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Eur. Pharm. J. 2019, 66(1), 1-3.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.2478/afpuc-2019-0005

EUROPEAN PHARMACEUTICAL JOURNAL

Total DNA methylation in the brain in response to decitabine treatment in female rats

Original research article/Review

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Received 18 March, 2019, accepted 30 May, 2019

Abstract Hypomethylating agent decitabine is being used in the treatment of certain types of leukaemia in combination with other anticancer drugs. Aberrant DNA methylation has been suggested to occur in pathological states including depression. Scarce data in male rats suggest antidepressant effects of decitabine. The main aim of our studies is to test the hypothesis that the inhibition of DNA methylation results in antidepressant effects in female rats. Before doing so, we decided to verify the effects of decitabine on DNA methylation in females. The findings demonstrate that the treatment with decitabine at the dose shown previously to inhibit DNA methylation in males, had no effect on total DNA methylation in two brain regions, namely the hippocampus and frontal cortex of female rats. In conclusion, the present study allows us to suggest that the effect of decitabine on DNA methylation in the brain is sex dependent.

Keywords *5-aza-2′-deoxycytidine – epigenetics – gender*

INTRODUCTION

Decitabine is an analogue of the pyrimidine base cytosine and it has a chemotherapeutic potential given by the ability to inhibit DNA methyltransferases (Zhou et al., 2018). DNA methylation is an epigenetic mechanism catalysed by DNA methyltransferases, which occurs by covalent attachment of methyl groups to the 5-position of cytosine residues of DNA, especially at the cytosine-guanine dinucleotides. Decitabine incorporates into nascent DNA strands during DNA synthesis, while irreversibly binding DNA-methyltransferases and inactivating them (Malik and Cashen, 2014). Increased DNA methylation is generally associated with the attenuation of the extent of gene expression. Recent studies have shown that the inhibition of DNA methylation may induce antidepressant-like effects in male rats. Sales and colleagues (2011) treated healthy male rats kept under standard conditions with different doses of decitabine and they reported antidepressant effects of repeated injections at the dose of 0.4 mg/kg.

The main aim of our studies is to test the hypothesis that the inhibition of DNA methylation results in antidepressant effects in female rats. Before doing so, we decided to verify the effects of decitabine on DNA methylation in females. We used the same dose described to be effective in male rats (Sales et al., 2011), but unlike these authors, we were working with rats exposed to low tryptophan diet known to induce depression-like behaviour.

MATERIAL AND METHODS

Female adult Sprague-Dawley rats were fed normal or low tryptophan diet for 10 days. The low tryptophan and control diets contained 0.04% and 0.2% of tryptophan, respectively (Franklin et al., 2015). Animals exposed to low tryptophan diet were treated either with decitabine (5-aza-2′-deoxycytidine) at the dose of 0.4 mg/kg or vehicle on day 8 and 9 of tryptophan depletion. Decitabine was injected intraperitoneally three times with the time interval of 5 h between the 1st and 2nd and 18 h between the 2nd and 3rd injection. On day 10 of tryptophan depletion, the animals were quickly decapitated and their organs were collected. All the procedures were

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approved by the Animal Health and Animal Welfare Division of the State Veterinary and Food Administration of the Slovak Republic.

After the isolation of DNA from the hippocampus and the frontal cortex, the DNA methylation was quantified using the DNA Methylation enzyme-linked immunosorbent assay (EIA) kit (Epigentek, USA), according to the manufacturer's instructions. The results were analysed by one-way ANOVA.

RESULTS

The treatment of female rats with decitabine had no effect on the total DNA methylation in the hippocampus. Similarly, there were no changes in DNA methylation in the frontal cortex of decitabine treated rats. The analysis by one-way ANOVA did not show significant effect of treatment either in the hippocampus ($F(2, 17) = 0.28695$, $p > 0.05$) or in the frontal cortex ($F(2, 17)=0.14242$, $p > 0.05$). Ingestion of low tryptophan diet was not associated with the changes in total DNA methylation (Fig. 1).

DISCUSSION

The present study was designed to verify the efficacy of decitabine to inhibit DNA methylation in female rats before investigating the behavioural consequences of decreased DNA methylation. The findings demonstrate that the treatment with decitabine had no effect on total DNA methylation in two brain regions. Obviously, present experimental design is not appropriate for further investigation of the impact of reduced DNA methylation on depression-like behaviour.

Hypomethylating agent decitabine is being used in the treatment of certain types of leukaemia in different doses and in combination with other anticancer drugs (Tiong and Wei, 2019). Aberrant DNA methylation has been suggested to occur in numerous disease states, including cancer, autoimmune diseases and neurodegenerative disorders (Kader et al., 2018). Depressive disorder in humans is also associated with changes in epigenetic mechanisms including DNA methylation. In particular, increased DNA methylation levels on selected genes, such as brain-derived neurotrophic factor (BDNF) gene, have been reported in patients with depression (Chen et al., 2017).

It may be argued that no dose response relationships were evaluated in the present experiments. The reason is that this was done previously by others (Sales et al., 2011), who investigated DNA methylation and behavioural parameters in male rats treated with 6 different doses of decitabine in the range of 0.1–0.8 mg/kg. According to their results, an optimal dose was the one used in the present study, namely 0.4 mg/kg. This dose of decitabine, described to induce the inhibition of DNA methylation in males (Sales et al., 2011) was ineffective in the present study in females. Surprisingly, very little

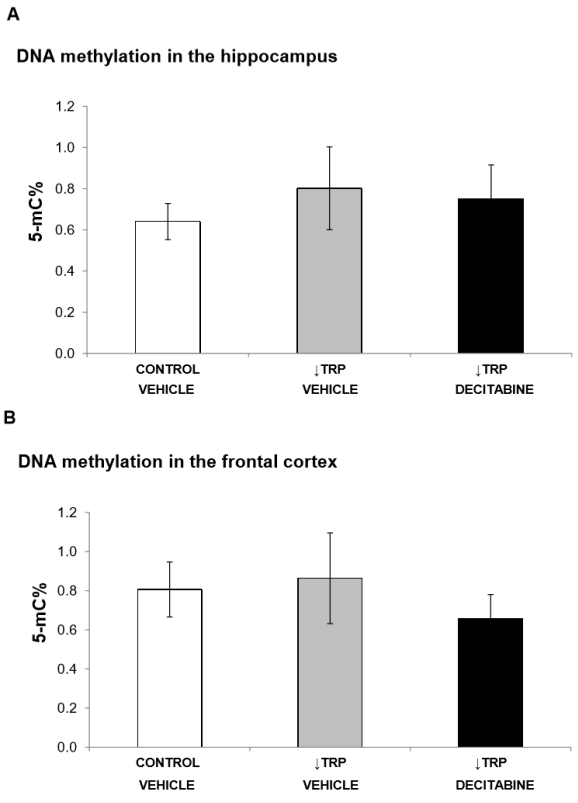

Figure 1. Total DNA methylation (A) in the hippocampus and (B) in the frontal cortex in female rats fed low tryptophan diet and treated with decitabine at the dose of 0.4 mg/kg or vehicle

information is available on potential sex differences in the action of decitabine or in total DNA methylation. There is a study in healthy humans showing reduced level of global methylation in women (Zhang et al., 2011). DNA methylation of an exon of the gene coding for BDNF in the prefrontal cortex was found to be higher in adult female compared to male rats maltreated during the development (Balze et al., 2013). Perinatal hypoxia-induced DNA methylation of certain genes in the hearts of adult offspring was lower in female compared to male rats. In vivo effects of decitabine treatment were not evaluated (Patterson et al., 2010).

Even though no information that allowed prediction of sex differences in the pharmacokinetics or pharmacodynamics of decitabine was available, the present study suggests that the effect of decitabine on DNA methylation in the brain is sex dependent.

ACKNOWLEDGEMENTS

The study was supported by the grant of Slovak Research and Development Agency under the contract No. APVV-15-0388 and VEGA 2/0042/19.

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Eur. Pharm. J. 2019, 66(1), 4-10.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.2478/afpuc-2019-0001

EUROPEAN PHARMACEUTICAL JOURNAL

Legal regulation of drug advertising and its restrictions in the conditions of the Slovak Republic

Original research article/Review

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Received 23 April, 2018, accepted 16 November, 2018

Abstract The question of drug availability is a key requirement for each country. Their deficiency can cause fatal consequences for the health of the population. For this reason, the production and distribution of medicines represents the economic potential of the state, which is also protected and regulated in the Slovak Republic. Drug distribution is also part of every market economy as it is the primary form of business-to-customer (B2C) offering. At first glance, the promotion of drugs might seem to be just marketing. But this area is under the scrutiny of the Slovak legislations. From the point of view of the systemic nature of law, advertising of medicinal products is regulated both in public law and private law. This is particularly the area of administrative law, commercial law and civil or criminal law, which must respect the often complicated penetration of European law into national law. The issue of ad management and the associated availability of medicines, in our terms, is at the centre of public interest. The main aim of the authors in this paper is to examine not only the European but especially the national legal regulation of the advertising of medicines in the context of the decision-making activity of the Slovak authorities supervising compliance with the legal restrictions on the promotion of pharmaceutical products. Another goal is to identify the problems in application practice and to propose ways to eliminate identified shortcomings by specific procedures. The authors, through scientific and doctrinal interpretation, examine selected statutes of the Law on Advertising and related legislation pertaining to the issue of drug advertising. Through expert literature, jurisprudence and the decision-making processes of the administrative authorities, they seek answers to practical application problems. At the end of the contribution, they critically analyse the identified shortcomings and propose appropriate measures to eliminate them.

Keywords *Advertising – management – medicine – restriction*

INTRODUCTION

The need to apply the principles of the market economy in the further development of Slovak economy raises the demand for information, knowledge and experience from functioning market economies. The underlying idea is to find ways, methods and procedures to achieve this goal. Therefore, there is an increasing need for marketing, used by business entities to further their business. Marketing is considered to be a particularly effective approach to finding optimal effects, and thus to achieving the goals of entrepreneurs. Targets are different, for example from the expansion of certain opinions on smoking, to the most common and most pronounced, aimed at maximizing business profits. Advertising is one of the main forms of communication and its role is to make a product or service aware to the customer, to distinguish them from other offers and in particular to convince the customer to buy the product. Successful advertising is, therefore, according to Payne (1996) one of the key factors in the success of marketing policy.

The primary legal regulation of advertising is found in the Constitutional Law of Slovak National Council no. 460/1992 Coll. The Constitution of the Slovak Republic as amended (hereinafter „the Constitution"). In particular, advertisement

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concerns Article 55 of the Constitution, which states that the economy of the Slovak Republic is based on the principles of a social and environmentally oriented market economy, while it protects and promotes competition. The last sentence of Article 55 refers to a more detailed adjustment of this issue in separate laws. The limitation of the guarantor's right to advertisements is only permissible for the prevention of unfair competition and the dissemination of false or misleading information.

Legal regulation of advertising can also be found in several European Union legislations, of which the Directive of the European Parliament and of the Council of 6 November 2001 no. 2001/83 / EC on the Community code relates to medicinal products for human use (hereinafter referred to as the Community Code). A special place also belongs to the European Parliament and Council Directive no. 2006/114 / EC of 12 December 2006 on misleading and comparative advertising (the Misleading and Comparative Advertising Directive).

From the point of view of national regulations, advertising is primarily regulated by Act no. 147/2001 Coll. on advertising and on the amendment of certain laws (hereinafter referred to as the „Advertising Act"), or in Act no. 513/1991 Coll. Commercial Code as amended (hereinafter referred to as the „Commercial Code"). The advertisement distributed in radio and television broadcasts is modified separately, in Act no. 308/2000 Coll. on Broadcasting and Retransmission as amended (hereinafter the „Broadcasting and Retransmission Act"). The advertising of pharmaceutical products (although according to the authors of this article) is regulated by 362/2011 Coll. on Medicinal Products and Medical Devices and on Amendments to Certain Laws, as amended (hereinafter the „Medicines Act"). In this case, it is mainly about adjusting the sponsorship of professional events or providing forbidden rebate.

In developed countries where advertising operates in a stabilized liberal market economy environment, the form of ethical codes has stabilized to regulate advertising ethics. Although they are legally binding rules on ad management, they are respected by special associations bringing together businesspeople in the field of advertising.

OBJECTIVE OF THE CONTRIBUTION AND METHODOLOGY

The main purpose of the authors of this contribution is to examine the European and national regulations on advertising of medicines in the context of the decision-making activities of Slovak administrations supervising compliance with the legal restrictions on the promotion of pharmaceutical products. Another goal is to identify problems in application practice, especially when deciding administrative authorities, and to propose ways to eliminate identified shortcomings by specific procedures.

Due to the nature of this contribution, several scientific methods of knowledge are applied, especially the logic method that can be used in all sciences. Furthermore, it is a method of abstraction, without which the post could be unobtrusive or chaotic in view of its wide range. The method of logical analysis as well as the analytical method for analysing the legal status and regulation of drug advertising is the necessary method for successful processing of the given issue. By using a comparative method, it is possible to gain knowledge and views on the legal regulations and the interpretation of individual institutes. Based on the scientific knowledge of valid and effective law and legal science, the doctrinal interpretation has also been used in some parts of the work.

In Slovakia, the subject of advertising has come to the forefront of legal science. By studying available literature, it is possible to conclude that there is not only professional literature, but also journalistic contributions in the field of legal regulation of advertising. In particular, journalist Tyrolová (2007), Vozár (2006) and the team of authors Siminská, Šimeková, Gyarfaš (2013), which are already out of date due to the rapid development of legislation in some parts, can be mentioned. In order to achieve the stated goal, it is necessary to review and provide a brief analysis of the decisions of the State Institute for Drug Control (SIDC) and the Ministry of Health of the Slovak Republic.

ADVERTISING LAW

The original legal basis for the adaptation of the general advertising requirements as well as the supervision of advertising was contained in Act no. 220/1996 Coll. about advertising effective until April 30, 2001. During the four years the law was in force, many shortcomings have emerged. The very definition of advertising itself was problematic; shortcomings appeared in restrictive measures to advertise some products, but also to supervise compliance with the law.

A relatively serious problem with regard to advertising law no. 220/1996 Coll. about advertising in Slovakia in 2000 arose in connection with the preparation of the European Code on Advertising, which made this law absolutely unsuitable for the current needs of regulation of the advertising market. In view of the adoption of several directives governing other areas of advertising, due to the need to implement European law in the Slovak legal order, it was abandoned because an amendment to the law was being considered. The result was the adoption of an effective law on advertising, where the legislation was based on four basic principles, such as legality, honesty, truthfulness and decency. Based on these basic ideas, general advertising requirements for products such as medicines and infant formula are formulated with specific terms and limitations set in advertising. It can be said that the legislator's intention was not to fully regulate the whole area of advertising by this law; they may also have retained the parts of the Law on Medicines, the Broadcasting and Retransmission Act or the Commercial Code governing

certain legal institutes and the dissemination of advertising in certain media.

