflect the current status of circulating metal content, they may

not represent a sufficient information on the burden of these elements in particular tissues [9].

As shown in our previous study, the endometrium, the innermost glandular tissue of the uterine cavity, can be a potential target of metal accumulation within the human body [10]. The detected metals included Cd, Cr, Ni and Pb, which represent an emerging group of potential metalloestrogens, compounds possessing the ability to bind estrogen receptors and give rise to estrogen agonist responses. So far they have been implicated in the aetiology of estrogen-dependent diseases such as cancers of the breast and endometrium as well as endometriosis [11–13]. As endometrial tissue is characterized by the high expression of both estrogen receptors, alpha and beta [14, 15], their potential interaction with selected metals is plausible. Interestingly, endometrial tissue with histological lesions was shown to contain higher levels of toxic metals, particularly Cd [10]. Altogether, the findings of our previous study highlighted the potential usefulness of quantitative analyses of endometrial metal content as an additional indicator of impairments of the menstrual cycle and fertility.

Aluminium (Al), which is the most ubiquitous metal in the earth crust, is not known to play any biological function. Moreover, it has been potentially implicated in induction of neurodegenerative diseases [16] and to act as a metalloestrogen [11]. Generally, Al is poorly absorbed in gastrointestinal tract (in range of 0.1–1.0% of oral dose) and in healthy subjects almost all of absorbed Al is most likely excreted readily from the body [17]. The remaining Al is unequally distributed with the highest content found in bones (approx. 41%), muscles (approx. 40%) and lungs (approx. 12%) [18]. The increased accumulation in humans including deposition in brain and toxic effects of Al can, however, occur in patients with renal failure or if the doses largely exceed the excretory capacity [19]. Ingestion with fruit juices or citric acid causes a marked increase in gastrointestinal absorption [20]. The exposure to Al in general population can be high as its compounds are widely used as food additives and cosmetic ingredients, and have medical implications as antacids, phosphate binders, buffered aspirins, vaccines and allergen injections [21]. Moreover, Al exposure has been shown to increase at least 30-fold over the last 50 years with currently 11 kg of Al being cast for every human each year [22]. At the same time, there is essentially no information on Al status in human tissues constituting the reproduction system, including endometrium.

The present study was undertaken in order to study the content of Al in the functional layer (shed during menstruation) of human endometrial tissue obtained from healthy female subjects and these with identified histological lesions. We also aimed to investigate the relation between tissue metal content and endometrial thickness, female age, place of living, history of cigarette smoking and diet. This is the first study to demonstrate that Al is present in endometrial tissue at the detectable levels and that this tissue can be a potential target of Al accumulation or a route of elimination of this element through periodic discharge.

Material and methods

Study group

Samples of endometrium were obtained from 25 white Caucasian females undergoing diagnostic or therapeutic curettage of the uterine cavity under general anesthesia in the Gynecologic and Obstetrical University Hospital in Poznań, Poland. The causes of intervention were abnormal excessive bleeding, bleeding after menopause or hypertrophy of the endometrium estimated in ultrasound examination (Aloka SSD3500, Japan). All procedures were performed as a routine medical treatment. Endometrial samples were obtained using a surgical stainless steel curettage instrument. During this procedure time of contact of a curettage instrument and sampled tissue did not exceed 1 s. To maintain diagnostic quality, samples were divided using plastic instrument into 0.2×0.2×0.2 mm pieces for metal content analyses (one sample per female due to limited tissue material) and the rest of the material was sent for routine pathological examination. The tissue samples utilized for metal analyses were first profusely flushed (to remove blood) and then immersed in sterile distilled water (Polpharma, Poland), and placed in cryogenic tubes (Nunc™, ThermoScientific, United States) and stored at -40°C prior to determination. None of the studied individuals were exposed to occupational sources of metals. Based on a short questionnaire, the place of living, smoking habits and diet of the investigated females were specified. The study was approved by the local bioethical committee of the Poznan University of Medical Sciences, Poznan, Poland and every patient undersigned the written consent.

Al content analyses

Tissue samples were handled using plastic instruments with special care taken to avoid any contamination. Thawed tissues were dried in an oven at 40°C, flushed twice with MilliQ water (Millipore, USA) to ensure the

removal of blood remnants, dried to a constant weight and then weighed. Complete digestion was performed with suprapure 14 mol/L HNO_3 (Sigma-Aldrich, Germany) in sealed plastic tubes using an oven (80 °C). The concentration of Al in the investigated samples was determined at 396.152 nm by a microwave induced nitrogen plasma atomic emission spectrometer (Agilent, USA) equipped with a nitrogen generator. The determination was performed in triplicate, values were averaged. The calibration was performed using standard analytical solution (Merck, Germany). Prior to the analysis, the detection method was validated as already described [10, 23]. Moreover, a control without any tissue but containing HNO_3 was performed in order to exclude the interference of any procedure step on metal content determination – all analyzed elements were below limits of detection. The final concentrations of Al was given as µg metal per kg of dry tissue.

Statistical calculations

The results were analyzed using STATISTICA 10.0 software (StatSoft, U.S.A.). Gaussian distribution was tested with Shapiro-Wilk's test, and because most of the data did not meet this assumption, non-parametric methods were employed. To evaluate differences between two independent groups the Mann–Whitney U test was used. Relations between two datasets were determined with Spearman's rank correlation coefficient. $P < 0.05$ was considered as statistically significant.

Results

Characteristics of the study group are presented in **Table 1.** The Al was detected in every analysed sample at mean (±SD) concentration of 5.89 (±4.42) µg/kg and 0.90–16.0 µg/kg range. There was no statistical difference in Al content between groups with recognized lesions and normal histological image. The overall mean Al concentration in endometrial tissue with particular histological image decreased in the following order: no lesions > polyposis > hyperplasia > atrophy (**Figure 1**). There was no correlation between metal content and endometrial thickness.

There was no significant relationship between the age of the women and the content of any studied metal. Moreover, females ⩽ 40 years old did not differ in endometrial metal contents from females > 40 years old (**Table 2**).

Table 1. Characteristics of the study group

Age (years) (mean ± SD)	45.0 ± 13.6
Endometrial thickness (mm) (mean ± SD)	10.5 ± 3.9
Histological image	
No lesions (n)	14
Lesions (n)	11
– endometrial simple hyperplasia (n)	4
– endometrial polyposis (n)	5
– atrophic endometrium (n)	2
Place of living	
⩽ 25 thousand residents (n)	3
25–100 thousand residents (n)	13
⩾ 100 thousand residents (n)	9
Diet (frequency of product consumption)	
– Fish very rarely (n)	9
– Fish rarely (n)	10
– Fish moderate (n)	5
– Meat rarely (n)	2
– Meat moderate (n)	7
– Meat often (n)	12
– Meat very often (n)	3
– Fruits moderate (n)	1
– Fruits often (n)	13
– Fruits very often (n)	10
Smoking history	
Non-smokers	12
Current smokers	3
Former smokers:	10
– Years of smoking (mean ± SD)	12.4 ± 12.5
– Smoking frequency range (cigarettes per day) (mean ± SD)	9.6 ± 7.4

SD – standard deviation

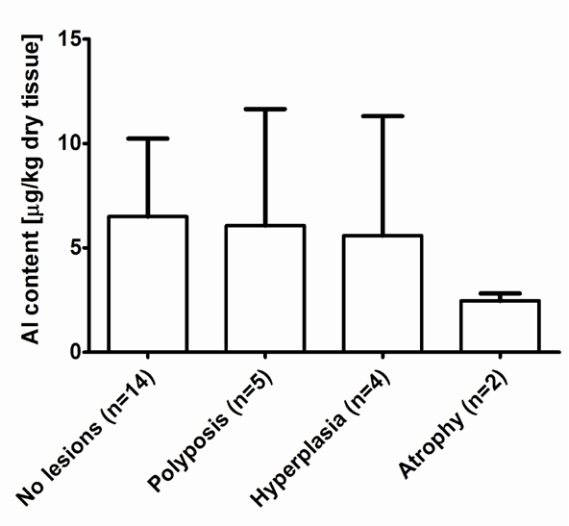

Figure 1. Level of Al content in different histological patterns of endometrial tissue. Columns represent mean, the bars represent standard deviation

No statistically significant correlations with years of smoking or number of cigarettes smoked per day and the metal content were found. Moreover, current and former smokers did not differ significantly in the metal content from non-smoking females. Nevertheless, an increased mean Al content was observed in female with smoking history (**Table 2**).

The lowest Al content was found in endometrium collected from female inhabiting areas ⩽ 25 thousand residents (**Table 2**) although a large disproportion in the number of individuals of each group excluded the possibility for representative statistical comparison and the differences in metal content between inhabitants of small, middle and large residence areas was insignificant. Moreover, there was no significant correlation between the exact number of residents and Al content.

The frequency of fruit, fish and meat consumption was also not found to be associated with Al content; no clear pattern for any of considered food product was demonstrated (**Table 2**).

Discussion

To the best of our knowledge, this is the first study to investigate and determine the Al content in endometrial tissue. This was mostly possible due to the use of highly sensitive MIP-OES technique although due to the preliminary nature of this research, relatively low sample size and several study limitations, one should treat the results cautiously. Firstly, the study lacks a real control group as due to ethical reasons we were unable to obtain samples from subjects having no medical indication for this procedure. The group of females with tissue not found to be histologically altered can be, however, treated as a provisional control. Secondly, the access to tissue sample for metal content analyses was largely limited and we were unable to collect more than one piece of tissue from each female. Such possibility would allow studying the homogeneity of Al

Table 2. Mean (±SD) content of Al (µk/kg dry tissue) in human endometrium in different female groups (no statistically significant differences were found)

Group		Al content
Age (years)	Female < 40 (n = 11)	6.9 (± 4.7)
	Female > 40 (n = 14)	5.0 (± 4.2)
Smoking habit	Non-smokers (n = 12)	4.8 (± 2.5)
	Current smokers (n = 3)	5.4 (± 5.0)
	Former smokers (n = 10)	7.1 (± 5.7)
	Current + current smokers (n = 13)	6.7 (± 5.4)
Place of living (number of residents)	< 25.000 (n = 3)	2.1 (± 0.8)
	25.000 – 100.000 (n = 13)	6.2 (± 3.5)
	100.000 (n = 9)	6.8 (± 5.8)
Diet – fish consumption	Very rarely (n = 9)	7.2 (± 5.3)
	Rarely (n = 10)	5.1 (± 3.8)
	Moderate (n = 5)	4.1 (± 5.3)
Diet – meat consumption	Rarely (n = 2)	10.2 (± 8.3)
	Moderate (n = 7)	4.4 (± 2.9)
	Often (n = 12)	5.1 (± 3.9)
	Very often (n = 3)	8.0 (± 6.3)
Diet – fruits consumption	Moderate (n = 1)	5.8
	Often (n = 13)	5.8 (± 4.8)
	Very often (n = 10)	5.5 (± 4.3)

content within the endometrial layer. Thirdly, it was impossible to use any other instrument to collect samples than curettage instrument made of stainless steel. Moreover, due to small sample size, it was not possible to cut the piece sufficient for metal analyses using the non-metal instrument. It is, however, unlikely that steel could introduce Al into the tissues during specimen collection and further, its contact time was very short and relatively equal for each patient. In **Table 3** we have provided the list of detailed steps which can help avoid metal contamination during human tissue processing in future research.

The Al content found in the present study was shown to vary between investigated females but did not exceed 16.0 μg/kg. Compared to other metals investigated previously in human endometrium, Al occurred at moderate level – lower than Cr, Pb and Zn but higher than Ni and Mn [10, 24, 25]. According to the current state of knowledge, the hematogenous route is most likely the main source of various metals for this tissue [25]. Since the functional layer of endometrium is shed during menstrual cycle, it can represent not only a target of Al accumulation but, additionally to defecation and urine secretion, a route of periodic discharge of contaminants circulating in the bloodstream. Further studies are necessary to elucidate these issues and should involve comparison of metal content between secretory and proliferation phases. As found, other metals, Zn and Cu tend to increase its content in endometrial tissue during menstrual cycle reaching the lowest levels during ovulation time and the highest at late secretory phase [26]. However in general the present study did not disclose any difference in Al content between lesioned and normal tissue, the atrophic endometrium revealed the lowest metal level. Because the size of each group of histological image (normal, hyperplasia, polyposis and atrophy) was small, further research is necessary to elucidate the accumulation of Al in different endometrial lesions including malignancy.

It is unknown whether the increased presence of Al in endometrial tissue can lead to any specific responses or alterations. Al is not known to play any biological role in living organisms and further, its excess can lead, at the cellular level, to a significant increase in reactive oxygen species and induce oxidative damage [27, 28]. A few recent studies have also revealed that Al can induce some alterations in female reproduction system. In rodents, the oral administration of Al has led to significant decrease of serum content of estrogen, progestogen, testosterone, follicle-stimulating hormone, and luteinizing hormone [29]. Moreover, the metalloestrogenic action of Al has been demonstrated in MCF7 human breast cancer cells, in which Al in form of Al chloride or chlorhydrate has been shown to displace [3H]-estradiol from cytosolic estrogen receptor (ER) [30]. The possible adverse effects of Al on endometrium would require further studies, for example relationship between metal content and ER expression in endometrial tissue or *in vitro* experiments using the human endometrial cell line (e.g. T HESCs).

Further studies are necessary to assess the relationship (if any) between serum and urine Al content and this found in tissues of reproduction system and to fully elucidate the potential factors that can influence it. The present study did not disclose any significant relationships between metal content and endometrial thickness, occurrence of lesions, female age, diet, place of living smoking habits. As diet appears to be a main source of Al exposure in humans, there is a need to conduct studies on larger group of participants, possibly representing different dietary habits and varying in the daily intake of this metal. The decreased mean content of Al in atrophic tissue and increased in females with smoking history requires further research involving larger group of participants.

Table 3. Recommendations for metal analyses in human tissue samples which undergo complete digestion

Collection	If possible use only plastic or other metal-free (e.g. made of zirconium carbide) instruments. Otherwise, use stainless-steel instruments and avoid longer contact with tissue sample.
Processing	Use ultra-pure or at least double distilled water to get rid of blood and blood remnants from tissue sample. Flush the tissue profusely at least 2-3 times. If stainless steel was used during collection and the sample is large enough, use metal-free instrument to cut the unaffected piece.
Handling	Use only plastic instruments to handle the tissue sample. Avoid any contact with metal surfaces.
Digestion	Use only suprapure acids. Test the metal content in the solution used to digest tissue samples to exclude potential interference with results.
Storage	The digested samples should be stored no longer than 2 months prior to analyses.
Analyses	Use standard solutions for instrument calibration. Use standard reference material appropriate for the analysed tissue (e.g. bovine liver).

Conclusion

The human functional layer of human endometrial tissue contains various concentrations of Al. The potential factors which can influence the content of this metal remain unknown. Further research is necessary to elucidate the relationship between specific endometrial lesions and metal content. Moreover, the potential effects of Al presence in endometrium should be addressed. It can be suggested that accumulation of Al in functional layer of endometrial tissue may represent an unique and so far unknown route of periodic discharge of toxic contaminants such as metals from female body.

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Conflict of interest statement
The authors declare that there is no conflict of interest in the authorship or publication of contribution.

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Do we need to improve breast cancer education? Attitude towards breast self-examination and screening programmes among Polish women

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ABSTRACT

Introduction. Breast cancer is a global health threat which requires systematic basic health education and increasing the public attention and awareness. Therefore, breast self-examination (BSE), however controversial, was largely promoted in many countries including Poland. Moreover, the national breast screening programmes (BSP) were made available to general public.

Aim. The study investigated the attitude towards BSE, BSP and breast cancer as a health threat among Polish women in relation to age, education, place of living and economical status.

Material and methods. An anonymous questionnaire was completed by 751 Polish women. The results were statistically elaborated.

Results. The majority of women declared to know how to perform BSE but only a small part (older females) practiced it regularly (once a month). National BSP were acknowledged by most of surveyed. The higher awareness of BSP was found for women with higher education and economical status whereas the lowest – for women inhabiting small villages and performing BSE rarely or never. Medical doctors and other medical staff were an insignificant source of BSP. Most of responders recognized breast cancer as a serious health threat in Poland.

Conclusion. The general awareness of BSP and BSE among Polish women is satisfactory, yet the percentage of females performing BSE on regular basis remains too low. There is a need to increase the contribution of medical staff in breast cancer education and control activities.

Keywords: breast cancer, breast self-examination, public awareness, cancer prevention.

Introduction

Breast cancer represents a global health problem with over 1.6 million cases and 522,000 deaths reported globally in 2012 [1]. Such a high prevalence enforces a need to gain public awareness and implement effective methods of prevention. Many countries offer breast screening programmes (BSP) to females between 50 and 70 years old, which relies on X-ray mammography [2–4]. This method has been proven to be highly sensitive and accurate but it cannot be used in younger women with dense glandular breasts, in whom the reported sensitivity usually does not exceed 50% [5, 6]. There is, however, a suite of other diagnostic tools, including optical mammography, shear wave elastography or ultrasonography, which can be helpful in detection of breast lesions at a younger female age [7–9].

Apart from medical imaging of breasts, the great emphasis has been put on the promotion of breast self-examination (BSE), which according to recommendations should be performed once a month, regardless of the female age [10]. It was, however, demonstrated that despite earlier detection of breast cancer,

BSE does not to reduce the mortality and additionally, generates a significant number of false-positive results [11, 12]. Moreover, it was even criticized for increasing the number of unnecessary biopsies and generating economical costs [11]. It was, however, shown that in women with BRCA1 and BRCA2 mutations, the BSE increases the sense of control and safety [13]. Contradictory to this, the other study found that in some women, BSE can increase depression level, anxiety and fear of cancer [14]. Some authors argue that BSE is an act of awareness and that there is no moral right to take away such inexpensive and always available tool from females [15]. Nevertheless, the BSE is not any longer recommended by many health authorities as a universal method of breast screening [16]. World Health Organization states, however, that BSE is advised to women at higher cancer risk [17]. As this risk can arise, inter alia, from genetic predispositions (mutations), large group of females, particularly in developing countries or nations with economical disproportions, may be unaware of them due to limited access to molecular genetic screening.

Despite the ongoing discussion on BSE usefulness, it was largely promoted (and still is) in many countries, as a part of health education [18]. In Poland, it was previously reported that women are aware of BSE but only one third of them actually perform it – the result concluded as unsatisfactory [19]. It can be predicted that young women performing BSE may reveal higher degree of health prevention and in future, be more keen to participate in national BSP based on X-ray mammography.

The present study aimed to investigate the current frequency of BSE among Polish women and factors which may influence it. Participation and awareness of national breast screening programmes and attitude towards the breast cancer as a health threat were also assessed. The results of this study characterize the current status of basic breast cancer education among Polish women and highlight the need for further improvement.

Material and methods

The study used an anonymous questionnaires addressing the following issues:

- the knowledge on how to perform BSE,
- the frequency of BSE performance,
- the awareness of national BSP,
- the source of information on BSP,
- the attitude towards breast cancer as a health threat.

The survey was conducted between 2012 and 2014 and was completed by total of 751 Polish women. Characteristics of the studied group were given in **Table 1**. The results were elaborated in regard to female age, education, economical status and place of living. The statistical analyses were performed with Statistica v.10.0 (StatSoft, Poland). Relations between indicated responses and age (normally distributed data) were assessed with parametric T-Student test. Comparison in the frequencies of given answers among different groups was assessed with Pearson's chi square test. $P < 0.05$ was considered as statistically significant.

Table 1. Characteristics of the surveyed group of women

Age	
(mean ± SD)	33.0 (±15.5)
range	18–84 years
Education (%)	
primary	75.5
secondary	23.9
higher	0.7
Place of living (%)	
village	14.5
< 100,000 residents	26.1
100,000–500,000 residents	7.1
> 500,000 residents	52.3
Economical status (%)	
very poor	1.1
poor	3.1
fair	43.5
good	44.6
very good	7.7

Results

Attitude towards BSE

Most of surveyed women declared to know how to perform BSE (79.6%) and from this group large percentage (88.0%) indicated to practice it but only one third (34.0%) did it regularly (once a month). The rest admitted to perform BSE rarely. Women performing BSE regularly were older than these performing it rarely (mean 30.7 vs 41.1 years old; $p < 0.001$). No significant differences in the attitude towards BSE were noted between women with higher and secondary education ($p > 0.05$). The economical status and place of living were also not found to be associated with knowledge and performance of BSE nor regularity of it ($p > 0.05$ in all cases).

Attitude towards BSP

The majority of surveyed women heard about BSP (87.3%). The main sources of this information includ-

ed TV and Internet. Medical doctors and other medical staff (nurses and midwifes) were rarely indicated as a source of information in that matter (**Figure 1**). Women performing BSE regularly heard about BSP significantly ($p < 0.05$) more often (92.9% of responders) than those who did it rarely (87.1%) or never (82.6%). Female education, place of living and economical status were not associated with the BSP awareness ($p > 0.05$).