As regards its formulation, the Advertising Act is divided into three articles, with the focus of the legal regulation contained in the first article. The opening clause of Section 1 of the Act contains its purpose in four areas: the general regulation of advertising requirements, the conditions for the advertising of certain products, the adjustment of legal protection against the effects of misleading advertising and comparative advertising in a form that is inadmissible, as well as the scope of the supervisory authorities compliance with the provisions of the law.

The Statute of Paragraph 2 of the Act defines the basic concepts that are most important from a regulatory point of view. This is advertising that the legislature defines as a presentation or other communication in any form relating to commercial, business or other gainful activity in order to put products on the market. For comparison, some authors (Kotler, 1992) in the field of marketing explain this term differently. By advertising, I understand every paid form of non-personal presentation and support of ideas, products or services paid by an identifiable sponsor.

The basic advertising criteria are based on competition law as well as the principles of ethics and consumer protection. In the first place, according to Olšovská et al., (2015), advertising must not be deceptive. This fact is considered in terms of its content, data, characters and information. These features are assessed in particular with respect to the people to whom they are assigned or who are affected. It is also unacceptable that, as a result of misleading content, addressees are misled to influence their economic behaviour on the market or even damage them.

The definition of misleading advertising is contained in Paragraph 45 of the Commercial Code, which modifies the misleading advertising in detail. In the view of the Supreme Court of the Slovak Republic (2000) no. 5Obo 138/2000, misleading advertising is one of the signs of unfair competition. In his opinion, it must be an advertisement capable of provoking an idea that is inconsistent with reality, with the possibility that it might come to deception. In addition, the competitor's conduct must be the ability to gain a competitive advantage at the expense of another competitor.

However, the definition of ad distribution, which the law describes as a natural person or a legal entity that propagates without further explanation, is problematic. According to some authors (Siminská et al., 2013), the dissemination of advertising of medicines (as opposed to the dissemination of other advertising) does not have to be the person who acts in his business activity. In their opinion, they rely on the decision of the Court of Justice of the European Union (2009) in Daamgard. The question was whether the concept of advertising of medicinal products also involved the dissemination of information by a person who is legally and effectively independent of the manufacturer of the medicinal product. The Court of Justice of the European Union stated, inter alia, that, in principle, it is not excluded that the dissemination of information by a person independent of the manufacturer of the medicinal product falls under the definition of advertising, not necessarily business conduct. A similar issue was dealt with in 2011 by the State Institute for Drug Control (2011) in connection with the advertising of medicines on railway wagons. It then decided that the advertising of medicinal products and the person sanctioned by the unlawfulness of such advertising may not be the holder of the registration of the medicinal product. However, it is essential that advertisers have a promotional intent.

In the context of the general advertising requirements contained in Statute 3 of the Advertising Act, advertising is restricted by prohibiting its appearance, which in its content violates the values protected by law. It is about protecting human life, health, the environment, freedom, conscience, socially recognized morality, property protection and so on. Particular emphasis is placed on advertising that affects minors, especially in connection with incitement to such behavior that may endanger their health, mental or moral development as well as displaying them in dangerous situations. Such limitations, in our opinion, are based on traditions and experience gained during ad development. In addition, they have a common international standard that is included in our terms and conditions in the Advertising Code of Ethics issued by the Advertising Advice Board as an association of advertising agencies in the Slovak Republic.

Additionally, the Advertising Act does not allow the advertisement to include personal data, data on the property of individuals without their prior consent. However, advertising itself to a particular addressee is not contrary to that provision, since the necessary personal data used to deliver the ad to the addressee are not part of the content of the ad as defined and are only attached to the ad.

For advertising by certain media, such as an automatic telephone answering system, telefax and e-mail, prior consent of their users is required. The purpose of such a limitation by the legislator is to prevent harassment and interference with the privacy or property rights of recipients of advertising. In the case of other means of communication, it must not be distributed directly if the addressee has refused delivery in advance. However, the method of refusing ad delivery is not defined in the law, which in practice means that the addressee can do it in any form (Dulová Spišáková et al., (2017). In real life, it's about marking the mailbox so the advertiser does not miss the ad. As a less practical and less used way of declining advertising is written notice to the postman or advertising agency. In this matter, the State Institute for Drug Control in 2014 began administrative proceedings in the matter of unlawful drug advertising in 2010. The reason for the opening of the proceeding was the form of advertising that was an imitation of a recipe. The registration certificate holder objected to the claim that the 2010 flyer, apart from the size, had nothing to do with the prescription. Different from this, for example, „Doctor Recommendation" or „Patient Reimbursement". Perhaps, in the opinion of the

administration, it was also a hidden advertisement. However, the administrative authority could not decide on the matter because more than three years had passed since the breach of the law and the administrative procedure had to be stopped (State Institute for Drug Control, 2014).

Advertising may not be distributed if it is inconsistent with good morals and if it presents products whose production, sale, supply or use is prohibited or if it does not meet the requirements of a specific regulation.

The original law on advertising did not allow for mutual comparison of products in advertising because, as is apparent from the explanatory memorandum, the proposer of the law was based on the principle of decency. At that time, it was not appropriate for anyone in the competition to deal with the other entrepreneur's affairs and to interfere with his interests without any special reason and compulsion. Allowing comparative advertising is understood by some authors (Ovečková et al., 2017) as a breakthrough of this principle, which was necessary especially in the context of harmonization of our law with European Union law, which allowed comparative advertising in Article 4 of the Misleading and Comparative Advertising Directive. In particular, due to the sensitivity of competition, special provisions are laid down in Statutes 4 of this Act. This means that comparative advertising is only allowed in a legally defined framework and other comparative advertising is inadmissible.

DRUG ADVERTISING

Medicines are special products that have a significant impact on human health and therefore their advertising required specific provisions and adjustments in the advertising law. Strictly restrictive advertising measures affect certain groups of medicines, those that are issued only on prescription or which are covered by health insurance and medicines that are not registered in the Slovak Republic. Medicines should not be presented in advertising to encourage their excessive use or use without the need for medical examination.

In accordance with Article 86, Paragraph 1of the Medicines Directive, advertising of medicinal products under Paragraph 8, Section 1 includes„any form of under-information, agitation or incitement to promote the prescription, delivery, sale or consumption of medicinal products" under-the-counter notification, agitation or incentives to promote prescription, delivery, sale or consumption of the drug. According to the Directive, the advertising of medicinal products also means, in particular, advertising to the general public, the advertising of medicinal products to persons qualified to prescribe them, or the issue of, visits to medical representatives responsible for the distribution of medicinal products to prescribers, the supply of samples. The national provisions essentially reproduce the wording Directive on the advertising of medicinal products (Tyrolová, 2007).

The provision of Statute 8, Paragraph 3 of the Advertising Act exhaustively lists what cannot be considered as their advertising. This includes, for example, labeling, written information for its users, correspondence that may be supplemented with material of a non-promotional nature needed to answer a specific question about the medicine, reference material and information on, for example, changes to the packaging of the medicine, to warn of undesirable effects pharmacovigilance or business catalogue and price list on the condition that it does not contain any information on medicinal products, information relating to health or human illness, unless it contains a direct or indirect reference to the medicinal product or disclosure of information containing only the name and price of the medicinal product or the medicinal product.

Article 88 of the Medicines Directive requires Member States not to allow the advertising of selected types of medicinal products. This restriction is also passed to the Advertising Act. It is unacceptable to promote medicines not registered in the Slovak Republic. The same prohibition applies to drugs containing narcotic drugs, psychotropic substances and preparations whose expenditure is bound by medical or veterinary prescriptions. The limitation also applies to medicinal products whose expenditure is not subject to medical prescription but is reimbursed on the basis of public health insurance. An exception to this ban is vaccination campaigns organized by the marketing authorization holder or the marketing authorization holder of the marketing authorization. However, the exemption is subject to the consent of the Ministry of Health of the Slovak Republic.

With regard to the decision of the State Institute for Drug Control no. 580/2014/600, it must be pointed out that advertising of medicinal products must encourage their rational use on the basis of objective information on the characteristics of the medicinal product without exaggerating its effect. The holder of the registration certificate, in violation of this obligation in the advertisement, has encouraged the use of such a medicine, which is explicitly intended for emergency situations only. For these reasons, the Supervisory Authority, by its decision, prohibited the dissemination of advertising and imposed a fine of EUR 10,000 on the infringer (State Institute for Drug Control, 2014). The violator appealed against the decision of the first-instance body. The Ministry of Health of the Slovak Republic (2014), as the appeal body, has confirmed the ban on advertising. However, the fine of EUR 10,000 was reduced to EUR 5,000 on the ground that the offender was the first offense.

Accordind to Benda-Prokeinova et al. (2017), other restrictions relate directly to the distribution of medicinal products intended for the public for the purpose of advertising, as well as the visits of physicians during their surgery hours to promote medicinal products. The physician is forbidden to accept these people during their office hours.

The provision of Statute 8, Paragraph 9 of the Advertising Act, which excludes advertising of medicinal products intended

for the public, may be considered to be of key importance, for example, giving the impression that medical examination or medical treatment is unnecessary, whether it offered a diagnosis or a method of treatment by correspondence. Medicines must not give the impression that their effects are guaranteed and are not accompanied by any undesirable effects or are better than or equal to the effects of another medicinal product. The rigidity of the legislation is also sharpened by sanctioning any indication in advertising that good health could be improved by taking the medicine or by indicating that a person's good health might be affected by not taking the medicine; this ban does not apply to the above-mentioned vaccination campaigns, addresses exclusively or mainly children.

In the case of drugs being promoted to people authorized to prescribe and issue them, the law prohibits them from supplying, offering and promising donations, monetary and material benefits or any other benefit. According to Žulová et al. (2018), the subject of legal regulation is also the possibility of attending promotional events, which must be strictly limited to its purpose and can only be provided to healthcare workers. However, in the opinion of the legislator, the law does not preclude direct or indirect treatment for events exclusively for professional and scientific purposes. However, the condition must be fulfilled that such a treatment will always be strictly limited to the main scientific purpose of the event and will not be given to other people as a healthcare worker.

Samples of drugs, other than those containing narcotic drugs and psychotropic substances, may be provided only on written request. The authorized entity is the person authorized to prescribe medicinal products. However, it may be provided with a maximum of two samples of the smallest package of the registered medicinal product per year, labeled „FREE SAMPLE – NOT FOR SALE" and the summary of product characteristics. These samples are also subject to control and registration by the marketing authorization holder.

Paragraph 8, Section 22 of the Medicines Act also places the holder of the marketing authorization decision on other obligations. This is, for example, the creation of a scientific service responsible for information on the medicines it puts on the market. In addition, he must make available or hand over to the State Institute for Drug Control a sample of each advertisement coming from his business, together with a statement of the intended person, of the method of distribution and the date of commencement of dissemination, of ensuring compliance of the advertising of his company's medicinal products with the requirements of advertising law and others. In practice, the question arises as to whether advertising regulation also applies to homeopathic medicines. The answer is in Paragraph 24, which clearly states that the Advertising Act also applies to this category of medicinal products, with the exception of medicinal products not registered in the Slovak Republic. However, only the information and data approved at the time of its registration may be used in the advertisement of homeopathic medicinal products.

PERFORMING SURVEILLANCE AND SANCTIONS FOR VIOLATING THE LAW

The competence of state supervision of compliance with the provisions of the Act on advertising in connection with the advertising of medicinal products is entrusted to the State Institute for Drug Control and the Institute for State Control of Veterinary Bioproducts and Medicines in the Supervision of Advertising of Veterinary Medicines. The Public Health Office of the Slovak Republic and the regional public health authorities supervise the advertising of cosmetic products as well as selected foods or infant formula, nutritional supplements and other products (Vozár, 2006).

The specific procedure of the supervisory authorities consists of measures they are imposing to ensure redress. First of all, if they decide to prohibit advertising, they are entitled to require from the advertiser to submit evidence of truthfulness of factual data in case of suspicion of misleading or inadmissible comparative advertising. The called entity is legally obliged, upon request, to provide proof to the supervisor on the truthfulness of factual advertising data within 15 days of receipt of the notice. If the proof is not submitted or is inadequate, the legitimate irrefutable assumption is that the comparative advertising is inadmissible and follows the imposition of a sanction by the administration. It is particularly important to have the authority to impose on the advertiser the obligation to post the decision of the supervisory authority if it finds a violation of the law. We emphasize that the authority to ban advertising has a supervisory authority even if „only" threatens to be an inadmissible comparative advertisement (Meszároš, 2018). This is the chosen method of administrative action.

In addition to such measures, the administrative body shall impose compulsory fines, the amount of which shall be graduated. The minimum fine of up to €1660 penalizes a person entitled to prescribe or dispense drugs if they violate the prohibition to receive a gift, a monetary or material advantage or other benefit. A fine of €3320 may be imposed on the infringer if the ad does not meet the requirements for public speaking, does not observe the principles of linguistic culture, grammar and spelling rules or steady professional terminology. For an ad containing anything that denigrates human dignity offends national feeling or religious sentiment, as well as any discrimination based on sex, race and social origin, the amount of the fine is set at EUR 66,400. The maximum fine of up to EUR 166,000 may be penalized by the infringer for inadmissible comparative advertising or for the advertisement of unregistered medicines, drugs containing addictive and psychotropic substances, or medicines bound by a medical or veterinary prescription.

When imposing a fine, the supervisory authority makes use of the opportunity of proper consideration while taking

into account the seriousness, duration, consequences of the unlawful act and whether it is a repeated violation of this law. However, the possibility of imposing a fine is subjectively time-limited for one year from the day the law enforcement authority became aware. The objective time was set for three years from the day of the violation. In practice, this means that the decision to impose a sanction must be valid within one year of the Office having learned of the breach of the sanction. This procedure is also based on the decision of the Supreme Court of the Slovak Republic (2011) no. 5Sžo 41/2011, according to which, if administrative authorities fail to impose a fine within one year, proceedings under Article 30 of the Administrative Procedure Code should be stopped. However, the supervisory authority must decide no later than three years after the unlawful act has been committed. Otherwise, proceedings must be stopped and the guilty party remains uncontrolled. The fine imposed is payable within 30 days from the date on which the decision to is valid. It is revenue to the state budget. In addition to the provisions of Paragraphs 10 and 11 of the Advertising Act, the Statutes of Act no. 71/1967 Coll. on Administrative Procedure (Administrative Procedure), as amended, which is a lex specialis of administrative procedural law.

If general advertising requirements are violated, e.g. the spread of misleading advertising, and inappropriate comparative advertising or advertising requirements for certain products, sanctions are increased. Chovancová (2016) finds that the higher rate of fine in this case is justified, since misleading and inadmissible comparative advertising is a serious violation of recognized competition principles, while deceptive advertising seriously damages the consumer.