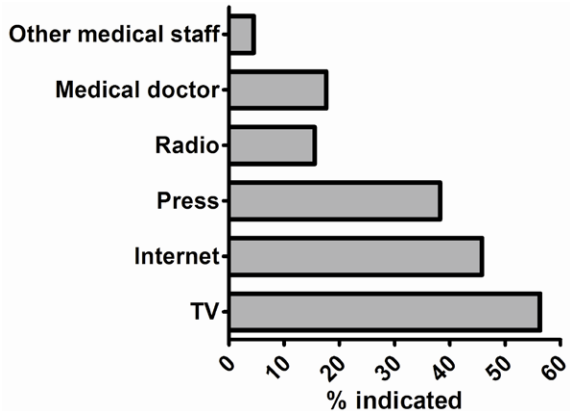

Figure 1. Sources of information on BSP among the surveyed group of women

Attitude towards breast cancer

Most of responders (89.7%) indicated that breast cancer is a significant health threat in Poland. This view was represented more often by women with higher education and better economical status, and with the lowest frequency by those who never performed BSE and inhabited villages and areas of less than 100,000 residents ($p < 0.05$ in all cases).

Discussion

The breast cancer educational programmes should be followed by systematic evaluation of public awareness and attitudes to highlight the points which may require improvement in gaining public attention and develop proper health prevention and cancer control activities. The results of present study indicate that surveyed group of Polish women were generally aware of health treats associated with breast cancer, knew how to perform BSE and were informed about BSP. On the other hand, only a small part of women practiced BSE regularly. The irregularity in BSE performance by Polish women was already reported in previous studies [19, 20] and highlights the continuous necessity to encourage female to self-monitor their breasts more frequently (idealy once a month). Women performing BSE regularly were reported to have significantly higher self-efficacy and increased health motivation [21]. Importantly, in our study, the group performing BSE regularly was more aware of national BSP and more often indicated that breast cancer represents a serious health threat. Altogether, even if BSE is disregarded as a medical procedure, it still has a value as a tool through which women can take charge of their health and develop desired health behavior. It is likely that women practicing BSE will more likely attend national BSP, gain knowledge on risk factors, discuss the limitations of screening methods with health professionals and generally, reveal pro-healthy activities. In that meaning, the function of BSE far exceeds its role as an inexpensive screening method, particularly in countries with lower economical status.

There are several reasons for which the women may not perform BSE regularly, the main being the fear for finding malignancy [21–23]. Moreover, some women find unnecessary to perform it due to no history of breast cancer in their family [24]. The genetic predispositions and heredity are among the most frequently recognized breast cancer risk factors by surveyed women [25–27]. There are, however, many other known factors that may increase risk of cancer development such as age, early menstruation, late menopause, dense breasts, diet rich in saturated fats, sedentary lifestyle, obesity, long-term use of hormone replacement therapy, alcohol abuse and exposure to radiation and chemical contaminants [28, 29] In fact, the direct cause of any individual breast cancer is unknown and may be a complex function of several co-occurring factors.

The health preventing programmes such as BSP should be promoted through a variety of sources including TV and radio advertisements, online and printed materials [30]. There is no doubt that medical staff should be actively involved in educating women about cancer risk factors, and to initiate screening programs aimed at early detection and intervention [31], yet the present study demonstrated that its role in BSP promotion is insignificant. Studies conducted in other countries, e.g. Turkey, United Arab Emirates, Jordania, demonstrated that health professionals play significantly greater role than in Poland (but still not a key one) through which women acquire information on breast cancer prevention [21, 32, 33]. This along with recent report on very low level of public trust in Polish physicians [34] raises serious concerns. Much effort should be put forward to improve the communication between patient and health professionals and to educate women, including those below the age of

increased breast cancer risk, on the screening methods and their limitations.

Conclusion

Polish women are generally aware of BSE and BSP, and recognize breast cancer as a serious health threat. Most of women, however, do not perform BSE regularly. The greater involvement of medical doctors and other professional staff in health education towards breast cancer prevention is necessary.

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CASE STUDY

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Clinical outcomes of conjugated linoleic acid supplementation in the overweight and the obese: a study protocol

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ABSTRACT

This is the protocol of a study aiming to assess the impact of conjugated linoleic acid (CLA) on body mass and composition, carbohydrate and fat digestion and absorption, total energy expenditure, lipid profile, polyunsaturated fatty acids levels, markers of endothelial dysfunction and mineral status in humans. Seventy-four adult volunteers (BMI ⩾ 25 kg/m^2) will be randomized to receive 3.0 g of 80% CLA (50:50 cis-9, trans-11 and trans-10, cis-12 isomers) or 3.0 g of linoleic acid daily for 3 months. A range of parameters will be measured at baseline and after the intervention.

Keywords: obesity, randomized, digestion, absorption, body composition, lipid profile, endothelial dysfunction, mineral status, breath test, 13C.

Introduction

Herein we present the protocol of a study aiming to assess the impact of conjugated linoleic acid (CLA) on body mass and composition, carbohydrate and fat digestion and absorption, total energy expenditure, lipid profile, polyunsaturated fatty acids (PUFA) levels, markers of endothelial dysfunction and mineral status in humans.

Basic Concept of the Project

Obesity-related morbidity and mortality burden societies worldwide [1]. Although many factors contribute to obesity, the dietary factors play a leading role. Among these, insufficient proportion of unsaturated fat seems to be of particular importance. CLA comprises of conjugated isomers of the 18-carbon polyunsaturated fatty acid. In many naturally occurring foods, such as dairy products and ruminant meats, cis-9, trans-10, and trans-10, cis-12-CLA can be found. On the other hand, CLA, present in small quantities in tissues, is not synthetized by the human organism. Although there are data that point towards the influence of CLA on adiposity, the mechanisms behind the observed effects remain unknown [2]. There is evidence supporting the influence of 50:50 mixture of cis-9, trans-10 and trans-10, cis-12-isomers in weight management [3]. The trans-10, cis-12-isomer seems to lead to body content of adipose tissue and increase insulin resistance at the same time [4]. However, the effect was not reported when the 50:50 mixture was investigated.

Research methodology

Study population

The study will comprise 74 adults with BMI ⩾ 25 kg/m^2 at the beginning of screening. Subjects will be instruct-

ed to maintain isocaloric diet and not to change their eating habits during the study period. Criteria for completion include consumption of 75% of the supplement provided. A summary of exclusion criteria is shown in **Table 1**.

Table 1. Exclusion criteria

Exclusion criteria
Subjects with: – history of chronic systemic disease (with the exception of hypertension) – celiac disease – type 2 diabetes – liver and/or pancreatic disease – current or recent (within the preceding month) treatment with CLA and agents interfering with fat digestion and/or absorption (chitosan, orlistat, green tea) – pregnancy.

Protocol

The subjects will be randomly assigned to receive CLA or placebo. Participants enrolled to the CLA group will be given capsules containing 3.0 g of 80% CLA (50:50 cis-9, trans-11 and trans-10, cis-12 isomers) daily for 3 months. Volunteers randomized to the placebo group will be given capsules containing 3.0 g of linoleic acid (LA) per day. Breath tests will be performed after an overnight fast with the use of Iris-Infrared Isotope Analyser (Wagner Analysen Technik, Bremen, Germany). Venous blood samples will be collected from each subject according to the standard already implemented in the institution after an overnight fast. The procedure will be performed before randomization and after 3 months of supplementation. The study protocol flowchart is shown in **Figure 1**. Statistical analyses will be performed to describe the studied populations; to determine the normality of distribution of parameter values in the groups; to compare the parameter values in the populations at baseline and after the study period; to compare observed changes of parameter values between the groups; to search for correlations between changes in parameter values. The level of significance will be set at $p < 0.05$.

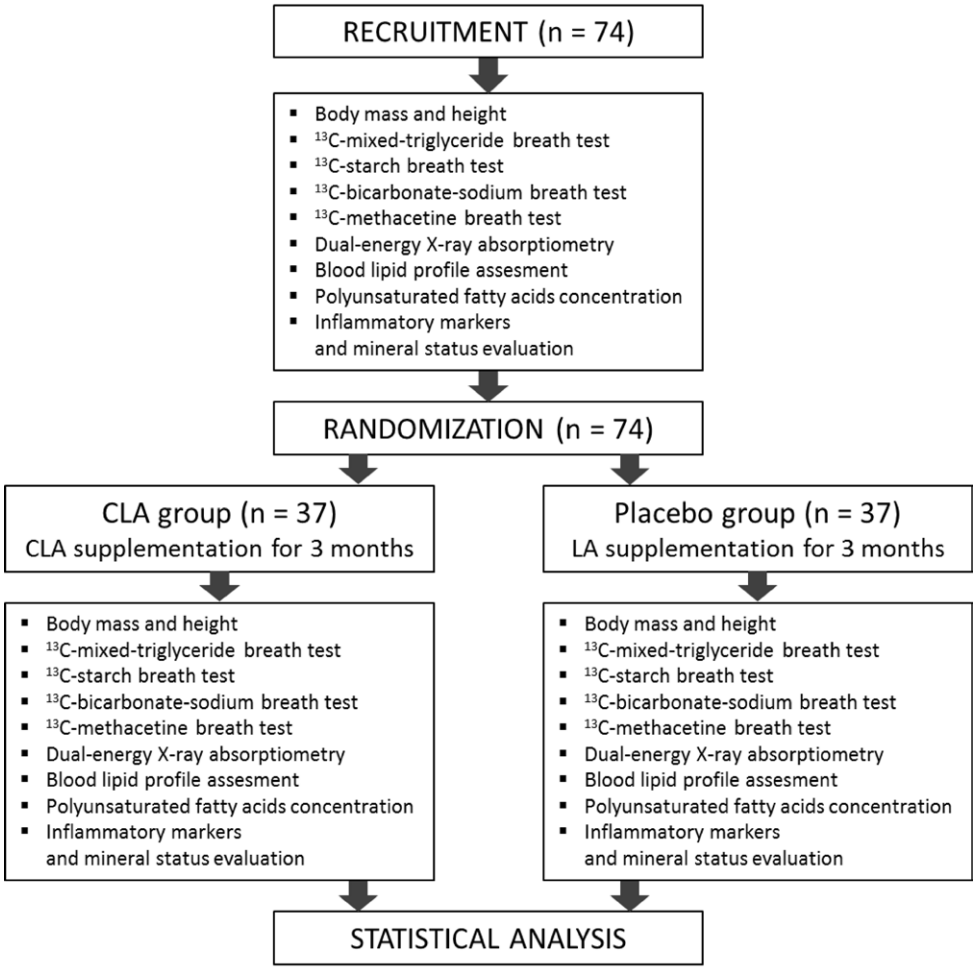

Figure 1. Study protocol

The following methodological approach will be consistently applied:

- Body composition will be measured by dual-energy X-ray absorptiometry (DXA).
- Fat digestion and absorption will be assessed by ^{13}C-mixed-triglyceride breath test. A dose of 150 mg of ^{13}C-mixed triglyceride will be administered with a standardized meal, that is 50 g roll with 12.5 g of butter (82% of fat). Breath samples will be collected before the test meal and every 30 minutes for 6 hours after ingestion.
- Total energy expenditure will be assessed by ^{13}C-bicarbonate sodium breath test. A capsule of ^{13}C-bicarbonate sodium will be administered orally after dissolution in 125 ml of warm fruit tea. Sixteen breath samples will be acquired from the patient over 3 hours.
- Liver metabolism will be assessed by ^{13}C-methacetine breath test (a dose of 75 mg ^{13}C-methacetin will be dissolved in 100 ml of fruit tea and administered orally. Breath samples will be collected before and every 10 minutes for 2 hours after ingestion.
- Carbohydrates digestion and absorption will be assessed by ^{13}C-starch breath test. A standard meal will consist of 50 g of cornflakes with 100 ml of low fat milk. The breath samples will be collected before and every 30 minutes for 4 hours after the test meal.
- Blood lipid profile will be determined by enzymatic methods (Olympus, Beckman Coulter, Pasadena, USA).
- Mineral status will be assessed in 24-hour urine collection and in hair samples.
- PUFA concentration in blood will be determined by gas chromatography-mass spectrometry (Agilent Technologies, Palo Alto, USA).
- Blood analyses related to endothelial dysfunction will be performed.