RESEARCH

As part of the comprehensive processing of the examined issue, we contacted the State Institute for Drug Control. Our aim was to analyse its decision-making activity for the period from 2013 to 2017. We found that in this period, the infringement proceedings started only 36 times. The reason was the actual finding of a potential breach of the holder of the certificate. Only in isolated cases, the administrative authority started proceedings at the initiative of a third party.

Year	2013	2014	2015	2016	2017
Number of decisions of SIDC in certain year	9	18	5	3	1
SIDC decided that the law was not violated	0	5	2	0	0
SIDC stopped proceedings	7	11	2	1	0
SIDC banned ad and imposed fine	2	2	1	2	1

The analysis of the decision shows that the decision-making process was the year 2014, when the state instituted only 18 cases of possible violation of the law. On the other hand, however, in 11 cases, the proceedings stopped because of the fact that the act was time-barred. In only two cases, state decided to prohibit advertising and imposed a fine. In the following years, the number of proceedings started to sharply decline, and in 2017 it was only deciding on a single violation of the law on advertising. As a result of the administrative procedure, the ban on advertising was imposed and a fine was imposed.

CONCLUSION

The result of the research was the revealing of certain flaws in the legal regulation of drug advertising, which can cause difficulties in practice. In our opinion, the legislator did not choose the appropriate way to adapt this commodity advertisement when the advertising of drugs was regulated in Paragraph 8 of the Advertising Act, but the promotion of other pharmaceutical production is included in the Medicines Act. We also see the problem that the Medicines Act in some cases interferes with the regulation of drug advertising without respecting its terminology. As an example, we mention the modification of sponsorship of professional events in the Medicines Act. This ignores the fact that support for scientific congresses falls under the definition of drug advertising and is regulated in the Advertising Act. We also have a negative perception of including drug samples under the definition of discounts in the Medicines Act, while drug samples are tax-deductible in the advertising law.

However, we have found that the legislator also caused another problem by entrusting the supervision of advertising to two different bodies. The first-degree supervisory authority under the Advertising Act is the State Institute for Drug Control and under the Medicines Act it is the Ministry of Health of the Slovak Republic. Probably only theoretically, a situation could arise that the holder of the certificate, by supporting a particular event, fulfills the factual nature of an administrative offense under the Medicines Law and also under the Advertising Act. In our view, they could be penalized for violating both laws and two different bodies. In practice, however, this approach would come across the principle of „ne bis in idem" („not twice in the same case"). This example points to the inconsistency and practical difficulties associated with the division of one type of regulation into two rules controlled by two different bodies. The chaos of legislation only confirms and multiplies the position of the Ministry of Health of the Slovak Republic. This central state administration body performs not only the function of the first-instance supervisory body but also the function of the appeal body against the decisions of the State Institute for Drug Control.

As can be seen from our findings, the fundamental problem of regulating and controlling drug advertising is seen in the provisions of Statutes 11, Paragraph 5 of the Advertising Act. This provision establishes an objective and subjective time period until the administrative authority has to make a decision on the violation of the law. From the analysis of the State Institute for Drug Control for the years 2013 to 2017, we found that, despite the proven violations of the Advertising Act, the administrative body had to seal the proceedings largely because of the expiration of time, and the culprit passed the sentence. A suitable solution to this problem can be found in an amendment to the provision of the law, which would be an objective time for the decision on the matter to be extended to five years and a subjective period to three years.

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Eur. Pharm. J. 2019, 66(1), 11-18.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.2478/afpuc-2019-0002

EUROPEAN PHARMACEUTICAL JOURNAL

Is gentamicin administered to individual patients in optimal doses already at the beginning of therapy?

Original research article/Review

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Received 24 September, 2018, accepted 11 January, 2019

Abstract ***Introduction*** A gentamicin dose, which the physicians select, frequently does not take any pharmacokinetic parameters into consideration.
Aim To analyse the results of therapeutic drug monitoring (TDM) of gentamicin for those patients who have not had the gentamicin dose adjusted at the beginning of therapy (first group) and for those patients who had the gentamicin dose adjusted at the beginning of therapy (second group).
Methods We acquired the basic data about patients from the requests for laboratory examination of levels of gentamicin. We measured all the gentamicin concentrations mentioned in this work using the FPIA method.
Results The monitored set included 379 hospitalized patients during a 4-year period. We divided the monitored set into 2 groups. First group was composed of patients without dose adjustment of gentamicin at the beginning of therapy, and the second group was composed of patients with dose adjustment of gentamicin by the clinical pharmacist at the beginning of therapy. In addition, the patients in each group were divided according to the body mass index (BMI). In the first group of patients, a low percentage of patients had both optimal levels (trough, peak levels). As for patients with BMI > 25 m^2/ kg, there were only 17 % such cases, and the patients with BMI ≤ 25 m^2/kg were only 18.8 %. In the second group, the patients had all trough and peak levels in optimal therapeutic range at obese patients, overweight patients and also at patients with normal weight ($p < 0.001$).
Conclusion Adjustment of dosage regimens immediately at the beginning of therapy will provide for administering sufficient doses of antibiotics at the beginning of therapy, which is a pre-condition for a successful anti-infective therapy. Therapeutic monitoring of levels allows for administration of sufficient dose of gentamicin without fear of any undesirable effects.

Keywords *Gentamicin – therapeutic drug monitoring – adjustment of dosage of gentamicin*

INTRODUCTION

Gentamicin has been used in clinical practice for more than 50 years due to its good bactericidal effect, which is dependent on its concentration, low resistance, synergy with beta-lactam antibiotics, and finally, also due to its low price (Martin et al., 2012). Nephrotoxicity and ototoxicity have discouraged physicians from frequently using gentamicin and other aminoglycosides in clinical practice. Current advances in proper administration have returned them again among the effective antibiotics against gram-negative bacteria that have a place in clinical practice (Durante-Mangoni et al., 2009). Gentamicin is used in combination with other antibiotics, mainly for the treatment of serious infections (Gómolka & Niemczyk, 2014). In Teaching Hospital Nitra, gentamicin is a frequently used antibiotic due to the abovementioned reasons. Most frequently, it is part of an anti-infective therapy at clinics and surgical departments. In the monitored set of 379 patients who had been administered gentamicin over the period of four years, the proportion of patients hospitalized at clinics and surgical departments was as high as 70.7%. In the recommendations of specialist infectious and surgical associations in North America, gentamicin is a part of combined antibiotic therapy, used in the treatment of

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intra-abdominal infections in children and adults (Solomkin et al., 2010). Patients in the monitored set were administered gentamicin in 92% in combination with other antibiotics. Another significant advantage of gentamicin treatment in the Teaching Hospital Nitra is a possibility of **therapeutic drug monitoring (TDM)** in the hospital pharmacokinetic laboratory and the follow-up interpretation of TDM results and proposal for dosage regimens by the clinical pharmacist.

ETHICAL APPROVAL

The study was approved by the Ethics Committee of the Teaching Hospital Nitra.

METHODS

Prospective monitoring *included* all adult patients for whom the serum levels of gentamicin were measured (trough and peak levels) during the period from 1st August 2010 to 1st August 2014 in the Pharmacokinetic Laboratory of the Teaching Hospital Nitra.

Patients

All the patients were hospitalized at different clinics and departments of the Teaching Hospital Nitra. We acquired the basic data about patients from the requests for laboratory examination of levels of gentamicin, which were sent to the pharmacokinetic laboratory with samples of biological material.
Demographic and biometric data were collected by means of the mentioned requests: age, gender, actual weight (ABW), height, serum creatine level (SCr), and information on dosage regimen for gentamicin. We calculated the BMI (body mass index) according to the formula. The pharmacokinetic parameters: Cocrofta-Gaulta Creatinine Clearance ($CrCl_{CG}$), total clearance of gentamicin (total Cl), the total distribution volume of gentamicin (total Vd), elimination rate constant (ke), biological half-life ($t_{1/2}$), and ideal weight (IBW) were obtained using the pharmacokinetic program *(Abbottbase Pharmacokinetic Program)*.
In this work, the set of patients was divided into two groups: The first group consisted of the patients who did not have the gentamicin dosage regimen adjusted at the beginning of therapy; the second group consisted of the patients who had the gentamicin dosage regimen adjusted immediately at the beginning of therapy according to the current pharmacokinetic parameters.

Laboratory methods

We measured all gentamicin concentrations mentioned in this work using the FPIA method on the analyser AxSYM of company ABBOTT in the Pharmacokinetic Laboratory of the Teaching Hospital Nitra.

Statistical methods

Patient demographic and pharmacokinetic parameters were represented by simple arithmetic mean, standard deviation, or confidence intervals (CI).
Description characteristics were calculated for demographic parameters and dose size. When comparing two groups, a two-sample t-test was used. For the pairwise linear regression (analysis) by the parametric linear least squares method, significance by the linear ANOVA method was evaluated, and consequently, the significance of the contrast and slope of the linear model was independently tested. To determine the statistical significance of association between nominal or ordinal variables arranged in the contingency table, Fisher's exact test and Yates's corrected Chi-square test were used, respectively.

Aim of the study

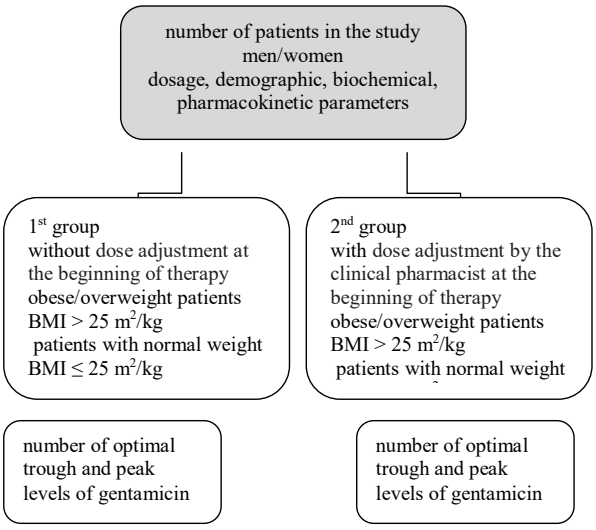
- To analyse the results of therapeutic monitoring of serum levels of gentamicin of patients who **have not had the gentamicin dose adjusted at the beginning of therapy**, according to the pharmacokinetic parameters
- To analyse the results of therapeutic monitoring of serum levels of gentamicin of patients who **had the gentamicin dose adjusted at the beginning of therapy**, according to the pharmacokinetic parameters
- Comparisons of results of determined levels of gentamicin at trough and peak levels of concentrations in both groups of patients regarding the pharmacokinetic parameters and dosage regimen for gentamicin

Design of the study

Design of the study is depicted in the following scheme, as shown in Figure 1.

RESULTS

Based on the inclusion criteria, the set included 379 patients, who had the trough and peak levels of gentamicin monitored. All the patients included in the prospective monitoring were hospitalized at the clinics and departments of the Teaching Hospital Nitra; in the total set of 379 pateints, there were 299 men (78.9%) and 80 women (21.1%). 268 patients (n = 379; 70.7%) were hospitalized at surgical clinics and departments, and 111 patients (n = 379; 29.3%) were hospitalized at clinics and departments of conservative disciplines. The highest number of patients in both groups represented the patients at the Surgery Clinic, the Clinic of Accident Surgery and the Orthopaedics and at the Infectious Disease Clinic. Patients were divided into two groups. The first group consisted of 204 patients, who did not have the gentamicin dosage regimen adjusted at the beginning of therapy; the second group consisted of 175 patients, who had the gentamicin dose



BMI – body mass index

Figure 1. Design of the study

regimen adjusted by the clinical pharmacist immediately at the beginning of therapy according to the current pharmacokinetic parameters. The total number and ratio (%) of patients hospitalized at individual clinics/departments in the first and second group is stated in Table 1. Demographic data and pharmacokinetic parameters of both groups are given in Table 2 and 3.

Difference in ratio of men in the first group (n = 204; 79.4%) and the second group (n = 175; 78.4%) was not significant. Age average in the second group of patients with dose adjustment at the beginning of therapy was significantly lower ($p < 0.05$); the other demographic data and serum creatine were not significantly different. Pharmacokinetic parameters that prove better elimination of gentamicin were significantly better ($p < 0.05$) in the second group of patients. For better comparison and assessment of dosage regimens and consequently determined levels, we further divided the first and second group of patients according to the BMI (body mass index) into two sub-groups. One sub-group included obese patients and overweight patients with BMI > 25 kg/

Table 1. The total number and ratio (%) of patients hospitalized at individual clinics/departments in first and second group.

Clinic/department	**First group n = 204 (%)** without dose adjustment of gentamicin at the beginning of therapy	**Second group n = 175 (%)** with dose adjustment of gentamicin at the beginning of therapy	Total **n = 379 (%)**
Clinic of Surgery	**84 (41.2)**	**99 (56.6)**	**183 (48.3)**
Clinic of Gynaecology and Obstetrics	1 (0.5)	4 (2.3)	5 (1.3)
Infectious Disease Clinic	**21 (10.3)**	**17 (9.7)**	**38 (10.0)**
Internal Clinic	13 (6.4)	5 (2.8)	18 (4.7)
Cardiology Clinic	22 (10.7)	4 (2.3)	26 (6.9)
Anaesthesiology and Intensive Care Clinic	10 (4.9)	4 (2.3)	14 (3.7)
Department of Dermatology	1 (0.5)	0	1 (0.3)
Clinic of Accident Surgery and Orthopaedics	**24 (11.7)**	**32 (18.2)**	**56 (14.8)**
Clinic of Neurosurgery	0	1 (0.6)	1 (0.3)
Clinic of Neurology	5 (2.5)	1 (0.6)	6 (1.6)
Department of Vascular Surgery	7 (3.4)	3 (1.7)	10 (2.6)
Department of Plastic Surgery	2 (1.0)	0	2 (0.5)
Department of Oncology	4 (2.0)	4 (2.3)	8 (2.1)
Department of ORL	4 (2.0)	1 (0.6)	5 (1.3)
Department of Urology	6 (2.9)	0	6 (1.6)

Table 2. Demographic parameters and biochemical parameters in first and second group.

Demographic parameters	**First group n = 204** without dose adjustment of gentamicin at the beginning of therapy	**Second group n = 175** with dose adjustment of gentamicin at the beginning of therapy	**p**
Age [year]	57 ± 17 (55–60)	53 ± 17 (51–55)	**p < 0.05**
Sex (men/women)	160/44	139/36	
Actual body weight (ABW) [kg]	84 ± 22 (81–88)	87 ± 19 (84–89)	0.238
Ideal body weight (IBW) [kg]	65 ± 8 (64–66)	65 ± 11 (64–67)	0.754
Height [cm]	174 ± 9 (173–175)	174 ± 9 (173–175)	0.273
Biochemical parameter			
Creatinine concentration [μmol/l]	84.2 ± 33.3 (79.6–88.7)	83.6 ± 20.6 (80.5–86.6)	0.823

Mean ± standard deviation (SD), (95% confidence interval), Two-choice t-test

Table 3. Pharmacokinetic parameters of gentamicin in first and second group.