The study was approved by the Bioethical Committee at Poznan University of Medical Sciences and adheres to the revised Declaration of Helsinki. Written consent to participation in the study will be obtained from all volunteers after providing full information.

The research project is supported by Nutricia Foundation (grant number 504-06-01103115-000-15-07588).

Expected results

Reports provide contradictory accounts of CLA effects. No influence of CLA on body weight or composition was found by Joseph et al. [5]. On the other hand, Gaullier et al, Pfeuffer et al., and Chen et al. observed a reduction in fat mass that could be attributed to CLA provision [6–8]. Beneficial changes in lipid levels were attributed to CLA in animal models [9, 10]. This study will verify whether CLA supplementation promotes lipid profile normalization in humans.

It was suggested that CLA may increase the basal metabolic rate, thermogenesis, and lipid oxidation [11, 12]. CLA could also lead to reducing adiposity by inhibiting heparin-releasable lipoprotein lipase [13]. This would result in increased apoptosis of preadipocytes.

The authors are aware that CLA was linked to hepatitis [14, 15]. A meta-analysis which found that CLA at a daily dose of 3.2 g leads to reduction in adiposity in humans also suggested that more research into CLA safety is warranted [16]. Therefore, the proposed study will also gather information on CLA supplementation safety.

In conclusion, this study will aim to reproduce the many reported benefits of CLA supplementation in humans.

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Author contributions
Contributions to conception and design: KŁ, AG, JKN, MDŻ, KG, PB, EF, EM, JW. Article drafting: KŁ, AG, JKN, EF, JW. Final approval of the version to be published: KŁ, AG, JKN, MDŻ, KG, PB, EF, EM, JW.

Conflict of interest statement
The authors declare that there is no conflict of interest in the authorship or publication of contribution.

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The role of microparticles in pathomechanisms of diabetic retinopathy – analysis of intercellular communication mechanisms in endothelial aging. Case control study in patients with metabolic syndrome, diabetes type 1 and type 2

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ABSTRACT

The project is proposed to explain the role of specific circulating microparticles (MPs) as conveyors in trafficking bio-active molecules in type 1 (T1DM) and type 2 (T2DM) diabetic patients with risk of diabetic retinopathy (DR) and in patients with metabolic syndrome (MS). The possible role of miRNAs as modulators of these processes (in switching on/off mechanism on the molecular level) is proposed. An increased number of MPs with respect to glucose concentrations and levels of proangiogenic factors in vivo (patients' plasma) is expected. The relationship between age of patents and MP content (cell membrane glycoproteins, phosphatidylserine or miRNA profile) is possible. MPs will be obtained from T1DM (n = 30) T2DM (n = 30), MS (n = 30) and controls (n = 30). Retinopathy in diabetic patients will be assessed by imaging method. Biological profile of MPs will be assessed in vitro by means of flow cytometry, molecular biology methods and cell proliferation assays.

Keywords: microparticles, endothelium, diabetic retinopathy, miRNA, vascular ageing.

General information

The project "The role of microparticles in pathomechanisms of diabetic retinopathy – analysis of intercellular communication mechanisms in endothelial aging" was awarded by the Polish National Science Center (NCN) in the 7th edition of OPUS competition under grant number 2012/07/B/NZ5/02510. The contract between NCN and Jagiellonian University Medical College (JUMC) was signed on 26th of June 2013 and the duration of the project is planned for 36 month, until 25th of June 2016.

The clinical part of the projects is a prospective case-control study in a group of diabetic patients with a high risk of retinopathy. The project is interdisciplinary in nature and requires a close co-operation of specialists in clinical and laboratory medicine and basic science.

This project aims to combine recent progress in molecular and cell biology with the new approach for understanding the sequence of events on molecular and cellular levels leading to diabetic retinopathy (DR). The project adds a new piece of puzzle to understand-

ing how hyperglycemic conditions affect retina, vascular (endothelial) and beta-cells to secretion of microparticles (MPs). It is assumed to characterize MPs released in this way (miRNA content), and compare this characteristic with *in vivo* conditions, with MPs obtained from metabolic syndrome, T1DM and T2DM patients.

Management

The Principal Investigator of this project is Associate Professor Ewa Stępień (PhD), a specialist in laboratory medicine (genetics) from Department of Medical Physics Jagiellonian University and Department of Clinical Biochemistry JUMC.

Co-investigators are: Professor Maciej Małecki (MD, PhD) from Department of Metabolic Disease JUMC, Iwona Szuścik (MD, PhD) from Private Ophthalmology Practice OKO-LASER Outpatient Clinic in Kraków, Associate Professor Bogdan Solnica (MD, PhD) and Aleksandra Tokarz (MSc) from Department of Clinical Biochemistry JUMC and Aleksander Żurakowski from Department of Interventional Cardiology, American Heart of Poland SA, Chrzanów.

The International Partner is Assistant Professor Francisco J. Enguita (BPharm, PhD) from Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Portugal.

Ethics

Bioethical Committee at JUMC accepted all project's protocols and forms, including information for patients form and consent form for participation in the research study on 24th October 2013. The permission No. KBET/206/B/2013 is valid until 31st of December 2016.

Finance

The project is mainly financed by a grant from NCN Life Science Panel in the 7th OPUS call dedicated to "Human and animal noninfectious diseases mechanisms, diagnosis and treatment of diseases, poisonings and injuries". The total amount of grant funding is 697,100 PLN (about 169,000 Euro). Grant funds were earmarked to purchase reagents for molecular biology, biochemistry and flow cytometry methods, disposable laboratory equipment, laboratory instrumentation for the conducting of proposed research and data analysis. In addition, grant funds were designed to cover personnel costs of project participants and costs associated with the dissemination of research results, i.e., through publications in peer reviewed journals and presentations at professional scientific conferences.

Research basic concept

Microparticles (MP) are small extracellular vesicles between 100 and 500 nm, released by the cells in a strictly regulated, cytoskeleton dependent process. In contrast, exosomes are smaller in diameter (between 40 and 100 nm) and they are released during constitutive or facultative exocytosis [1]. MPs are produced during shedding process in response to some stressing factors like: hypoxia and injury or due to inflammation [2]. A number of cell types associated with the vascular system have been shown to release MPs: platelets, lymphocytes, macrophages, vascular endothelial cells and others (smooth muscle, retina, progenitor and cancer cells) [3, 4]. MPs have been suggested to mediate local inflammation, thus they play a pivotal role in vascular diseases, such as atherosclerosis or DR [5, 6].

Endothelial dysfunction occurs when perturbed homeostatic endothelium disrupts vascular competence. These disruptions result in reduced vasodilatation, increased proinflammatory and prothrombotic property of the vascular network [7]. New insight in endothelial dysfunction, including the process of angiogenesis alteration is emerging from studies on vascular microvesicles such as endothelial MPs. It was previously documented that MPs derived from activated or apoptotic endothelial cells induce apoptosis in circulating angiogenic cells, and impair the atheroprotective and angiogenic function of the endothelium [8]. Impaired endothelial function is thought to be a denominator of pathogenesis of microvascular complications in T1DM and T2DM. It was documented that DR is associated with increased intima-media thickness (IMT) and endothelial dysfunction measured as concentrations of von Willebrand factor (vWF) and s-ICAM-1 [10] and elevated circulating ADMA [9].

MPs of endothelial, platelet, photoreceptor, and microglial origin were identified in vitreous samples and increased MPs of different origin in patients with DR may contribute to disease progression. The pro-inflammatory and pro-coagulation effects of MPs are mediated by specific lipid composition and/or by the transfer of pro-inflammatory factors from the cells of origin [11]. Moreover, endothelial microparticles (EMPs), released from apoptotic endothelial cells (ECs), influence cell repair of glucose damaged ECs by transferring microRNA [13].

Research objectives

The study primary objective is to characterize the profile of circulating MPs containing cell derived microRNA. This should allow to explain the mechanisms triggering undesirable events: thrombosis, apoptosis and degenerative vasculogenesis, which lead to DR.

One of the proposed mechanisms of DR is increased MP release from platelets and endothelial cells triggering TF in patients with complicated diabetes [14]. Apart from up-regulating effect on the expression of growth factors and chemokines, TF may directly promote ocular angiogenesis by the activation of MAPK- and protein kinase C-dependent signalling (non-canonical pathways). Another mechanism proposes the transfer of secondary messenger molecules (receptors, lipids) mainly from immune cells (lymphocytes T), which regulates vascular function [15, 16]. It was proposed, that failed angiogenesis in metabolic diseases is regulated by down-expression of vascular growth factors, *via* miRNA [17].

The secondary objective is the confirmation whether the exposure of endothelial cells to MPs generated in hyperglycemic conditions can induce calcium influx, cytoskeleton reorganization, VE-cadherin expression and catenin-beta translocation. It is important to distinguish the specific elements in this process, especially MPs content (miRNA) and their phenotype (origin) in the regulation of this machinery. This part of a study will be performed *in vitro*, in a cell culture model.