Pharmacokinetic parameters	**First group n = 204** without dose adjustment of gentamicin at the beginning of therapy	**Second group n = 175** with dose adjustment of gentamicin at the beginning of therapy	**p**
Creatinine clearance (ABW) [ml/min/1.73 m^2]	94.0 ± 40.5 (88.4–99.6)	93.1 ± 30.1 (88.6–97.3)	0.590
Creatinine clearance (IBW) [ml/min/1.73 m^2]	74.5 ± 35.7 (69.6–79.5)	72.7 ± 28.9 (68.4–77.0)	0.798
Total clearance [l/h]	4.5 ± 2.0 (4.2–4.8)	5.3 ± 2.1 (5.1–5.7)	**p < 0.05**
Total distribution volume V_d [l]	16.2 ± 2.1 (15.9–16.5)	16.5 ± 2.0 (16.2–16.8)	0.210
Elimination rate constant k_e [h^{-1}]	0.288 ± 0.165 (0.265–0.310)	0.325 ± 0.123 (0.307–0.344)	**p < 0.05**
Half-life $t_{½}$ [h]	3.01 ± 1.42 (2.81–3.20)	2.45 ± 1.04 (2.30–2.61)	**p < 0.05**

Mean ± standard deviation (SD), (95% confidence interval), Two-choice t-test

m^2. The other sub-group included patients with normal body weight with BM I ≤ 25 kg/m^2.

In the first group of patients without dose adjustment of gentamicin at the beginning of therapy, gentamicin was administered in the dosage regimen once daily only for 9 (n = 135; 6.7%) obese and overweight patients and for 6 patients with normal body weight (n = 69; 8.6%). In the second group of patients with dose adjustment by the clinical pharmacist at the beginning of therapy, the dosage regimen once daily was preferred for 125 obese and overweight patients (n = 128; 97.6%) and for all 47 patients with normal body weight (n = 47; 100%). Gentamicin was the most frequently administered in regimen 320 mg every 24 hours to both groups of patients and 360 mg every each 24 hours in the group of obese and overweight patients. Comparison of dosage regimens in individual groups of patients divided according to BMI is depicted in Figure 2 and Figure 3.

Daily doses of gentamicin considering the pharmacokinetic parameters, which were calculated for the patients of second group with BMI > 25 kg/m^2 and with BMI ≤ 25 kg/m^2 at the beginning of therapy, were significantly higher ($p < 0.001$) in contrast to the first group of patients. Patients of first group without dose adjustment of gentamicin considering the pharmacokinetic parameters were administered lower doses at the beginning of the therapy and most patients were under dosed (Table 4).

In the second group of patients with dose adjustment of gentamicin at the beginning of therapy, all trough and peak

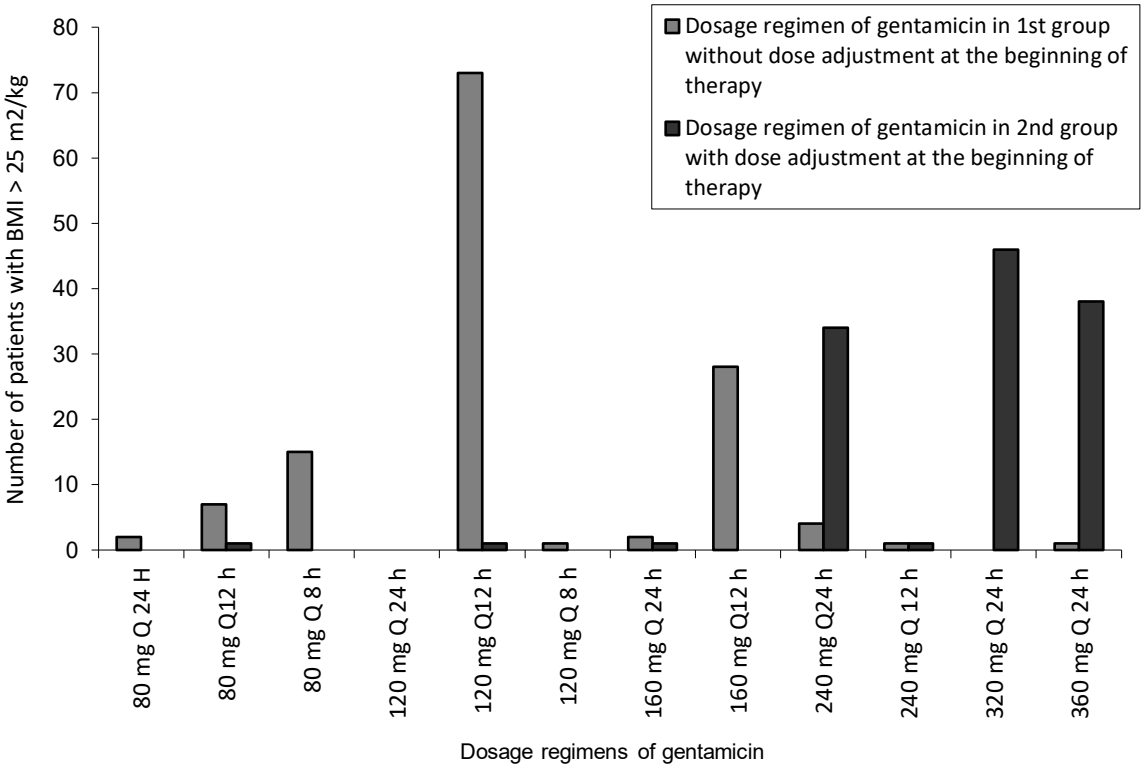


Figure 2. Stratification of patients with BMI > 25 m²/kg according to daily doses of gentamicin in first and second group.

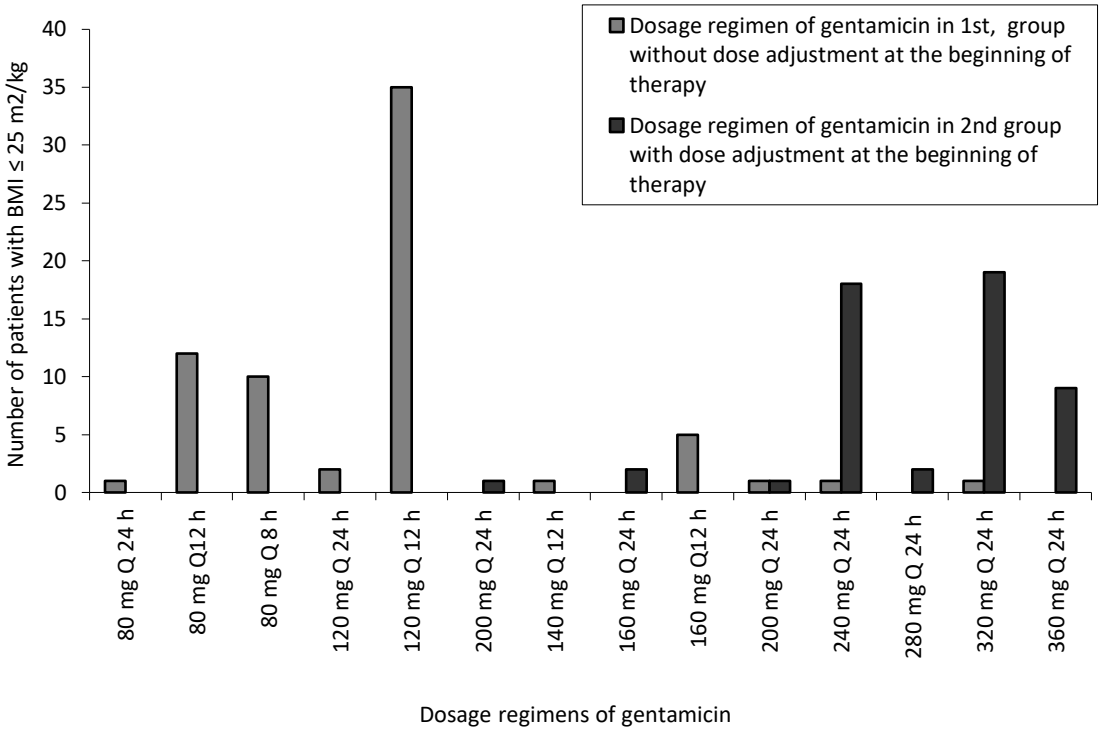


Figure 3. Stratification of patients with BMI ≤ 25 m²/kg according to daily doses of gentamicin in first and second group.

Table 4. Comparison of daily doses of gentamicin in first and second group.

Daily doses of gentamicin [mg/kg/day]	**First group n = 204** without dose adjustment of gentamicin at the beginning of therapy	**Second group n = 175** with dose adjustment of gentamicin at the beginning of therapy	**p**
Patients with BMI > 25 m^2/kg	2.7 ± 0.6 (2.6–2.8) n = 135	3.3 ± 0.6 (3.1–3.4) n = 128	**p < 0.001**
Patients with BMI > 25 m^2/kg	3.5 ± 0,9 (3.3–3.8) n = 69	4.3 ± 0.8 (4.1–4.6) n = 47	**p < 0.001**

Mean ± standard deviation (SD), (95% confidence interval) ANOVA test

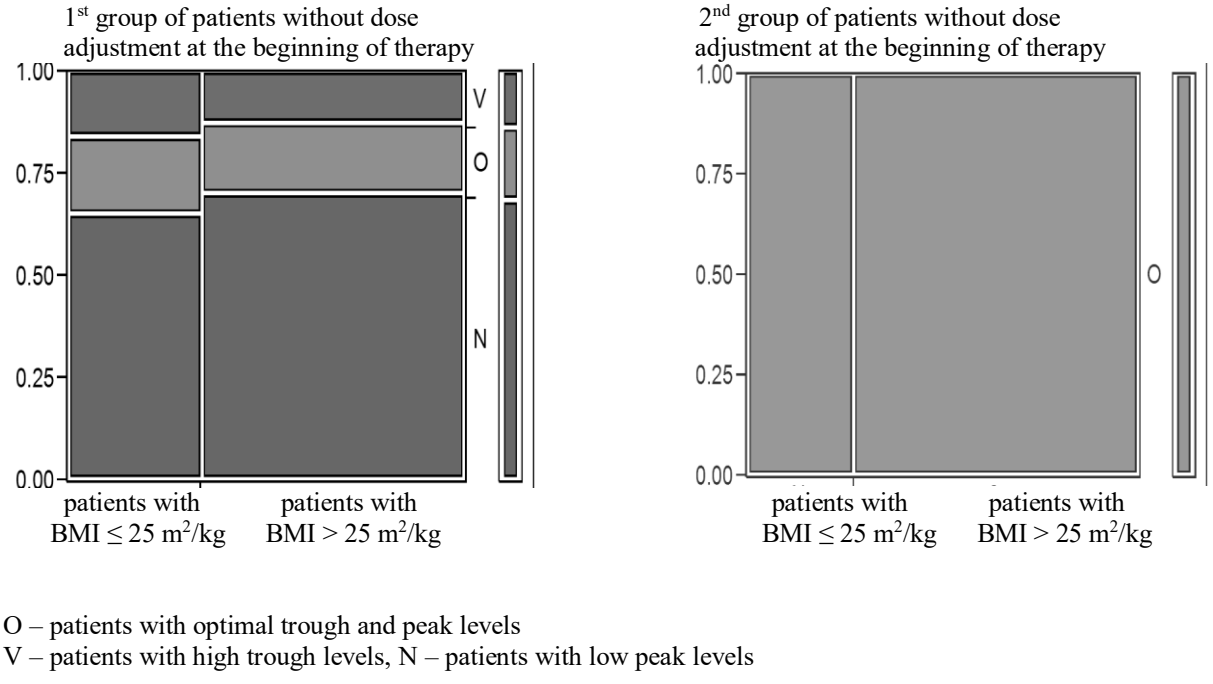


Figure 4. Contingency analysis. Number of patients with optimal levels of gentamicin in first and second group.

levels were in optimal therapeutic range for patients with BMI > 25 m^2/kg and with BMI ≤ 25 m^2/kg. In contrast with the first group of patients without dose adjustment at the beginning of therapy, in which only low percentage of patients had both optimal levels. For patients with BMI > 25 m^2/kg, it was only 17% and patients with BMI ≤ 25 m^2/kg only 18.8%. Ratio of optimal levels in both groups is depicted in the contingency analysis (Figure 4).

Differences between both groups in average trough and peak levels were highly statistically significant. Patients with dose adjustment at the beginning of therapy reached significantly lower trough levels and higher peak levels ($p < 0.001$), which is desirable for efficiency and safety of treatment by gentamicin (Table 5).

Adjustment of gentamicin dose for an individual patient at the beginning of the therapy allowed to reach the target values of gentamicin concentrations better and faster.

Success of gentamicin dose adjustment by the clinical pharmacist at the beginning of the therapy with regard to reaching efficient levels is statistically significant. If a correct dosage regimen is selected, there is a high chance to reach optimal peak level, which is also confirmed by the result of statistical calculation: OR is 193.59 (95% CI 46.55–805.09).

DISCUSSION

For all the patients of the second group with dose adjustment by the clinical pharmacist at the beginning of the therapy at first **determination of levels,** trough and peak levels of gentamicin concentrations reached the desired optimal therapeutic range (100%) in contrast with the first group of patients without dose adjustment at the beginning of therapy, where only 17.7% patients reached the optimal values at both levels.

As early as in the work of Thomson et al. (1996), patients with dose adjustment of gentamicin at the beginning of therapy –

Table 5. Comparison of trough and peak levels of gentamicin in first and second group.

Optimal range	**First group n = 135** without dose adjustment of gentamicin at the beginning of therapy with BMI > 25 kg/m^2	**Second group n = 128** with dose adjustment of gentamicin at the beginning of therapy with BMI > 25 kg/m^2	**p**
Trough levels [mg/l] < 2 mg/l	0.96 ± 0,75 (0.83–1.1)	0.43 ± 0.26 (0.38–0.47)	**< 0.001**
Peak levels [mg/l] 5–10 mg/l, >10 mg/l - once daily dosage	4.55 ± 2.26 (4.17–4.94)	10.03 ± 2.94 (9.51–10.54)	**< 0.001**
	First group n = 69 without dose adjustment of gentamicin at the beginning of therapy with BMI ≤ 25 kg/m^2	**Second group n=47** with dose adjustment of gentamicin at the begining of therapy with BMI≤ 25 kg/m^2	**p**
Trough levels [mg/l] < 2 mg/l	1.08 ± 0.98 (0.84–1.32)	0.44 ± 0.31 (0.35–0.53)	**<0.001**
Peak levels [mg/l] 5–10 mg/l, >10 mg/l - once daily dosage	5.03 ± 2.30 (4.47–5.58)	11.30 ± 2.88 (10.46–12.13)	**<0.001**

Mean ± standard deviation (SD), (95 % confidence interval) ANOVA test

their levels reached in high percentage optimal therapeutic range. The Thomson's study compared a group of 50 patients who were administered gentamicin only in empirically selected dose and 50 patients who were administered gentamicin according to elaborated recommendations. In the group of patients whose dosage regimen of gentamicin reflected the recommendations, peak levels reached significantly higher values (7.2 ± 1.9 vs. 5.7 ± 1.8 mg/l) and 96% of them had optimal both levels. Only 59% of patients who were administered gentamicin in empirically selected dose had optimal both levels.