Research plan

The proposed project will be composed of two stages: Clinical stage – selection of individuals to 4 study groups T1DM (n = 30) T2DM (n = 30), MS (n = 30) and controls (n = 30) and analysis of classical risk factors and biomarkers; Basic stage – quantification

STEP	TASK	METHODS	TIME
PATIENTS RECRUITMENT	ALLOCATION OF PATIENTS TO SPECIFIC GROUPS	CLINICAL EXAMINATIONS RETINA CONTROL BIOCHEMICAL ANALYSIS	1 MAY 2013– 31 MARCH 2014
PATIENTS MICROPARTICLES ANALYSIS	OBTAINING OF MPs FROM CLINICAL SAMPLES	FLOW CYTOMETRY ANALYSIS miRNA ANALYSIS	1 JULY 2013 – 31 NOVEMBER 2014
CELL CULTURE MICROPARTICLES	INDUCTION OF MPs IN RETINOBRALSTONA ENDOTHELIAL AND BETA CELL LINES	FLOW CYTOMETRY ANALYSIS miRNA ANALYSIS	1 MAY 2013-31 MARCH 2015
BASIC RESEARCH	CELL CULTURE ANALYSIS OF MPs BIOLOGICAL ACTIVITY	TUBULE FORMATION AND MIGRATION ASSAY, CELL IMAGING, GENE EXPRESSION	1 JANUARY 2013 - 30 JUNE 2015
VERYFICATION OF WORKING HYPOTHESIS	COMPARISSION OF CLINICAL AND BASIC DATA	STATISTICAL AND DATA MINING ANALYSIS	1 MARCH 2014 - 30 JUNE 2015
DISSEMINATION OF RESULTS	RESEARCH PAPERS AND POSTERS PREPARATION	PARTICIPATION IN CONFERENCES, PUBLISHING	1 JUNE 2014 – 1 MAY 2016

Figure 1. Work plan of the study "The role of microparticles in pathomechanisms of diabetic retinopathy - analysis of intercellular communication mechanisms in endothelial aging. Case control study in patients with metabolic syndrome, diabetes type 1 and type 2"

of MPs, miRNA profiling, in *vitro* investigation. Time course of particular tasks is presented on **Figure 1**.

Research methodology

Study population

Up to 90 patients and 30 control subjects in age between 25–65 years will be enrolled into this study. All participants will be classified into 4 groups matching the inclusion criteria (**Table 1**). MS was will be defined according to the revised ATPIII criteria that require at least three of the following components: (1) abdominal obesity (waist circumference ⩾ 102 cm for men or ⩾ 88 cm for women), (2) triglycerides ⩾ 150 mg/dL, (3) HDL cholesterol ⩽ 40 mg/dL for men or 50 mg/dL for women or lipids lowering therapy, (4) systolic/diastolic blood pressure ⩾ 130/85 mmHg or receiving drug treatment, and (5) fasting plasma glucose ⩾ 100 mg/dL [18].

Assessment of clinical outcomes

Length of time from entry to the study to when the clinical event will occur is planned no less than 2 years. Clinical outcomes will be assessed in primary and secondary end-points (**Table 2**).

Sample collection

All blood samples will be drawn at the same time of the day (between 08:00 and 10:00 am) with venipuncture with > 21-gauge needle in the antecubital vein following the application of a tourniquet. The first 2–3 ml of blood will not be included for MP analysis (used for additional analysis). Citrate (for MPs and miRNAs) or EDTA (hematology, HbA1C, biomarkers) anticoagulation will be used. For biochemistry, serum samples will be collected. The blood samples will be separated to obtain Platelet Poor Plasma (PPP) or serum and frozen at -80°C until analysis for miRNAs and selected biomarkers. Additionally, urine samples will be collected.

Table 1. Eligibility and inclusion criteria for patients enrolled to the project "The role of microparticles in pathomechanisms of diabetic retinopathy – analysis of intercellular communication mechanisms in endothelial aging. Case control study in patients with metabolic syndrome, diabetes type 1 and type 2"

Type 1 Diabetes Mellitus (T1DM) n = 30	Type 2 Diabetes Mellitus (T2DM) n = 30	Metabolic Syndrome (MS) n = 30	Control group (CG) n = 30
Inclusion criteria			
Male and female subjects in age between 25–65 years			
Subjects who will give their informed consent to participate in the study, both in the enrolment and later follow-up.			
Diabetes type 1	Diabetes type 2	Metabolic syndrome according to revised ATPIII criteria [18]	apparently healthy
BMI from 18.5 to 24.9/m^2 BMI from 25.0 to 40.0/m^2	BMI from 18.5 to 24.9/m^2 BMI from 25.0 to 40.0/m^2	BMI from 30 to 40.0 kg/m^2	BMI from 18.5 to 24.9 kg/m^2
Diabetic Retinopathy (DR) assessed by the colorful photographic documentation of two 45° retinal fields	Diabetic Retinopathy (DR) assessed by the colorful photographic documentation of two 45° retinal fields	–	–
Insulin resistance is not an exclusion criterion	Insulin resistance is not an exclusion criterion	Insulin resistance	–
Exclusion criteria			
Acute Coronary Syndrome (ACS), acute Ischemic Stroke (IS) or critical limb ischemia will be excluded from this study. A previous cardiovascular event (MI or IS) has to be at least 6 months prior to the study enrollment.			
History of cancer, renal and liver failure, and past or present systemic inflammation (hs-CRP > 10 mg/l), such as active chronic arthritis or phospholipid syndrome.			
Known or suspected bacterial or viral infection			
Treatments with steroidal and non-steroidal anti-inflammatory drugs, bariatric surgery			
Pregnancy or Hormone Replacement Therapy (HRT)			
Morbid obesity > 40.0 0 kg/m^2	Morbid obesity > 40.0 0 kg/m^2	Morbid obesity > 40.0 0 kg/m^2	–
–	–	–	Statin treatment

Table 2. Clinical outcomes of the project

Primary end-points	An anatomic feature that is measured at the end of a study to assess the progression of DR: measure the area of choroidal neovascularization, submacular leakage and hemorrhage
Secondary end-points	Statistically and clinically relevant differences in visual function at more than one time point

Laboratory analysis

Standard blood tests including biochemistry (glucose, lipids, creatinine) will be performed. For biomarkers, ELISA and molecular biology methods are planned. MP enumeration and phenotype analysis will be performed by flow cytometry. Genetic analysis of miRNA profile and gene expression will be done by means of quantitative PCR methods or next generation (NG) sequencing. Cell culture methods will be used for assessment of MP induced endothelial proliferation and angiogenesis.

Statistical analysis and methodology

The hazard ratio for data summary and the risk assessment will be performed. The linear regression models will be used for the continuous variable analysis, like biomarkers and MPs enumeration. MicroRNA will be correlated with biomarkers levels and the MPs number using multiple regression models. The Cox proportional hazard regression model will be used for DR parameters with respect to MPs and miRNA covariates. Additionally data mining analysis will be performed to reveal any data relationships.

Expected results

The clinical part of this study will be directed to develop new methods and systems for diagnostics and treatment of endothelial dysfunction related to pathological endothelial proliferation/neovascularization, senescence and other hyperglycemia related defects that cause deficiencies of ocular system:

- development and application of universal diagnostics protocols,
- discovery of new biomarkers,
- manufacturing of new diagnostic tests,
- targeting of the disease (DR).

The further basic research phase of this study may have an important role in the promoting the new experiments on circulating MPs and miRNAs and may have a high impact on the future technologies which apply new biomarkers in prophylaxis, treatment and prognosis of DR. In this stage we expect:

- to verify the hypothesis about the mechanisms involved in the processes related to DR on the cellular level: cell-to-cell communication *via* MPs as transferring vesicles of regulatory molecules including miRNAs and proangiogenic proteins,
- to bring a new insight into the relation between MP exposure, endothelial cell cytoskeleton reorganization and activation of cell signaling,
- to explain the role of MPs in neovascularization on the molecular level.

Acknowledgements

Conflict of interest statement

The authors declare that there is no conflict of interest in the authorship or publication of contribution.

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The project is mainly financed by a grant from NCN Life Science Panel in the 7th OPUS call dedicated to "Human and animal noninfectious diseases mechanisms, diagnosis and treatment of diseases, poisonings and injuries". The total amount of grant funding is 697,100 PLN (about 169,000 Euro).

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REVIEW PAPER

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Progress in study of *Cannabis sativa* leaves extracts without psychotropic cannabinoids in animal model of neuropathic pain

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ABSTRACT

Neuropathic pain is a type of chronic pain caused by a lesion or disease of the somatosensory nervous system. Current therapy for this pain includes the use of pharmacological and nonpharmacological methods but due to the fact that a lot of therapy does not produce the analgesic results, it is necessary to search for new and more effective pharmacological strategy in relief of this type of pain. One of the interesting natural sources of compounds against this type of pain is extract of *Cannabis sativa* without psychotropic cannabinoids. Medicinal properties of *C. sativa* have been explored for centuries. It is well established that active compounds of this herb act through two cannabinoid receptors (CB1, CB2) as endocannabinoid system in the central nervous system. The present review addresses the recent advances in the study of pharmacological mechanisms on cellular and receptor level underlying non-hallucinogenic cannabinoid analgesic effect. In recent years, results of studies allow to state that special plant extract of *C. sativa* (without psychotropic cannabinoids) may be a promising source of drug used to relieve neuropathic pain.

Keywords: cannabinoids, analgesic effect, animal models.

Introduction

The International Association for the Study of Pain [1] defines neurophatic pain (NP) as pain initiated or caused by a primary lesion or dysfunction in the nervous system, thus NP may be caused by any disease or injury to the nervous system. A nerve lesion leads to serious changes in the nervous system and makes it distinct NP from other chronic pain types that have an intact nociceptive system [2]. Pain occurring in acute or chronic diseases, including NP, are a very common challenge in medical care. Thus, pharmacotherapy of this pain is one of the top priorities in developed countries. Currently, it is estimated that 7–8% of the general population in Europe suffer from NP [3], but in 30 to 40% of patients

with diabetes have symptoms suggesting neuropathy [1]. Moreover, different type of pain occur in as many as 90% of patients during cancer [4] and it is predicted that at least 15–20% of patients may suffer from NP during the course of the cancer [5]. The research issue becomes even more complex and socially urgent due to the fact that the anatomical classification of NP includes over 50 types of diseases associated with the pathogenesis of pain [2], for example phantom pain, trigeminal neuralgia, carcinoma-associated paraneoplastic peripheral neuropathy, acute or inflammatory polyradiculoneuropathy (Guillain-Barre syndrome), diabetic mononeuropathy, alcoholism, amyloidosis, multiple sclerosis, traumatic brain injury, Parkinson's disease, mastectomy. According Gondim et al [6], peripheral neuropathy in inflammatory bowel disease also belongs to NP. In particular, the inflammatory bowel diseases are an increasing challenge for pharmacotherapy, and this is because of contraindications to conventional analgesics. Due to the fact that a lot of therapy does not produce the expected results in the treatment of NP, it is necessary to search for new and more effective pharmacological solutions in relief of this type of pain [2, 7]. This is even more important, due to the fact that opioids and nonsteroidal anti-inflammatory drugs are among the most commonly used drugs in clinical practice, their use subsequently can induce unexpected drug interactions and/or several types of these medicines often produce several adverse reactions [8]. Nowadays, beside typical analgesics, also antidepressants, anticonvulsants [9], capsaicin, and memantine are used in pain relief [1]. In the recent years, the problem becomes even more complex because chemotherapy (e.g. use of such drugs as cisplatin, paclitaxel, vincristine) can also induce sensory neuropathies as an adverse drug effect (neurotoxicity) [10].

Impact of cannabinoid receptors in pain

A very interesting option in this field may be plant extract from *Cannabis sativa* L. This is important for NP therapy, because results of many researches showed occurrence of high density cannabinoid receptors (CB1 and CB2) in many areas related to pain (endocannabinoid system, ED) [11, 12]. It was shown that deregulation of the ED underlies several neurological disorders including chronic pain [13]. In human brain two endocannabinoids were found out, N-arachidonoyl ethanolamine (anandamide), the first discovered and best studied endogenous lipid-signaling ligand and 2-arachidonoyl glycerol (2-AG) which acts through cannabinoid receptors [14–16]. It is important to note, that other chemical compounds may belong to endocannabinoids such as dihomo-γ-linolenoylethanolamide, docosatetraenoylethanolamide, and possibly the CB1-selective agonist – 2-arachidonylglyceryl ether (noladin ether). [9]*N*–arachidonoyl dopamine, *N*-oleoyl dopamine, and oleamide [17].

Studies have reported a similar distribution of CB1 cannabinoid and mu-opioid receptors in sites which are strongly involved in antinociception as the dorsal horn of the spinal cord [18], the caudate putamen, dorsal hippocampus, substantia nigra [19, 20], raphe nuclei, central medial thalamic nuclei and hypothalamus [21, 22]. A growing body of literature attests to the interaction between opioids and cannabinoids at the receptor and signal transduction levels [23]. Another study in animal model showed that cannabinoid receptor agonists increased proenkephalin gene expression in the caudate-putamen, nucleus accumbens, paraventricular and ventromedial hypothalamic nuclei and also medial mamillary nucleus [24]. Moreover, Vigano et al. [23] concluded that CB1 receptors may be necessary for the expression of several effects of opiates, so CB1 antagonists may offer a novel approach for treating opiate addiction and the cannabinoids use may help relieve the effects of opiate withdrawal. Several studies showed, that agonists for CB1 receptor exert an anti-inflammatory, anti-nociceptive and anti-hyperalgesic properties in the central and peripheral nervous system levels [25]. It was demonstrated furthermore that activation of peripheral CB2 receptors generates an antinociceptive response in situations of inflammatory hyperalgesia and NP by inhibiting the release of proinflammatory factors in non-neuronal cells located near nociceptive neuron terminals [26]. CB2 receptors are mainly located in the immune system, therefore representing a target in inflammatory pain processing [27]. Recent results of the research provided evidence of the involvement of cannabinoid receptors in the supraspinal modulation of pain in different models with use intra-cerebral microinjections of cannabinoid ligands or positive modulators for CB1 receptors [27]. The study has demonstrated that synaptically released glutamate, as a result of pain, stimulates mGlu5 receptor inducing endocannabinoid release, which in turn activates presynaptic cannabinoid CB1 receptor. Moreover, it was explained that CB1 receptor activation expressed on presynaptic GABAergic terminals reduces the probability of neurotransmitter release thus dis-inhibiting the periaqueductal grey-rostral ventromedial medulla-dorsal horn (PAG-RVM-DH) antinociceptive pathway.

According to Palazzo et al. [27], cannabinoids may increase a glutamate release (maybe as consequence of GABA decrease) and require a glutamate receptor activation to induce the antinociception phenomena.

Neuroinflammation and NP

Very often the NP is disproportionately enhanced in intensity (hyperalgesia) or altered in modality (hyperpathia or allodynia) in relation to the stimuli leading to changes in some factors coupled with inflammation [28]. The inflammatory response initiates a cascade of events inducing the concentration and activation of innate immune cells at the site of tissue injury and as a consequence an infiltration of damaged peripheral nerves by mast cells, granulocytes, macrophages and T lymphocytes which leads to release of immunoactive substances such as cytokines, neurotrophic factors, and chemokines initiating a local actions and can result in a more generalized immune response [29]. Proinflammatory cytokines, eg. interleukin-1beta (IL-1ß) and tumor necrosis factor-alpha (TNFα) secreted by activated glial cells in the spinal cord and the brain play probably prominent role in inflammation-induced nociception [30]. Recent study showed that a chronic constriction injury led to increase of TNFα level in the sciatic nerve [31]. These proinflammatory cytokines exert their actions, at least partially, through the activation of the transcription factor, nuclear factor kappaB (NF-kappaB), which, in turn, regulates the transcription of many inflammatory mediators by binding to the promoter region of various genes, [eg. tumor necrosis factor α (TNFα), interleukin 1α (IL1α), cyclooxygenase-2 (COX-2), inducible NO-synthase (iNOS) and proteases (eg. matrix metalloproteases (MMPs)) [32–34]. It is well documented that the dysregulation of NF-kB activity is associated with risk of neurodegeneration [35] and with alteration of synaptic plasticity [36, 37]. In dorsal root ganglia and the spinal cord of rats an increased number of activated NF-kB immunoreactive neurons and astrocytes was observed [36, 38]. Several studies indicate that the NF-kB activation pathway plays a crucial role not only in the immune responses, inflammation, and apoptosis but also in the development and processing of pathological pain [39]. The results indicate that NF-kappaB has an impact on nociceptive transmission and processing and that a number of substances that inhibit the NF-kappaB-activating cascade are capable of reducing the nociceptive response in different animal models. Therefore, a modulation of specific participants in the NF-kappaB signal transduction might exert a useful approach for the development of pain release drugs [40]. Both experiments performed in in vivo model [41] and studies done in patients during acute migraine attacks [42] have revealed also the possibility of the NF-kB activation by transcriptional regulation of iNOS. Moreover, results from Wu et al. [43] suggest that reciprocal changes in the expression of ZAS3 (a member of zinc finger protein family) and NF-kappaB proteins might generate NP after peripheral nerve injury [43]. Recent experiments showed that glial NF-κB inhibition reduces galanin and CGRP expression, which are neuropeptides that correlate with pain behavior and inflammation after peripheral nerve injury [44, 45].

Other receptor mechanism of NP pathogenesis

A relationship was demonstrated between the N-Methyl-D-Aspartate receptor (NMDAR)-mediated neuronal excitation and nociceptive behavior. There is considerable evidence that activation of NMDAR contributes to the mechanism of pathological pain, hence a NR2B-containing NMDA receptor is one of the best potential targets for NP [46, 47]. It is in line with information that hypoactivity of the spinal cannabinoid system results in an NMDA-dependent hyperalgesia and thus may participate in the etiology of certain chronic pain conditions [48]. Moreover, previously it was shown that NMDA-antagonists are effective in decreasing autotomy (self-injurious behavior) and relieving neuropathic symptoms in other types of experimental peripheral neuropathies [49], but based on the systematic review of clinical trials, no conclusions can yet be made about the efficacy of NMDA receptor antagonists on NP [50].

Phytochemistry and biological activities of *Cannabis sativa* extracts

The *Cannabis sativa* L. has been known for centuries as a psychoactive plant and they has been used for thousands of years in ethnomedicine to relieve human suffering. The plant produces over 421 chemical compounds, including about 80 terpeno-phenol compounds named phytocannabinoids [51] and these cannabinoids and hemp extracts may exert different and promising pharmacological activities. Several studies have shown that the most important psychoactive compound of cannabis extract is considered to be Δ9-tetrahydrocannabinol (THC) [52–55]. However, there are also well known phytocannabinoids with very weak or no psychotro-

pic effects. These include cannabidiol (CBD) [56], cannabigerol (CBG), cannabichromene (CBC), D9-tetrahydrocannabivarin (D9-THCV), cannabidivarin (CBDV), D9-tetrahydrocannabinolic acid (D9-THCA) and cannabidiolic acid (CBDA) [51], and also the main compound of essential oil – beta-caryophyllene [57]. Phytochemical analysis of 11 cannabis varieties proved chemical differences between Cannabis spp. [58]. Many recent studies with use gas chromatography (GC) analyzing cannabinoids and terpenoids have been performed for chemotaxonomic purposes [58]. Chemotaxonomic evaluation of cannabis has led to the recognition of three chemotypes [59]:

1. Chemotype I – narcotic hemp, where delta 9 THC and carboxy THC to CBD and carboxy CBD ratio is much higher than 1 (> 1),
2. Chemotype II – intermediary hemp, where delta 9 THC and carboxy THC to CBD and carboxy CBD ratio is about 1,
3. Chemotype III – fibrous hemp, where delta 9 THC and carboxy THC to CBD and carboxy CBD ratio is much lower than 1 (< 1).

In accordance with review of Izzo et al. [51], non-psychotropic phytocannabinoids exert multiple pharmacological effects and the most recently investigated mechanisms of their biological actions involve the modulation of the endocannabinoid system, transient receptor potential channels, the peroxisome proliferator-activated receptor GPR55, the putative abnormal-CBD receptor, 5-hydroxytryptamine receptor subtype 1A, glycine a1 and a1b receptors, the adenosine membrane transporter phospholipase A2, lipoxygenase (LOX) and cyclooxygenase-2 (COX-2) enzymes, and Ca2+ homeostasis. At present, it was shown that D9-THCV is a CB1 receptor antagonist [60]. It was found out that at low doses (<3 mg/kg) it antagonises D9-THC effects, but it acts as a CB1 agonist at higher doses (10 mg/kg) in vivo in mice [51]. In the study performed by Bolognini et al. [61] it was shown that D9-THCV can activate CB2 receptors in vitro and decrease signs of inflammation and inflammatory pain in mice partly via CB1 and/or CB2 receptor activation. In mouse models of carrageenan-induced inflammation and inflammatory pain and formalin-induced hyperalgesia, D9-THCV suppressed signs of these pathological processes [61]. In detail, the THCV reduced the formalin-induced pain behaviour when administered at a dose of 5 mg·kg^{-1} and suppressed carrageenan-induced signs of inflammation and inflammatory pain in mice when it was injected 30 min before carrageenan. This investigation has demonstrated that the anti-edema activity exhibited by THCV appeared to be CB2, but not CB1 receptor mediated, its anti-hyperalgesic activity seemed to be mediated by both CB1 and CB2 receptors in the formalin model, but by neither of these receptors in the carrageenan model. Therefore it means that peripheral antinociception without CNS effects is consistent with the peripheral distribution of CB2 receptors [62]. Further research directed at identifying the mechanisms underlying these in vivo effects of THCV, is needed.