Cox et al. (2011) compared trough levels of aminoglycoside antibiotics in therapeutic range in the group of patients, in which the dose for individual patients was consulted and in the group without any consultation. In the group where the dose was adjusted based on consultations, the patients had significantly higher percentage of trough levels in optimal range (59% vs. 89%). Only 40% of patients had optimal initial dose in the group without consultation; in the group with the consulted dose, it was more than 80% of patients. Fonzo-Christie et al. (2014) evaluated the implementation of the gentamicin dose recommendations for new-borns in 1 year-long prospective monitoring. One-year long study included two groups of new-borns. In the first group, the recommendations of dosage regimens for individual patients were implemented; in the second group, these recommendations are not applied. Trough concentrations were compared in both groups in the study. Occurrence of target concentrations was significantly higher in the group in which the recommended doses were applied (once daily, prolonged interval) (68.5% versus 33.0%). Probability of greater number of optimal concentrations also increased with the use of the recommended dosage regimens once daily.

For most patients in our prospective study, administration with a prolonged dose interval once daily was preferred with gentamicin dose adjustment. Administration once daily maximized the concentration-dependent effect of aminoglycosides, as well as the post-antibiotic effect. This regimen is generally accepted in hospitalized patients on basic beds, as well as in patients hospitalized in intensive care units. Target concentrations are more easily reached by the dosage regimen with prolonged interval. In the meta-analysis of Barza et al. (1996), reduction in the risk of nephrotoxicity was confirmed, but there was no significant difference in reducing the risk of ototoxicity and decreasing mortality. Higher efficiency was demonstrated in patients without pre-existing renal insufficiency, and finally, administration once daily was lower in cost. The dosage regimen with the prolonged interval reduces the probability of high trough concentrations responsible for any adverse events (Nezic et al., 2014). Radigan et al. (Radigan et al., 2010) prefer administration of aminoglycoside antibiotics over a prolonged interval even in the critically ill patients in intensive care units. In treatment by gentamicin, the benefit of a regimen with prolonged interval is greater when combined with therapeutic monitoring of levels, particularly in critically ill patients who have difficulty achieving effective peak concentrations (Wong et al., 2014).

Results of this work, in line with the results of studies by other authors', have significantly demonstrated the importance of individualized dosage regimens at the beginning of the therapy and the importance of therapeutic monitoring of levels.

CONCLUSION

Gentamicin, as an aminoglycoside antibiotic has still its place in the anti-infective therapy of hospitalized patients. Safe and effective administration of gentamicin requires reaching optimal residual and peak levels within the desired therapeutic range. Adjustment of dosage regimens by the clinical pharmacist for individual patients immediately at the beginning of therapy will provide for administering sufficient doses of antibiotics at the beginning of therapy, which is a pre-condition for a successful anti-infective therapy. Therapeutic monitoring of levels enables administration of sufficient doses of gentamicin at the beginning of therapy without fear of any adverse events. On the other hand, TDM identifies the usage of inadequate dosage regimens, which do not take the pharmacokinetic parameters into consideration and decreases the risk of under dosing.

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Eur. Pharm. J. 2019, 66(1), 19-27.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.2478/afpuc-2018-0006

EUROPEAN PHARMACEUTICAL JOURNAL

Pharmacy employees' self-rated knowledge, use and attitudes toward homeopathy: A comparative survey in Sweden and Germany

Original research article/Review

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Received 14 April, 2018, accepted 1 June, 2018

Abstract **Background:** Homeopathy is being increasingly practiced within different medical areas of use. Homeopathic medicines are sold in German pharmacies, whereas the assortment of Swedish pharmacies does not include homeopathic medicines. Despite differences between Sweden and Germany, homeopathic medicines are classified as drugs in both countries.
Objective: The aim of this study was to compare the pharmacy employees' self-rated knowledge, use and attitudes toward homeopathy in Sweden and Germany.
Methods: A quantitative web-survey was sent to 30 pharmacies in Sweden and 30 pharmacies in Germany, which were selected by using a multi-stage clustering sampling. The questionnaire contained closed-ended rating scales. To compare the self-rated knowledge, use and attitudes toward homeopathy of Swedish and German pharmacy employees, chi-square tests and Mann-Whitney tests were performed in SPSS.
Results: A total of 209 pharmacy employees answered the survey (108 in Sweden and 101 in Germany). German participants estimated their knowledge higher than the Swedish participants ($p < 0.01$). In both countries, most participants thought that pharmacy employees should have knowledge about homeopathy. Although most Swedish participants stated that they receive questions about homeopathy, the German pharmacy employees receive questions about homeopathy more frequently ($p < 0.01$). Swedish participants reported less experience of own use of homeopathic medicines and less belief in their effectiveness as compared to the German participants ($p < 0.01$). However, in both countries, most participants stated that homeopathic medicines should be sold in pharmacies.
Conclusion: As pharmacy employees should act professionally to advice customers on all drugs, increased homeopathic knowledge in pharmacy employees could potentially improve pharmaceutical practice.

Keywords *Homeopathy – pharmacy – attitudes – use – knowledge – Sweden – Germany*

INTRODUCTION

Background on homeopathy

Complementary and alternative medicines (CAM) include homeopathy (Rosser 2004), which is based on the principle of similarities meaning that a substance that causes symptoms in a healthy person is given to a sick person that has the same symptoms. The effect is said to increase with increasing dilutions (Molski 2011), which is the main controversy to the conventional medicines. Although homeopathy meets great scepticism (Ernst 2010), it is increasingly used in western countries (Betti et al. 2013). Placebo-controlled trials have shown that there is weak evidence for a specific effect of homoeopathic medicines (Shang et al. 2005). However, such trials may not be reliable, as homeopathy intends to treat each person individually, which requires great knowledge (Vithoulkas 2017). Evidence suggests that the highly diluted homeopathic medicines cannot be considered just placebos, but the pharmacodynamics of homeopathic medicines are not fully understood (Bellavite et al. 2014b; Bellavite et al. 2014a). Homeopathy is increasingly practiced all over the world within different medical areas of use (Betti et al. 2013; Relton et al. 2017). The most frequent areas of use are oncology and obstetrics (Trimborn et al. 2013; Harren, Georgi, and Hackethal 2011; Kalder and Knoblauch 2011), but also general medicine (Harren, Georgi, and Hackethal 2011) and veterinary medicine (Hektoen 2005).

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CAM including homeopathy in Germany and Sweden

No studies about pharmacy employees' knowledge, use and attitudes toward homeopathy exist and only few studies report on health care professions' knowledge, use and attitudes on CAM including homeopathy.
In Germany, CAM is most commonly recommended by physicians, midwives and pharmacists (Kalder and Knoblauch 2011; Maisch and Hübner 2014). Studies on health care professionals' attitudes and knowledge about CAM have shown that two-thirds of all employees are interested in CAM. In contrast, participants rated their own knowledge in CAM as inadequate (Trimborn et al. 2013; Conrad et al. 2014; Längler et al. 2013; Maisch and Hübner 2014).
With regard to Sweden, the small amount of data on health care professions' view on CAM indicates that there is more scepticism among health care professions (Bjerså, Forsberg, and Fagevik 2011) as well as less knowledge (Bjerså, Victorin, and Olsén 2012) compared to the German health care professions. CAM is less frequently recommended to patients in Sweden (Bjerså, Forsberg, and Fagevik 2011; Bjerså, Victorin, and Olsén 2012) than in Germany (Kalder and Knoblauch 2011; Maisch and Hübner 2014).
Health care professionals' attitudes toward CAM may differ from their attitudes toward homeopathy, as CAM comprises a wide range of therapies (Trimborn et al. 2013; Molassiotis et al. 2005). As homeopathy is among the most frequently used treatments in CAM (Molassiotis et al. 2005), it is of importance to study the attitudes of healthcare professionals including pharmacy employees towards homeopathy specifically.

Differences between Germany and Sweden

In Germany, CAM including homeopathy is widely used for different complaints by the general population (Bücker et al. 2008). Homeopathic medicines have long been recommended and prescribed by German physicians and sold in German pharmacies, whereas the assortment of Swedish pharmacies does not include homeopathic medicines. Swedish physicians were not allowed to recommend homeopathic medicines until the year 2011 (Küchler 2011; European-Committee-for-Homeopathy-(ECH) 2017). In Sweden, the use of CAM including homeopathy has increased (Nilsson, Trehn, and Asplund 2001), but is still less frequent compared to Germany (Bücker et al. 2008) that has a longer history of homeopathy (Rosser 2004). Despite these differences regarding the use of homeopathic medicines in Germany and Sweden, today homeopathic medicines are classified as drugs in both countries regarding to the applicable rules of the European Union (EU) relating to the medicinal products for human use (2001/83/EG) (Community 2004).
In view of the above, this survey was conducted in both Sweden and Germany to compare the self-rated knowledge, use and attitudes of pharmacy employees to analyse differences in the pharmaceutical practice regarding homeopathy in these two countries. As Sweden and Germany have the same regulations on homeopathic medicines but differ in respect to the pharmacy assortment and the historical background regarding homeopathy, a comparison of Swedish and German pharmacy employees' experiences with homeopathic medicines in their daily work may be useful in the discussion if homeopathic medicines should be included in the pharmacy assortment or not. To gain knowledge about pharmacy employees' knowledge, use and attitudes on homeopathy is important, as these insights could be used to improve the daily work in pharmacies.

AIM

The aim of this study was to compare the pharmacy employees' self-rated knowledge, use and attitudes toward homeopathy in Sweden and Germany.

METHODS

Study design

A quantitative survey method was chosen to examine the self-rated knowledge, use and attitudes about homeopathy among the pharmacy employees in Germany and Sweden. The selected design is a cross-sectional study aiming to collect data on the entire population under study (Bowden 2011; Ejlertsson 2014), which is an appropriate design for the purpose of this study, because large representative populations can be used (Bowden 2011). This is important for this study, because it is addressed to the pharmacy employees in two different countries requiring more participants to be able to make comparisons between them.

Questionnaire design

The questionnaire was translated into Swedish and German. Google Forms was used to create an online survey that was available in October 2017. The questionnaire contained four sections. The aim of the first section was to summarize the background information of the participants, and it commenced with the close end nonintrusive way (Ejlertsson 2014). The second section contained Likert-scale questions regarding the knowledge about homeopathy. Section three commenced with Likert-scale questions about attitudes on homeopathy and section four was about the use of homeopathic medicines to gain insight into how widely homeopathic medicines are used in Sweden and Germany. Descriptive graphic rating scales with 3–5 possible alternatives were used, as this type of scaling provides a valid and reliable measure of self-assessed perceptions (Tesler et al. 1991). All questions were close ended with one single choice. Sub-divisions such as often/sometimes/seldom/never or some/moderate/good/very good were based on the responders' self-estimation, as they were not pre-defined.

Reliability and validity

To increase the reliability and validity, pre-tests were performed. As reliability refers to the extent a study will give the same result if it was repeated (Kazdin 2009; Creswell 2009), two pharmacists that work at a Swedish pharmacy as well as seven persons from different health professions (three from Sweden and four from Germany) were asked to answer the survey two months before it was sent out. After three weeks, they answered the survey again and similar results were obtained, which means that no differences could be observed in any of the questions. To address face validity, which refers to whether the scale items represent the proposed concepts that the questionnaire is intended to as well as content validity, which refers to whether the method includes the different facets of the concepts (Kazdin 2009; Creswell 2009), some of the questions were modified to be clear after the pre-tests. Modifications included the more precise description of the questions and rating alternatives such as 'No effect or just placebo' instead of 'No effect' added to the question 'What do you think about the efficacy of homeopathic medicines?' in the attitudes section.

Data collection and participants

To achieve an even geographical distribution of pharmacies and to include the pharmacies that are located both in larger cities as well as in the countryside, the multi-stage clustering sampling method was used for the selection of pharmacies in both countries. This refers to the sampling in multiple stages where randomly selected subsets of a cluster get smaller at each stage (Cochran 1977). In the present study, this method involved the selection of three pharmacies in ten counties in both Sweden and Germany, which made up 60 pharmacies in total (Figure 1).

To each of the 60 selected pharmacies, an inquiry to distribute the survey to the drug counselling employees of that pharmacy was sent via email. The email containing a link to the survey including study information to participants was sent to the contact email-address of all the selected pharmacies, because all employees working with drug counselling at the pharmacies are possible responders. No reminding emails were sent out.

By using this method, no information about drop-outs could be obtained. The number of possible respondents could not be exactly calculated, because the number of drug counselling employees is not known for all the pharmacies. The approximate number of possible respondents was estimated to a total of about 450 assuming an average of 7.5 drug counselling pharmacy employees at each of the 60 selected pharmacies. The number of participants that are required to achieve the study aims was estimated to at least 208 participants. This number was calculated by using Raosoft® online sample size calculator (Raosoft.Inc. 2004) based on the estimated population size of 450 and a

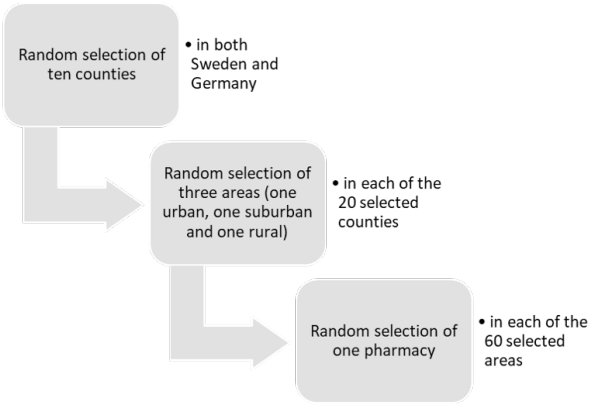

Figure 1. Selection of pharmacies.

response distribution of 50% assuming a 5% error marginal and 95% confidence level. The aim of the estimated sample size was to propose the sample size that is required to study the differences between the two countries (Whitley and Ball 2002). To achieve the estimated sample size, all the drug counselling pharmacy employees were included; so, no exclusion criteria were chosen. However, this implies that those in sick leave, parental leave or those not being active in their profession are not included, although they could contribute with their work experience; thus, the convenience selection method was used.

Data analysis

Descriptive statistics was used to categorize the survey participants' demographic information and to summarize the data. To explore if there were differences between Germany and Sweden regarding the pharmacy employees' self-rated knowledge, use and attitudes on homeopathy, non-parametric tests were performed to compare the German and Swedish pharmacy employees. The significance level was set to 5%. For the group comparison of categorical nominal variables, a chi-square test was performed to test if the distribution of observations differs between German and Swedish pharmacy employees. To test for group differences regarding the ordinal variables of the scaled questions, a Mann-Whitney U-test was performed to compare the distributions of ranks between the German and Swedish pharmacy employees. All analyses were performed in IBM SPSS Statistics 23 (IBM© Corp. 2015).

Ethics

The participation in this survey was anonymous and participants were informed about the aim of the study and that the data will be used for research purposes. The authors have no conflict of interest. The present study required no ethical approval according to the Act in Swedish law concerning Ethical Review of Research involving Humans (SFS 2003:460) from the Ministry of Education and Cultural

Table 1. Descriptive statistics of Swedish and German survey respondents.

		Sweden (n = 108)	Germany (n = 101)	p-value
Profession	Pharmacist	77 (71.3 %)	63 (62.4 %)	> 0.05[a]
	Pharmaceutical technician	31 (28.7 %)	38 (37.6 %)	
Years working	< 1 year	5 (4.6 %)	10 (9.9 %)	> 0.05[a]
	1 – 10 years	49 (45.4 %)	34 (33.7 %)	
	> 10 years	54 (50 %)	57 56.4 %)	
Age	< 26 years	14 (12.9 %)	10 (9.9 %)	> 0.05[a]
	26 – 35 years	33 (30.6 %)	24 (23.8 %)	
	36 – 45 years	30 (27.8 %)	23 (22.8 %)	
	46 – 55 years	16 (14.8 %)	26 (25.7 %)	
	56 – 65 years	13 (12.0 %)	13 (12.9 %)	
	> 65	2 (1.9 %)	5 (4.9 %)	
Gender	Female	81 (75.0 %)	70 (69.3 %)	> 0.05[a]
	Male	26 (24.1 %)	31 (30.7 %)	
	Others	1 (0.9 %)	0 (0 %)	

a: Chi-Square test

Affairs and according to the non-binding recommendations of the Central Ethics Committee in Germany.

RESULTS

A total of 209 pharmacy employees answered the survey. In Sweden, 108 (51.7% of total respondents) pharmacy employees answered the survey and in Germany, 101 (48.3% of total respondents) participated. In both Sweden and Germany, most participants were middle-aged female pharmacists. Descriptive statistics for the Swedish and German survey respondents are summarized in Table 1. As shown in Table 1, the background variables did not differ between the countries.

Self-rated knowledge about homeopathy

In both countries, participants think that pharmacy employees should have knowledge about homeopathy, but the German pharmacy employees think that this is more important compared to the Swedish employees (Table 2). German pharmacy employees estimated their knowledge significantly higher than the Swedish pharmacy employees (Figure 2) and they also had received significantly more education about homeopathy (Table 2).

Use of homeopathic medicines

German pharmacy employees receive questions about homeopathic medicines more frequently than the Swedish pharmacy employees and they also recommend

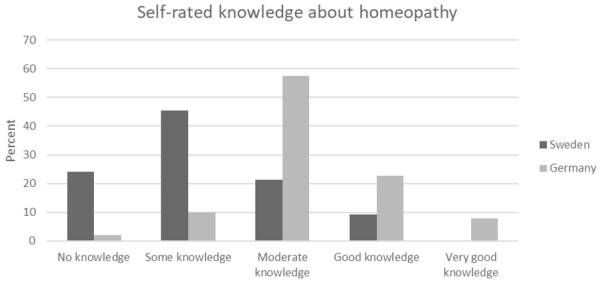

Figure 2. Pharmacy employees' self-estimated knowledge about homeopathy (p < 0.01, Mann-Whitney U-test).

homeopathic medicines more frequently compared to the Swedish pharmacy employees (Table 3). Regarding the question if participants have used homeopathic medicines by themselves or given to their children or pets and the effect they have experienced, there was a significant difference between Sweden and Germany, as the Swedish pharmacy employees have less personal experience of using homeopathic medicines (Figure 3).

The question about having friends or someone in the family who use homeopathic medicines also revealed significant differences between Sweden and Germany, as the German participants knew more homeopathy users. However, in Sweden, most participants had some or several friends or family members that use homeopathic medicines (Table 3).

Attitudes about homeopathy

As shown in Figure 4 and Table 4, the view about homeopathy differs significantly in Germany and Sweden. In Sweden,

Table 2. Self-rated knowledge about homeopathy.

		Sweden (n = 108)	Germany (n = 101)	p-value
Was homeopathy included in your educational studies?	Don't know	4 (3.7 %)	5 (5.0 %)	< 0.01[a]
	Yes	5 (4.6 %)	22 (21.8 %)	
	Yes, a little	39 (36.1 %)	55 (54.4 %)	
	No	60 (55.6)	19 (18.8 %)	
Should pharmacy employees have knowledge about homeopathy?	Don't know	4 (3.7 %)	2 (2.0 %)	< 0.01[a]
	Yes, it is important	35 (32.4 %)	54 (53.4 %)	
	Yes, some knowledge could be useful	58 (53.7 %)	32 (31.7 %)	
	No	11 (10.2 %)	13 (12.9 %)	

a: Chi-Square test

Table 3. Use of homeopathic medicines.

		Sweden (n = 108)	Germany (n = 101)	p-value
Questions about homeopathy from customers	Often	2 (1.9 %)	33 (32.6 %)	< 0.01[a]
	Sometimes	24 (22.2 %)	52 (51.5 %)	
	Seldom	49 (45.4 %)	14 (13.9 %)	
	Never	33 (30.6 %)	2 (2.0 %)	
Recommendation of homeopathy to customers	Often	0 (0 %)	30 (29.7 %)	< 0.01[a]
	Sometimes	3 (2.8 %)	36 (35.6 %)	
	Seldom	17 (15.7 %)	19 (18.8 %)	
	Never	88 (81.5 %)	16 (15.9 %)	
Friends or family members that use homeopathy	Don't know	22 (20.4 %)	1 (1.0 %)	< 0.01[b]
	Yes, several	16 (14.8 %)	72 (71.3 %)	
	Yes, some	43 (39.8 %)	24 (23.7 %)	
	No, no one	27 (25.0 %)	4 (4.0 %)	

a: Mann-Whitney U-test
b: Chi-Square test

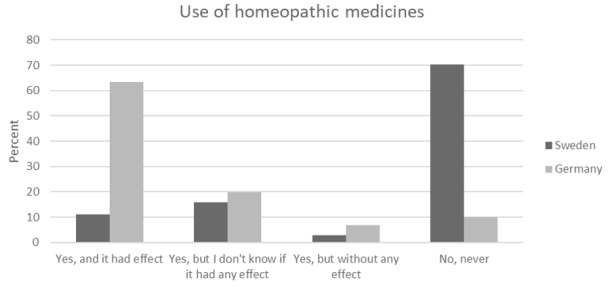

Figure 3. Pharmacy employees' experiences of using homeopathic medicines (p-value < 0.01, Mann-Whitney U-test).

most participants don't believe that homeopathic medicines have any effect, more than placebo, whereas most German participants believe that homeopathic medicines could have good effect in many diseases/conditions (Figure 4).

Regarding the question about the fact that homeopathic medicines are classified as drugs, more German participants think that this is right and similar results were obtained when participants were asked if homeopathic medicines should be sold in pharmacies (Table 4). Although most Swedish participants stated that homeopathic medicines should be sold in pharmacies, there is still a significant difference between Sweden and Germany regarding this question,

Table 4. Attitudes about homeopathy.

		Sweden (n = 108)	Germany (n = 101)	p-value
Is it right that homeopathic medicines are classified as drugs?	Don't know	35 (32.4 %)	8 (7.9 %)	< 0.01[a]
	It is right	34 (31.5 %)	66 (65.3 %)	
	It is wrong	39 (36.1 %)	27 (26.7 %)	
Should homeopathic medicines be sold in pharmacies?	Don't know	16 (14.8 %)	5 (5.0 %)	< 0.05[a]
	Yes	65 (60.2 %)	80 (79.2 %)	
	No	27 (25.0 %)	16 (15.8 %)	

a: Chi-Square test

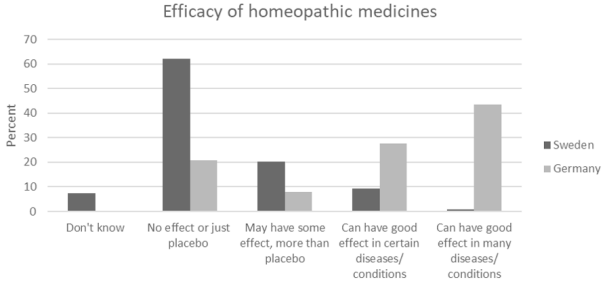

Figure 4. Pharmacy employees' attitudes about the efficacy of homeopathic medicines (p < 0.01, Mann-Whitney U-test).

as more German participants stated that homeopathic medicines should be sold in pharmacies compared to the Swedish participants (Table 4).

DISCUSSION

This study explored the self-rated knowledge, use and attitudes about homeopathy among the Swedish and German pharmacy employees. The results show that the Swedish and German pharmacy employees differ significantly regarding their self-rated knowledge, use and attitudes about homeopathy.

Self-rated knowledge

Although Swedish participants differ from the German participants regarding their self-rated knowledge and education about homeopathy, most of the Swedish participants think that some knowledge could be useful. These findings may indicate that there is a knowledge gap regarding homeopathy among the Swedish pharmacy employees and that knowledge about homeopathy could be needed for development in the profession. A desire for more continuing medical education on CAM including homeopathy was expressed by 85.2% of paediatric oncologists in a German survey about attitudes on CAM in paediatric oncology (Maisch and Hübner 2014). However, in the present study, the participants were not asked if they are satisfied with the amount of education about homeopathy they had received or if they wish to continue education on homeopathy to develop in their profession.

In a survey from 2005, the Canadian citizens were asked about their use of natural health products including homeopathy. In this survey, 27% of participants stated that they wanted to buy natural health products at pharmacies and 43% said that they completely trusted pharmacists for advice on natural health products. These results indicate that consumers of natural health products including homeopathic medicines expect pharmacists to have knowledge about these products (Natural Health Products Directorate 2005). Related to the findings from the present study, the higher self-estimated knowledge in Germany could be explained by the fact that the German pharmacy employees are expected to have knowledge about homeopathy.

Use

Even though homeopathic medicines are not sold in the Swedish pharmacies, about 70 % of the Swedish participants reported that they receive questions about homeopathy. This may reflect that there is a demand for homeopathic medicines in Sweden and that customers may wish consultation about homeopathy from pharmacy professionals. As evidence suggests, the pharmacy employees are expected to have knowledge about natural products including homeopathy (Natural Health Products Directorate 2005), which may also apply to countries where homeopathic medicines are not sold in pharmacies such as Sweden.

Swedish pharmacy employees have less experience of using homeopathic medicines than the German participants. Interestingly, in both countries, the majority that answered that they have used homeopathic medicines have also experienced effect indicating that pharmacy employees that have own experience with the use of homeopathic medicines are more likely to believe in homeopathy. This explanation is

in accordance to the findings from the previous mentioned study about attitudes on CAM in paediatric oncology, as homeopathy was among the CAM therapies with which physicians had most own experience and the personal experience of CAM had a significantly positive influence on their attitudes and the wish for further continuing medical education (Maisch and Hübner 2014).
The demand on homeopathic medicines in Sweden is confirmed by the finding that a majority of participants had some or several friends or family members that use homeopathic medicines, although the use of such medicines is more prevalent in Germany (Bücker et al. 2008).

Attitudes

The results from this study demonstrate that there are great differences between the Swedish and German pharmacy employees regarding their view about homeopathy. However, 22% of the German participants don't believe in the efficacy of homeopathy showing that the German participants are not a homogenous population regarding their attitudes about the efficacy of homeopathy. Another interesting finding is that about 10% of the Swedish participants stated that they don't know if homeopathic medicines are effective or not, compared to 0% in the German populations. That some Swedish participants had no opinion about the efficacy of homeopathic medicines may be associated with the lower rated knowledge among the Swedish participants.
Notwithstanding the difference between the German and Swedish participants regarding the question if homeopathy should be sold in pharmacies, most of the Swedish participants stated that homeopathic medicines should be sold in pharmacies. Although the reasons behind that are not known, this finding may be important in the discussion if homeopathic medicines should be included in the Swedish pharmacy assortment. It could influence attitudes on homeopathy if homeopathic medicines are sold in pharmacies or not. In Sweden, homeopathic medicines are not sold in pharmacies, which may lead to a lower valuation of homeopathy in Sweden. On the other hand, the fact that homeopathic medicines are sold in pharmacies may cause overestimation of homeopathic medicines in Germany. According to Amanda Henderson, a researcher in nursing practice, attitudes influence medical knowledge, which applies power during health practice. She refers to Foucault who states that power is constituted by social practices and that attitudes formulate knowledge. He means that attitudes may determine the methods of health care practice and that this power is controlled by social practices and prejudices (Henderson et al. 1994). Related to the findings from the present study, the differences between German and Swedish pharmacy employees may originate in the different history of homeopathy (Rosser 2004) as well as cultures and healthcare systems that might not be comparable (Bjerså, Forsberg, and Fagevik 2011; Bjerså, Victorin, and Olsén 2012).

Strengths and limitations

To our knowledge, this is the first survey regarding the pharmacy employees' self-rated knowledge, use and attitudes toward homeopathy. The study was conducted in two countries that differ in respect to the pharmacy assortment regarding homeopathy, which enables comparisons between different pharmaceutical practices.
A limitation of this study is the relative small study population. In addition, no information about the response rate or the geographical distribution could be obtained by using the chosen survey distribution method. Since the actual potential participants were not known, the response rate hence could not be calculated. This could negatively affect the generalizability of the findings, because the characteristics of non-responders could theoretically differ from responders (Kazdin 2009; Creswell 2009). Moreover, a back translation was not conducted, which might have increased the risk for dissimilarities between the Swedish and the German versions of the questionnaire. Noteworthy is that the translation into both languages started from the English version of the questionnaire so any back-translation should have been into the English language.
Regarding the study design, it should be noted that cross-sectional studies, like any other research design has limitations. Observational studies cannot provide explanations of causalities nor do they provide an in-depth explanation of a phenomenon. Causal relationships cannot be proven, because time relations between variables are not studied in a cross-sectional design. However, the cross-sectional design has the potential to give associations and generate prevalence data (Bowden 2011; Ejlertsson 2014).

Implications and suggestions for the future

Findings from this study are important, because it may be useful to understand that pharmacy employees' self-rated knowledge, use and attitudes about homeopathy differs in the two countries that have a different history of homeopathy but similar regulations, because these insights can be used to analyse and improve pharmaceutical practice. To make comparisons between the pharmacy employees in these countries is especially important regarding the fact that homeopathic medicines are included in the German pharmacy assortment but not in the Swedish. It could be discussed if homeopathic medicines should be included in the Swedish pharmacy assortment or not. It could also be discussed if Swedish pharmacy education should focus more on homeopathy. To address these questions, future studies should evaluate if the Swedish pharmacy employees wish more education about homeopathy as well as the reasons why homeopathy should be sold in pharmacies or not. Such studies would be of great importance, as homeopathy is increasingly used in the Western World (Betti et al. 2013; Relton et al. 2017).