Results of Gertsch et al. [63] research proved that another non-psychotropic compound, (E)-beta-caryophyllene [(E)-BCP], which is one of the most abundant in the plant essential oil, has been shown to selectively target the CB2 receptor at nM concentrations and to act as a full agonist. Moreover, Gertsch et al. [63] demonstrated that (E)-BCP (500 nM) inhibited the lipopolysaccharide (LPS)-induced proinflammatory cytokine expression in peripheral blood and (E)-BCP at 5 mg kg^{-1} strongly reduced the carrageenan-induced inflammation in mice. These data confirmed that different CB2 receptor-selective ligands, including CB2 receptor agonists, are able to inhibit carrageenan-stimulated edema formation in mice. According to Bento's results [64] it was found also that the (E)-BCP reduced cytokine levels (tumor necrosis factor-α, keratinocyte-derived chemokine, and macrophage-inflammatory protein-2) in a culture of macrophages stimulated with lipopolysaccharide. Klauke et al. [65] confirmed that (E)-BCP exerts its analgesic effects in mouse models of inflammation and NP (formalin-induced inflammation model) and a model of NP, which involves the partial ligation of the sciatic nerve in male wild type (CB2^{+}/$^{+}$) and CB2 $^{-}$/$^{-}$ mice). It was observed that analgesic effects of (E)-BCP were absent in CB2^{-}/$^{-}$ mice and blocked by the CB2 antagonist SR144528. Moreover, using von Frey test and Hargreaves test they showed, that in mechanical allodynia (E)-BCP exerted a strong effect at a dose 10 mg/kg, but in second test only 1 mg/kg gradually reduced thermal hyperalgesia.

Another major psycho-inactive component of cannabis, CBD has been more thoroughly investigated. CBD has exerted analgesic and anti-inflammatory, antioxidant, neuroprotective and pro-apoptotic activities and might predict a possible future use for the treatment not only of pain, but also of neurodegenerative disorders, ischemia and cancer [51]. Additionally, Zuardi et al. [66] showed that the antipsychotic action of CBD was similar to atypical antipsychotics such as clozapine. Moreover, CBD has exerted anxiolytic-like effects by activating post-synaptic 5-HT(1A) receptors in the periaqueductal gray matter [67].

However, among published studies the most strong pharmacological effect showed that CBD has substantial anti-nociceptive and anti-inflammatory activities. Previously, Evans et al. [68] and Formukong et al. [69] found that CBD was more effective than other natural cannabinoids in the phenylbenzoquinone (PBQ)-induced mouse writhing test. They concluded that the compound was about 360 times more potent than aspirin and 590 times more potent than THC [68, 69]. There are also inconsistent results of studies, for example Sanders et al. [70] showed that orally administered CBD was inactive in the acetic acid stretching model and CBN was only effective at high concentrations. In the study performed by Costa et al. [71] in both neuropathic (sciatic nerve chronic constriction) and inflammatory pain (complete Freund's adjuvant intraplantar injection) in rats' model, it was found that treatment with CBD at dose of 2.5–20 mg/kg (neuropathic model) and at 20 mg/kg (adjuvant-injected rats) from day 7 to day 14 after the injury, reduced hyperalgesia to thermal and mechanical stimulation. A reduction was also demonstrated in the content of several mediators, e.g. prostaglandin E2 (PGE2), lipid peroxide and nitric oxide (NO) after CBD treatment. On the basis of these results one may conclude that the CBD exerts the therapeutic potential in NP. Moreover, when CBD was administered orally (5–40 mg/kg) once a day for 3 days after the carrageenan-induced inflammation in the rat, it exerted not only anti-inflammatory effect but also six hours after carrageenan injection, the lower doses of CBD abolished a hyperalgesia [72]. It was calculated that CBD at a dose of 5 mg/kg reduced edema to 50%, at 7.5, 5 and 10 mg/kg to about 65% and to 100% after 40 mg/kg. These authors demonstrated also that CBD in the dose of 10 mg/kg non-significantly reduced COX activity (21%) and the doses of 20 and 40 mg/kg of CBD brought COX activity down to the level of non-inflammed tissues. Although there is an increasing number of experimental works and clinical studies explaining the mechanism of extracts obtained from *Cannabis sativa*, the data defining whether its biologically active compounds influence the expression at mRNA and/or protein level is still insufficient. It was shown for example that oral administration of ajulemic acid (1'-1'-dimethylheptyl-THC-11-oic acid; AjA), a cannabinoid acid devoid of psychoactivity, reduces joint tissue damage in rats with adjuvant arthritis. Zurier et al. [73] in an *in vitro* study (on peripheral blood and synovial fluid monocytes – PBM and SFM – isolated from healthy subjects and from patients with inflammatory arthritis, respectively) have revealed that AjA in doses 0–30 microM did not influence TNFα production by activated cells but AjA reduced levels of IL-1β mRNA in a concentration-dependent manner which might be helpful to explain the therapeutic effects of AjA in the animal model of arthritis [73]. In CBD treated mice a significant reduction of plasma levels of the pro-inflammatory cytokines, IFN-gamma and TNFα was observed [74]. Furthermore, Costa et al. have examined whether CBD inhibited the production of nociceptive and inflammatory mediators involved in development and maintenance of NP and inflammation. In rats orally treated with CBD (2.5–20 mg/kg to neuropathic and 20 mg/kg to adjuvant-injected rats) from day 7 to day 14 after the injury no reduction in NF-kappaB activation and TNFa content was observed [71]. There has been some evidences on the efficacy of analgesic effect of cannabinoids originating not only from different animal models [75], but from clinical trials which included e.g. patients with peripheral neuropathy in inflammatory bowel disease [6], sclerosis multiplex [76] and also in cancer pain [77].

Preparations containing the *C. sativa* extract

In 2005, Ministry of Health of Canada has approved SATIVEX® for the adjunctive treatment of symptomatic relief of NP in multiple sclerosis in adults. SATIVEX® contains Tetranabinex® and Nabidiolex® – *Cannabis sativa* extracts. In this product the principal active components are 2.7 mg of THC and 2.5 mg of CBD.

It is worth emphasizing, that several studies concerning assessment of the Sativex analgesic activity have been carried out [78, 79]. Recently, a double-blind, randomized, placebo-controlled clinical study showed significant difference between Sativex treatment group and placebo. This product demonstrated the potent analgesic effect and it was generally well tolerated [78]. Other clinical studies largely affirm that NP patients derive benefits from cannabinoid treatment, but evidence to date suggests that abuse on Sativex is likely to occur [79]. This indicates the need to develop new products derived from cannabis extract devoid of THC, therefore further studies should be performed in this field.

Summary

According to several experimental and review articles [80] currently it can be considered that progress in

understanding the physiological effects and pharmacological activity of phytocannabinoids may provide new therapeutic opportunities in the treatment of NP. There are some evidences provided both by animal and clinical studies on the efficacy of analgesic effect of different cannabinoids, both hallucinogenic (delta9-tetrahydrocannabinol – THC) and non-hallucinogenic (cannabidiol, delta9-tetrahydrocannabivarin, beta-caryophyllene) compounds. Clinical studies largely affirm that NP patients derive benefits from cannabinoids treatment e.g in peripheral neuropathy, in inflammatory bowel disease, sclerosis multiplex and with cancer pain. It indicates the need to develop a new product derived from cannabis extract that will be devoid of THC, and further studies should be performed in this field. Some aspects of the analgesic and anti-inflammatory effects of *Cannabis sativa* extracts containing non-psychotropic plant-derived cannabinoids seem to be interesting in context of discovery and development of drugs for the treatment of NP.

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Conflict of interest statement
The authors declare that there is no conflict of interest in the authorship or publication of contribution.

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REVIEW PAPER

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High mobility group box 1 protein in the central nervous system

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ABSTRACT

High-mobility group box 1 protein (HMGB1) is a multifunctional protein originally identified as a nuclear transcription modifier. Two pathways are leading to HMGB1 release to the extracellular space i.e. active secretion triggered by noxious stimulation and passive leakage due to necrotic membrane damage. Binding with receptors for advanced glycation end products (RAGE) as well as Toll-like receptor 2 (TLR2) and TLR4 leads to nuclear factor κB (NF-κB) activation and proinflammatory reaction in target cells. Secretion of cytokines, upregulation of adhesion molecules and chemoattraction are triggered by the extracellular HMGB1. Such ubiquitous and numerous protein plays a role in pathogenesis of many common diseases like sepsis, rheumatoid arthritis and pneumonia. Central nervous system (CNS) disorders are also mediated by HMGB1. Multiple studies highlight pivotal role of HMGB1 in acute pathologies of CNS like cerebral ischemia, aneurysmal subarachnoid hemorrhage as well as chronic degenerative disorders such as Alzheimer's disease and multiple sclerosis. Wide range of HMGB1 antagonists are currently investigated as novel therapeutic agents in sepsis, colitis and stroke. This review article provides basic information about HMGB1 protein and its role in the pathogenesis of CNS diseases.

Keywords: high mobility group box 1, amphoterine, HMGB1, central nervous system, stroke.

Introduction

High-mobility group box 1 (HMGB1) is a multifunctional protein and a promising target for new therapies. Novel discoveries in this field are gradually translated into clinically useful tools. At Poznan University of Medical Sciences Department of Neurosurgery, HMGB1 protein levels and its prognostic potential in subarachnoid hemorrhage patients is currently investigated. Dynamic development of proteomics and minimally invasive brain monitoring will allow us in the future to routinely identify entire proteome (set of proteins) of cerebrospinal fluid in order to accurately predict treatment outcome [1]. Every cell nucleus in our body consists of $5x10^6$ copies of HMGB1 [2]. Loosely bound to the chromatin, HMGB1 is modifying transcription of various genes i.e. for steroid hormones, p53 and nuclear factor κB (NF-κB) [3]. Knock-out rats die shortly after the birth because of massive organ failure and hypoglycemia [4]. HMGB1 as a damage-associated molecular pattern (DAMP) leak out to extracellular space during necrosis to initiate immune response as well as repair processes. It was found that HMGB1 work as a chemoattractant and mitogen [5]. Second release pathway was found in the stimulated immunological cells (macrophages, monocytes), which are capable of active secretion of HMGB1 [6]. Discovery of HMGB1 involvement in pathogenesis of such common diseases like rheumatoid arthritis, sepsis, pneumonia and ischemic stroke demonstrates plurality of functions carried out by this protein [7].

Material and methods

A systematic review of PubMed and Scopus databases was carried out according to keywords: HMGB1, high mobility group box 1, central nervous system inflammation diseases. Articles were selected based on their accuracy and informative character.