CONCLUSION

Pharmacy employees in Germany had a higher self-rated knowledge about homeopathy, more experiences of using homeopathic medicines and they had more positive attitudes about homeopathy compared to the pharmacy employees in Sweden. In both countries, most participants stated that homeopathic medicines should be sold in pharmacies. Pharmacy employees should act professionally to advice customers on all drugs, which include homeopathic medicines in countries where the homeopathic medicines are regulated as drugs. Therefore, increased homeopathic knowledge in pharmacy employees could potentially improve pharmaceutical practice. If homeopathic medicines should be sold in pharmacies or not remain a discussion issue that could be addressed in future studies.

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Eur. Pharm. J. 2019, 66(1), 28-31.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.2478/afpuc-2019-0008

EUROPEAN PHARMACEUTICAL JOURNAL

Polar Phenolic Compounds in Peppermint Rhizomes and Leaves

Original Paper

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Received 9 May, 2019, accepted 18 September, 2019

Abstract Peppermint belongs to one of most popular medicinal plants in pharmacy as well as in the food industry.
Aim: For the conventional usage, the aerial part, especially leaves, is used. This investigation was aimed at the determination of phenolic compound in peppermint rhizomes infusion and the comparison with the phenolics in leaves infusions.
Methods: For the separation and identification of the phenolic compounds, the Sykam HPLC-DAD connected with Microsaic 4500MiD®, a single quadrupole mass spectrometer, was used.
Results: Three compounds in rhizomes and eight compounds in leaves were identified and quantified. In rhizomes, rosmarinic acid was determined as the main secondary metabolite, but its content was three times lower than that in leaves. Infusion of peppermint leaves was richer in flavonoids content with eriocitrin as a major phenolic compound.
Conclusion: Rhizomes of peppermint may also be used as a potential source of rosmarinic acid and caffeic acid derivatives.

Keywords *Mentha – Rhizomes – Leaves – HPLC-DAD – MS-single quadrupole – Rosmarinic acid*

INTRODUCTION

The genus *Mentha* L. belongs to the large family of Lamiaceae, subfamily Nepetoidae. A lot is known about the usage of aerial parts, especially because of the menthol-rich essential oil and phenolic compounds such as rosmarinic acid and eriocitrin. Peppermint has been reported to possess many biological activities, for example, digestion-stimulating, choleretic, antiseptic, secretolytic, antibacterial, antiviral, antispasmodic, antioxidant, anti-inflammatory, myorelaxant, and analgesic effects (Mckay & Blumberg, 2006; Lawrence, 2007). The use of mints is mostly due to the presence of two groups of secondary metabolites: essential oil components (monoterpenes, sesquiterpenes) and phenolic compounds (flavonoids and phenolic acids) (Mimica-Dukic & Bozin, 2008). Less is known about peppermint rhizomes, which are produced in high quantity every year (Fialova et al., 2012).
The aim of this study was to compare the leaves and rhizomes of peppermint from the side of phenolic compounds using Sykam HPLC-DAD connected with the Microsaic 4500 MiD® mass spectrometer.

MATERIALS AND METHODS

Leaves and rhizomes of *Mentha piperita* were collected from the Medicinal Plant Garden of the Faculty of Pharmacy, Comenius University in Bratislava. Leaves were collected at the flowering time and rhizomes in spring. The plants were dried in the drying room at 30–32 °C.. Voucher specimens are deposited at the Department of Pharmacognosy and Botany, Faculty of Pharmacy, Comenius University in Bratislava, Slovakia.
Infusions of dried leaves or rhizomes of *M. piperita* L. were prepared according to Pharmacopoeia Bohemoslovaca 4th edition (PhBs IV, 1987). Each infusion was lyophilized separately. For the HPLC analysis, 5 mg of lyophilizate was dissolved in 1 mL of water of HPLC quality.

Qualitative analysis by HPLC-DAD-MS

The HPLC-DAD analyses were performed using an HPLC system (Sykam, Eresing, Germany) equipped with a pump

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Table 1: Phenolic compounds in lyophilizates of rhizomes and leaves of peppermint.

Plant part	Compound	RT (Sykam)	[M-H] *m/z*	Identified/proposed Structure
Peppermint rhizomes	1	29.7	609	Hesperetin-7-*O*-rutinoside (hesperidin)
	2	30.6	359	Rosmarinic acid
	3	32.1	717	Caffeic acid tetramer
Peppermint leaves	1	25.9	595	Eriodictyol-7-*O*-rutinoside (eriocitrin)
	2	26.5	593	Luteolin-7-*O*-rutinoside
	3	27.6	461	Luteolin-7-*O*-glucuronide
	4	28.6	577	Apigenin-7-*O*-rutinoside (isorhoifolin)
	5	29.3	717	Caffeic acid tetramer (salvianolic acid B?)
	6	29.6	609	Hesperetin-7-*O*-rutinoside (hesperidin)
	7	30.6	359	Rosmarinic acid
	8	32.2	717	Caffeic acid tetramer

RT, retention time.

(S1125), an autosampler (S5250), a column oven (S4120), PDA detector (S3345), and Clarity Software. The HPLC system was connected in a series to mass spectrometer 4500MiD® (Microsaic Systems plc, Woking, the UK), a single quadrupole with a mass range of 1400 m/z equipped with ESI source (spraychip®). HPLC separation of the peppermint leaves or rhizomes lyophilizate was carried out on a TELOS LU C18 (2), 250x4.6 mm ID, 5um (KINESIS, Cheshire, the UK), at a temperature of 30°C and a flow rate of 0.8 mL/min. Water (pH 2.59 with HOAc, Merck, Germany) and MeCN (MS grade, Honeywell, Riedel-de-Haen, Seelze, Germany) were used as mobile phase A and B, respectively. The following gradient program was used: 10% B (0 min), 15% B (10 min), 30% B (20 min), 40% B (40 min), 90% B (45 min), and 10% B (50 min), followed by a column cleaning and re-equilibration step (Fialová et al., 2015). The MS parameters were given as follows: negative ion mode, tip voltage, –750.0 V; nebulizer flow, 2,500.0 ml min^{-1}; vacuum interface voltage, 40.0 V; tube lens voltage, 10.0 V; plate lens voltage, 5.0 V; ion guide voltage, 1.0 V; count time, 0.08 ms; and Software Masscape. N_2 was used as a nebulizing gas.

Quantitative determination of constituents by HPLC-DAD

The quantitative determination of phenolic compounds in *Mentha* leaves or rhizomes lyophilizates was provided by the method of external standards. The compounds in infusions were measured at two different wavelengths (280 and 320 nm). We used rosmarinic acid for the quantification of both the compounds and caffeic acid derivatives, eriocitrin, and hesperidin for the quantification of flavonoid glycosides (see Table 2). Chromatographic standards for rosmarinic acid and hesperidin were purchased from Sigma-Aldrich (St. Luis, USA) and for eriocitrin were purchased from HWI pharma service (Rülzheim, Germany). The calibration curves of rosmarinic acid were prepared at 320 nm, whereas those of eriocitrin and hesperidin were prepared at 280 nm. The calibration curves were obtained by injection of known concentrations (5 – 100 ppm). All three standards showed good linearity. The following r^2 values were obtained: for eriocitrin, r^2 = 0.9999 and regression curve y = 7.7359x; for hesperidin, r^2 = 0.9997 and regression curve y = 8.8442x – 0.3869; and for rosmarinic acid, r^2 = 0.9998 and regression curve y = 17.805x + 16.698. For eriocitrin, LOD was 1.31 μg·mL^{-1} and LOQ was 3.97 μg·mL^{-1}. For hesperidin, LOD was 2.28 μg·mL^{-1} and LOQ was 6.92 μg·mL^{-1}. For rosmarinic acid, LOD was 1.92 μg·mL^{-1} and LOQ was 5.81 μg·mL^{-1}. The results were expressed in μg mL^{-1} of water infusion. The examinations of secondary metabolites in mint rhizomes and leaves lyophilizates were performed in triplicate. The quantitative results were calculated from calibration curves, expressed as mean values and standard deviation (SD).

RESULTS AND DISCUSSION

Aerial parts of mints are used in food as well as traditional and conventional medicines all over the world. The most famous species is unambiguously the peppermint, aided by the menthol content in essential oil. Secondary metabolites are also important contributors of peppermint usage. Lyophilizates of the aerial part of peppermint are rich in phenolics such as rosmarinic acid, eriocitrin, luteolin glycosides, apigenin glycosides, and caffeic acid derivatives. All of these compounds influence the medicinal properties of peppermint extracts (dry or liquid). The major compounds

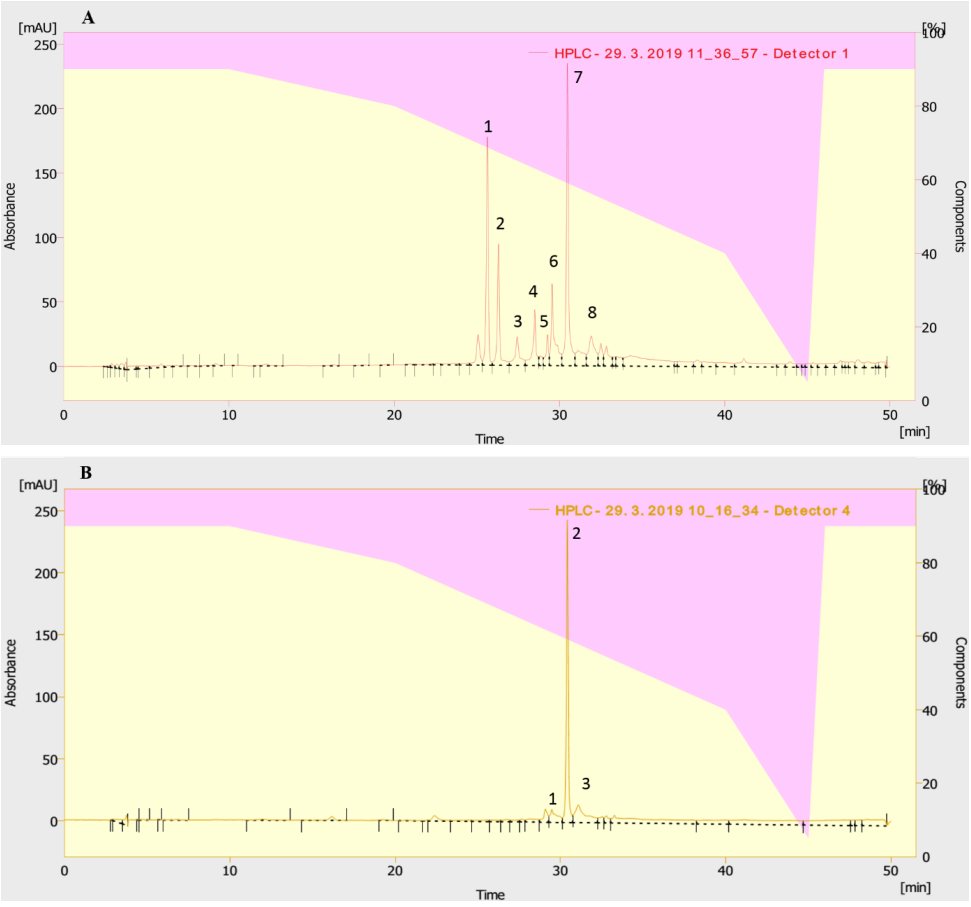

Figure 1: (A) HPLC-DAD chromatogram of peppermint leaves infusion (λ = 280 nm); (B) HPLC-DAD chromatogram of peppermint rhizomes infusion (λ = 320 nm).

Table 2: Quantitative abundance of polar phenolic compounds in infusions of M. piperita rhizomes and leaves (μg.mL^{-1}).

Compounds	Mass concentration (μg.mL^{-1})* ± SD	
	Rhizomes	Leaves
Eriodictyol-7-*O*-rutinoside (eriocitrin)[a]	-	**349.5 ± 10.52**
Luteolin-7-*O*-rutinoside [a]	-	204.1 ± 9.02
Luteolin-7-*O*-glucuronide [a]	-	58.8 ± 0.77
Apigenin-7-*O*-rutinoside (isorhoifolin) [a]	-	59.1 ± 3.88
Caffeic acid tetramer (salvianolic acid B?) [c]	-	24.1 ± 3.08
Hesperetin-7-*O*-rutinoside (hesperidin) [b]	19.8 ± 2.48	37.47 ± 3.02
Rosmarinic acid[c]	**93.8 ± 8.15**	286.4 ± 5.69
Caffeic acid tetramer[c]	7.99 ± 1.61	25.0 ± 1.48

**Values (μg.mL^{-1} in liquid extract) are presented as means ± standard deviation (n = 3), calculated as external standards: aeriocitrin, bhesperidin, crosmarinic acid.*

are eriocitrin and rosmarinic acid (Areias et al., 2001; Dorman et al., 2009). The question was if the underground parts are also rich in these compounds and in what amounts. Recently, we found out that the antioxidant activities of leaves and rhizomes are comparable (Fialová et al., 2012). Using liquid chromatography connected to 4500 MiD® mass spectrometer, the separation and identification of phenolic compounds of *Mentha* leaves and rhizomes lyophilizates was performed (see Table 1). Three phenolic compounds in rhizomes and eight in leaves samples were identified by comparison with authentic standards and/or literature. The resolution of caffeic acid tetramer (leaves: peak 6) was not clear. Anyways, we suggest

according to literature and previous analyses that peak 6 could be identified as salvianolic acid B. All compounds have been described previously in the genus *Mentha* L. (Areias et al., 2001; Dorman et al., 2009; Dorman et al., 2003; Fialová et al., 2009).

The quantitative analysis was performed using the HPLC-DAD by the method of external standards (eriocitrin, hesperidin, and rosmarinic acid). The results are displayed in Figure 1 and Table 2. Mint's rhizomes are not as rich in phenolic compounds as its leaves. The major compound in rhizomes' infusion was rosmarinic acid (93 $\mu g.mL^{-1}$), but its content was three times lower than that in leaves (286 $\mu g.mL^{-1}$). As in previous studies, eriocitrin (eridictyol-7-*O*-rutinoside) was identified as the main phenolic compound in the infusion of peppermint leaves.

CONCLUSIONS

By using the HPLC-DAD connected to MS (4500MiD®, a single quadrupole) we analyzed the infusions of peppermint leaves and rhizomes. We identified and quantified one flavonoid and two caffeic acid derivatives in rhizomes and five flavonoid glycosides and three caffeic acid derivatives in leaves. The main component in rhizomes is the phenolic compound rosmarinic acid. Despite of its three times lower content than that in leaves, rhizomes may be considered as a potential source for pharmaceutical research and for use in the food industry. It could be beneficial to prepare and study other kinds of peppermint rhizomes extracts (also non-polar).