The history of HMGB1

HMGB1 was first identified in 1973 as a nuclear protein by Johns and colleagues [8,9]. This most numerous protein in the nucleus was firstly named HMG1, because of its affiliation to high mobility group (HMG) protein family. All its members have in common rapid migration in electrophoretic polyacrylamide gel [10]. HMGB1 coding gene is located on chromosome 13 [11]. Nomenclature revision was carried out in 2001 and new name (HMGB1) was established [12]. In 1991 Heikki Rauvala and colleagues have discovered membrane-bound protein form involved in neurite out growth promotion during fetal central nervous system (CNS) development [13]. Wang and colleagues carried out another significant research in 1991 identifying HMGB1 as a late cytokine mediator of sepsis. Moreover, administration of neutralizing anti-HMGB1 antibodies significantly improved survival in mice [6]. These discoveries triggered great interest to further investigate HMGB1 protein.

The structure of HMGB1

HMGB1 is a 25 kDa protein of 215 amino acids (**Figure 1**). It consists of 2 homologous, helical and positively charged, DNA-binding domains, namely A box and B box. Both of them are partially covered by a negatively charged acidic tail composed exclusively of glutamic and aspartic acids [7]. In the extracellular space B-box is essential to initiate inflammatory mediator production [14]. On the other hand, A-box is an antagonist of B-box [15]. Both domains appeared early in phylogenies, probably even before animalia and plantae kingdoms split, which means HMGB1 is an evolutionary old invention [16]. Numerous acetylation and phosphorylation sites were identified in HMGB1. Some of them are located in nuclear localization signals (NLS), amino acids sequences responsible for nuclear localization of protein [17]. During programmed cell death – apoptosis HMGB1 is also released, but oxidation of cysteine in position 106 prevents it from receptors binding and unnecessary immune system activation [16].

HMGB1 is released during sudden or programmed cell death

Thanks to Wang and colleagues we know that extracellular HMGB1 can initiate immunological response in surrounding cells [6]. There are two major pathways leading to HMGB1 release (**Figure 2**). First, common for all nucleated cells, is a passive leakage of protein caused by cell membrane damage during necrosis. This pathway is characteristic for endogenous damage-associated molecular pattern molecules (DAMPs) and can initiate sterile inflammation around necrotic tissues i.e. in ischemic stroke [18]. Second pathway of HMGB1 release is an active secretion from immunological cells (macrophages, neutrophils, microglia) but also from other types of cells (endothelium, astrocytes). This pathway is activated in infectious inflammation i.e. sepsis, where HMGB1 release is triggered by LPS stimulation [19]. It turned out that HMGB1 released passively and actively vary in structure, the actively secreted protein undergoes acetylation and phosphorylation prior to release, while the passively leaked out protein is non-modified [20]. LPS stimulation is activating cytosolic enzymes responsible for these modifications. Constant circulation of HMGB1 between nucleus and cytosol (with strong nuclear predominance) is interrupted and HMGB1 is redirected to the extracellular space. Modified by activated enzymes, HMGB1 is secreted in unique fashion by secretory lysosomes [17]. Extracellular HMGB1 binds with the three main types of receptors i.e. receptor for advanced glycation end products (RAGE), Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) [21]. RAGE receptor is expressed on surface of macrophages, monocytes, microglia, neural and endothelial cells [22]. These types of cells are numerously represented in CNS. Binding of HMGB1 and other ligands to RAGE receptor, like S100 protein family members, results in chemoattraction, growth stimulation, immune cell differentiation and increased expression of

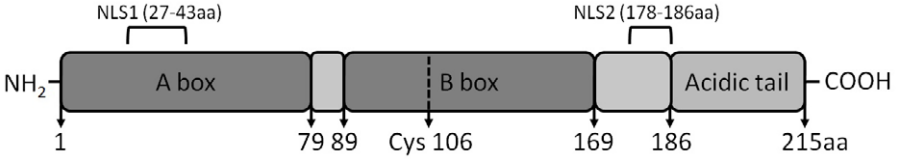

Figure 1. The structure of HMGB1. HMGB1 is a protein of 215 amino acids organized in two positively charged DNA binding domains (the A and B boxes) and a negatively charged acidic C-terminal tail composed of the aspartic and glutamic acids. The two nuclear localization signals susceptible to posttranslational modifications are also depicted. HMGB1 receptors binding affinity depends on oxidation of cysteine in position 106. Abbreviations: aa, amino acid; NLS, nuclear localization signal

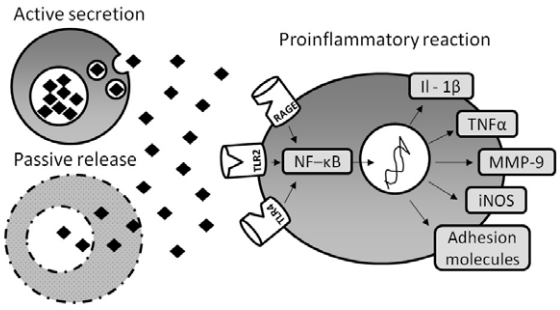

Figure 2. Major HMGB1 release pathways, target receptors and triggered reactions. Active secretion of HMGB1 from immunological (macrophages, neutrophils, microglia) and other types of cells (endothelium, astrocytes) is triggered by infectious as well as sterile stimulants (DAMP). Passive leakage of protein is caused by cell membrane damage during necrosis. Binding with three main receptors (RAGE, TLR2 and TLR4) leads to secretion of proinflammatory cytokines (Il – 1β; TNFα), upregulation of adhesion molecules, metalloproteinases (MMP-9) and nitric oxide synthase. All those reactions are mediated by activated nuclear factor κB. Abbreviations: DAMP, damage-associated molecular patterns; RAGE, receptor for advanced glycation end products; TLR, Toll-like receptor; Il, interleukin; TNF, tumor necrosis factor; MMP, matrix metalloproteinase; NF-κB, nuclear factor kappa B; iNOS, inducible nitric oxide synthase

RAGE and TLR2 receptors [23]. Toll-like receptors are pivotal for innate immunity. They recognize pathogens associated molecular pattern molecules (PAMPs). Their activation leads through NF – κB pathway to cytokine production and cell activation [24]. Inside nucleus HMGB1 carry out structural functions. Along with histone H1, HMGB1 regulates access to the genetic material and expression patterns of proteins [25, 26].

HMGB1 in central nervous system

HMGB1 is mainly investigated for its damaging impact on CNS, but this protein also mediates physiological processes in brain tissue. HMGB1 was found to be essential for proper forebrain development in rats. Interactions between HMGB1 and RAGE receptors are crucial for axonal sprouting and neurite out growth [13]. On the other hand, intraventricular injection of HMGB1 in rats causes general sickness syndrome manifested by fever, allodynia, change in animal behavior and weight loss due to anorexia [27]. Increase of interleukin – 1β (Il-1β) and tumor necrosis factor α (TNF-α) concentration in brain tissue is another consequence of HMGB1 administration [28, 29]. HMGB1 takes part in pathogenesis of many neurological disorders. Most common of all CNS pathology, ischemic stroke is extensively investigated for HMGB1 involvement. In primary cultures of mouse neurons and glia cells, HMGB1 was found to promote chronic neuroinflammation mediated by NF – κB pathway. Same cell cultures under glucose and oxygen deprivation release HMGB1 to the extracellular space [30]. Increased level of HMGB1, leaked out form dying cells, was found in rats cerebrospinal fluid and plasma as well as in stroke patients plasma [31, 32]. Also, HMGB1 promotes glutamic acid (excitatory neurotransmitter) release from gliosomes as well as sensitizes neurons to the glutamate mediated injury *in vitro* [30, 33]. Glutamate in high concentration causes uncontrolled calcium influx to neurons and protease, nuclease and phospholipase activation. This process called excitotoxicity is crucial for ischemic stroke pathogenesis [34]. Immune residual cells of CNS – microglia are considered to be the main source of proinflammatory cytokines released during ischemia. HMGB1 was found to trigger synthesis of matrix metalloproteinases (MMPs) and nitric oxide [35, 36]. Also astrocytes, activated by HMGB1, secrete inflammatory mediators i. e. TNF-α [32, 37, 38]. CNS is supplied by a dense system of blood vessels. Endothelial cells respond to HMGB1 stimulation by enhanced expression of intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin. This process promotes transendothelial migration of inflammatory cells and blood-brain barrier leakage [39]. Macrophages located around vessels and inside meninges respond to HMGB1 stimulation in the same fashion as microglia cells i.e. by producing proinflammatory cytokines, proteolytic enzymes and migration towards increasing HMGB1 concentration [40]. HMGB1 is involved in pathogenesis of chronic diseases like Alzheimer's disease (AD). High concentration of this protein was found in hippocampus in AD animal model. Combination of HMGB1 with β-amyloid prolongs lysis of beta amyloid [27]. Elevated HMGB1 levels in serum and cerebrospinal fluid were found among patient suffering from meningitis [41], subarachnoid hemorrhage [42, 43] or CNS trauma [44].

HMGB1 in therapy

There are two main directions in therapy targeting HMGB1, prevention of protein release from cell or blockade of HMGB1 binding with receptors. According to those two pathways, therapeutic agents may be divided into two groups. Inhibition of active secretion is characteristic for ethyl pyruvate, epigallocatechin gallate (EGCG) and oxaliplatin [45, 46]. Second and more numerous group of binding inhibitors include one of HMGB1 domains (A box), which is antagonistic to proinflammatory B box. Other members of this group are the soluble version of RAGE receptor (sRAGE), glycyrrhizic acid, simvastatin and atorvastatin [23]. Apart from CNS diseases, HMGB1 targeting therapies are already investigated in treatment of septic shock. Main advantage of anti-HMGB1 agents in treatment of this condition is their delayed administration [47]. Also recombinant A box, anti-RAGE antibodies as well as EGCG were proposed as effective agents in combating this lethal disease in animal model [48]. Ethyl pyruvate administration ameliorated colitis in mice and reduced intestinal cytokine production [49]. Single injection of cisplatine prevented HMGB1 secretion and caused transient symptom amelioration in collagen type II inducted arthritis (animal model of human rheumatoid arthritis) [45]. CNS diseases therapy is mainly focused on ischemic stroke. After inhibition of HMGB1 expression or by HMGB1 neutralization with antibodies, reduction of stroke tissue area was achieved in rats brain [50]. Also atrovastatin administration was found to be efficient in necrosis prevention [51]. Therapy with anti-HMGB1 antibodies was investigated in traumatic brain injuries [52]. The same agent was used efficiently in experimental treatment of multiple sclerosis in animal models. Amelioration of symptoms and slower progression of disease was achieved [53].

Conclusion

HMGB1 is an abundant and multifunctional protein strongly involved in both acute and chronic inflammation. Location and posttranslational modifications of this protein determine its function. HMGB1 may be a structural nuclear protein or extracellular mediator of sterile and infectious inflammation. Hopefully, the knowledge we are now gathering will help us in the future to design new therapies.

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Conflict of interest statement

The authors declare that there is no conflict of interest in the authorship or publication of contribution.

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Chapter in the book

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