ACKNOWLEDGMENTS

This work was supported by the Grant Agency of Ministry of Education, Science, Research, and Sport of the Slovak (grant no. VEGA-1/0290/16) and by the Slovak Research and Development Agency under the contract No. APVV-15-0123.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Eur. Pharm. J. 2019, 66(1), 32-37.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.2478/afpuc-2019-0007

EUROPEAN PHARMACEUTICAL JOURNAL

Hippocampal electrophysiological responses and changes in oxidative stress marker and serum lipid profile to pharmacological and non-pharmacological treatments of high-fat-fructose diet induced metabolic syndrome

Original paper

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Received 14 April, 2018, accepted 1 June, 2018

Abstract The aim of our study was to evaluate the possibility of influencing the risk factors of metabolic syndrome (MetS) and metabolic cognitive syndrome. As a model of MetS, we used high-fat-fructose diet (HFFD) fed hypertriacylglycerolemic (HTG) rats. Control group included HTG rats fed with HFFD during 8 weeks (HFFD8). Furthermore, we tested the effect of pharmacological and non-pharmacological therapies. Non-pharmacological therapy, which we chose, was a change in diet from HFFD (5 weeks) to standard one (3 weeks) and thus caloric restriction (HFFD5+3). The drug we used was rosmarinic acid (RA; 100mg/kg), which we administered to rats after 5 weeks of HFFD once a day for consecutive 3 weeks with current change in diet to standard one (HFFD5+3+RA) or during lasting last 3 weeks of HFFD (HFFD8+RA). After 8 weeks of experiment, lipid peroxidation markers, lipid profile of blood serum, and neuronal transmission and synaptic plasticity (long-term potentiation [LTP]) in hippocampal sections were evaluated *in vitro*. We observed a significant effect of dietary change in lipid profile (decreased total cholesterol and low-density lipoprotein cholesterol [LDL-cholesterol] and increased high-density lipoprotein cholesterol [HDL-cholesterol]). The combination of pharmacological and non-pharmacological treatments caused a decrease in total cholesterol, LDL-cholesterol, and lipid peroxidation in blood serum. Change in HFFD to standard diet without treatment resulted in slight improvement in neuronal transmission in the hippocampus and caloric restriction alone also had positive effect on LTP maintenance. Our results suggest that combination of pharmacological and non-pharmacological approaches had better impact on the biochemical parameters of MetS in blood serum, but weak impact on neuronal functions in the hippocampus, where the expected positive effect was achieved only by caloric restriction.

Keywords *metabolic syndrome – high-fat-fructose diet – hippocampus – cholesterol – oxidative stress – rosmarinic acid – caloric restriction*

INTRODUCTION

Metabolic cognitive syndrome is a condition when metabolic syndrome (MetS) can lead to deterioration of cognitive abilities. Cognitive skills include memory, spatial memory, learning, executive functions, attention, language skills (Frisardi et al., 2010). MetS parameters such as obesity, dyslipidemia, hypertension, insulin resistance, inflammation, and oxidative stress may cause changes in the physiology that can affect cognition. The mechanisms responsible for this link are not clear. Mechanisms associated with systemic inflammation and inflammation in the nervous system, insulin resistance and oxidative stress in the brain, atherosclerosis, endothelial impairment and impaired capillary reactivity in the central nervous system, and abnormal lipid metabolism in the brain are discussed (Stoeckel et al., 2016). Rosmarinic acid (RA) is a phenolic acid that is contained in several plants used in medicine, mainly belonging to the *Laminaceae* family. It has several physiological effects, such as antioxidant, anti-inflammatory, antiviral, antibacterial, antidepressant, anticarcinogenic, chemopreventive, and neuroprotective effects (Bhatt at al. 2013). RA passes the blood–brain barrier through receptor-mediated transfer, combined with the ligand CRM197. RA then easily enters brain endothelial cells by endocytosis (Kuo and Rajesh, 2017). Recently, it was found that non-pharmacological approach such as changing diet

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habits or caloric restriction can reduce insulin resistance and increase physical fitness and overall metabolic health, which appears to reduce the risk for cardiovascular diseases (Grundy et al., 2013).

MATERIAL AND METHODS

Experimental design

We used Prague hereditary hypertriacylglycerolemic (HTG) rats from the Department of Toxicology and Breeding of Laboratory Animals of the Institute of Pharmacology and Toxicology, Centre of Experimental Medicine, Slovak Academy of Sciences (IEPT CEM SAS), Dobrá Voda. The experiments were approved by the State Veterinary and Food Administration of the Slovak Republic and the Ethical Committee of IEPT CEM SAS. At the beginning of the experiment, the animals were 10 weeks old. The animals had *ad libitum* access to food and water and light cycle 12/12 (12 hours dark, 12 hours light). We divided the animals into four groups: HFFD8 group: HTG rats were fed 8 weeks with modified diet (high-fat-fructose diet; HFFD); HFFD5+3 group: HTG rats were fed 5 weeks with HFFD and then 3 weeks with standard diet; HFFD8+RA group: HTG rats were fed 8 weeks with HFFD and last 3 weeks RA (100 mg/kg) was administered to them; HFFD5+3+RA group: HTG rats were fed 5 weeks with HFFD and then 3 weeks with standard diet along with the administration of RA (100 mg/kg). RA was administered once a day on crackers. Pellets of modified diet contained 1% cholesterol, 7.5% pork lard, and 10% fructose.

Determination of oxidative damage

Malondialdehyde (MDA), as marker of lipid peroxidation, was determined by the double heating method of Drapper and Hadley (1990). The principle of the method was spectrophotometric measurement of solution staining during the reaction of tertiary butyl alcohol with MDA.

Determination of lipid profile

Erba Lachema Ltd (Brno, Czech Republic) kits were used to determine the lipid profile from the blood serum. We measured the levels of total cholesterol, low-density lipoprotein cholesterol (LDL-cholesterol), and high-density lipoprotein cholesterol (HDL-cholesterol). Absorbance of the resulting colored compound was measured spectrophotometrically.

Electrophysiological measurement of neuronal function in rat hippocampus *in vitro*

Neurotransmission was determined by recording and digitizing electrically induced responses of hippocampus. We used artificial cerebrospinal fluid (ACSF) composed of 124 mmol/l of NaCl, 3.3 mmol/l of KCl, 1.25 mmol/l of KH_2PO_4, 2.4 mmol/l of $MgSO_4$, 2.5 mmol/l of $CaCl_2$, 26 mmol/l of $NaHCO_3$, and 10 mmol/l of glucose and saturated with 95% O_2 + 5% CO_2 at a pH of 7.4. Hippocampal slices (400-μm thick) were stimulated by bipolar stainless steel wire electrode. Electrically evoked responses were recorded using glass microelectrode filled with ACSF (3–5 MΩ) in the *stratum radiatum* of the rat hippocampus. We assessed the amplitude of excitatory postsynaptic potential (EPSP) as a measure of neuronal transmission. Long-term potentiation (LTP) was elicited by high-frequency stimulation (100 Hz, 1 s) and recordings continued next 40 min after train induction.

Statistical evaluation

The data were statistically evaluated using the GraphPad Prism6 Software (GraphPad, La Jolla, USA). Data were expressed as means ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to evaluate the difference among all experimental groups (using the Bonferroni multiple comparison test). The limit of $p<0.05$ was considered as statistically significant difference.

RESULTS

Total cholesterol increased significantly in all groups after 5 weeks of HFFD. Changing the diet to standard caused a decrease in its levels, which is comparable to the pre-treatment values. HDL-cholesterol decreased after consumption of HFFD diet and had a tendency to increase again as a result of both pharmacological and non-pharmacological approaches as well as their combination, but the combination did not produce a further increase in the effect. LDL-cholesterol levels increased after 5 weeks of HFFD and decreased after changing diet as well as decreased in the HFFD5+3+RA group (Table 1).

Lipid peroxidation was determined by the increase in MDA in blood serum after 5-week consumption of HFFD in all 4 groups. The combination of pharmacological and non-pharmacological therapies resulted in a decrease in lipid peroxidation, but the values were still elevated compared to the original ones. A change in diet or RA treatment alone did not cause a significant improvement (Figure 1A). No change in lipid peroxidation was observed in the cortex (Figure 1B).

During the recordings of electrically evoked responses in hippocampal slices *in vitro*, we found that diet change alone caused tendency to increase the amplitude of EPSP, (Figure 2A). LTP was damaged in HFFD8, HFFD+RA, and HFFD5+3+RA groups, whereas it persisted (134 ± 22%) in hippocampus of rats whose diet was changed to standard (Figure 2B).

DISCUSSION

MetS is a cluster of risk factors such as obesity, dyslipidemia, hypertension, oxidative stress, inflammation, elevated glucose levels, or insulin resistance. These metabolic disturbances can cause cognitive decline throughout several

Table 1: Cholesterol profile in blood serum of rats (mmol/l).
*HFFD, high-fat-fructose diet; HFFD8, HTG rats fed 8 weeks with HFFD; HFFD5+3, HTG rats fed 5 weeks with HFFD and then 3 weeks with standard diet; HFFD8+RA, HTG rats fed 8 weeks with HFFD and during last 3 weeks of this diet treated with rosmarinic acid; HFFD5+3+RA, HTG rats fed 5 weeks with HFFD and then 3 weeks with standard diet and treated with rosmarinic acid. Data are expressed as means ± SEM; n = 10. *p<0.05 versus same group before the diet, **p<0.01 versus same group before the diet, ***p<0.001 versus same group before the diet, #p<0.05 versus same group after 5 weeks of diet, ##p<0.01 versus same group after 5 weeks of diet, ###p<0.001 versus same group after 5 weeks of diet, ap<0.05 versus HFFD8 at the same time, bp<0.05 versus HFFD8+RA at the same time.*

	Total cholesterol			HDL-cholestero			LDL-cholesterol		
	Before diet	After 5 week	After diet	Before diet	After 5 week	After diet	Before diet	After 5 week	After diet
HFFD 8	1.84 ± 0.05	2.62 ± 0.08***	3.13 ± 0.11***	0.78 ± 0.03	0.50 ± 0.03***	0.61 ± 0.01*	0.40 ± 0.02	1.19 ± 0.05***	1.53 ± 0.09***##
HFFD 5+3	1.76 ± 0.04	2.92 ± 0.11***	2.19 ± 0.04###ab	0.77 ± 0.05	0.33 ± 0.01***	0.50 ± 0.04***#	0.38 ± 0.02	1.06 ± 0.09***	0.60 ± 0.09###ab
HFFD 8+RA	1.99 ± 0.04	2.78 ± 0.16***	2.98 ± 0.09***	0.84 ± 0.02	0.46 ± 0.02***	0.54 ± 0.03***	0.37 ± 0.03	1.11 ± 0.06***	1.41 ± 0.05***#
HFFD 5+3+RA	1.99 ± 0.08	2.70 ± 0.13***	2.23 ± 0.07###ab	0.83 ± 0.03	0.53 ± 0.02***	0.64 ± 0.04**	0.33 ± 0.02	1.26 ± 0.06***	0.57 ± 0.05###ab

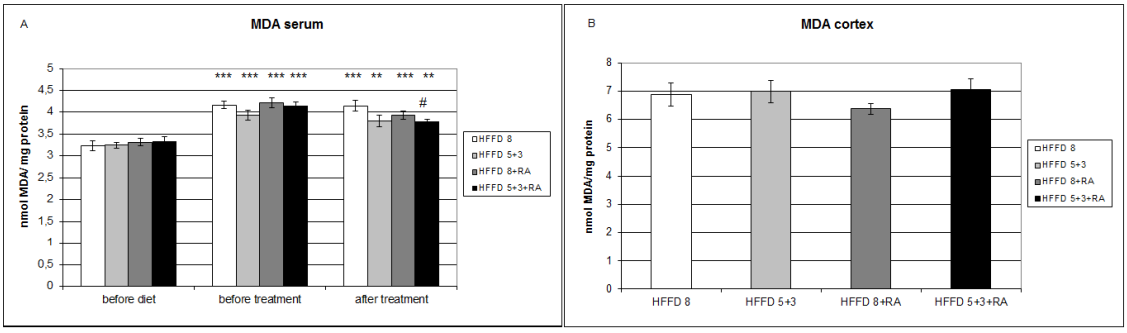

*Figure 1: Markers of lipid peroxidation in (A) blood serum and (B) cortex (mmol/mg protein). HFFD, high-fat-fructose diet; HFFD8, HTG rats fed 8 weeks with HFFD; HFFD5+3, HTG rats fed 5 weeks with HFFD and then 3 weeks with standard diet; HFFD8+RA, HTG rats fed 8 weeks with HFFD and during last 3 weeks of this diet treated with rosmarinic acid; HFFD5+3+RA, HTG rats fed 5 weeks with HFFD and then 3 weeks with standard diet and treated with rosmarinic acid; MDA, malondialdehyde. Data are expressed as means ± SEM; n = 10. **p<0.01 versus same group before the diet, ***p<0.001 versus same group before the diet, #p<0.05 versus same group after 5 weeks of diet.*

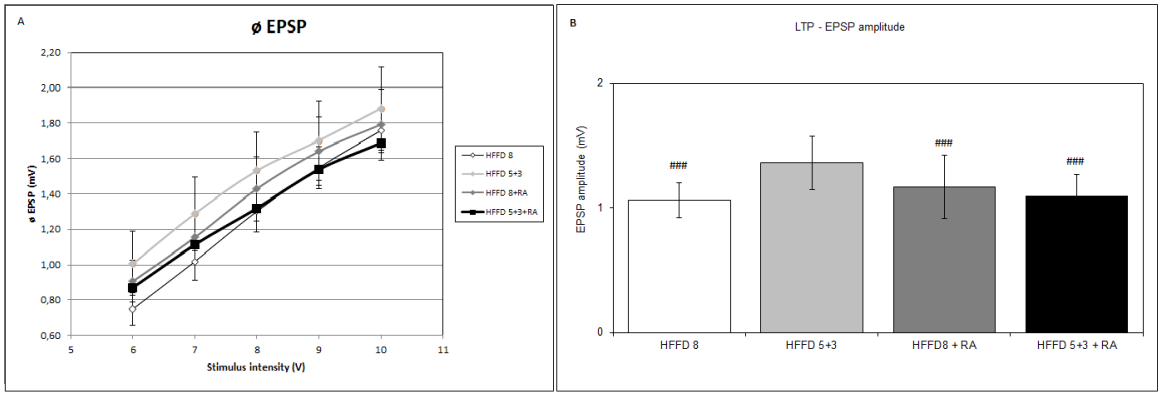

Figure 2: Electrically induced responses from hippocampal slices expressed as (A) excitatory postsynaptic potential (EPSP) amplitude (mV) (stimulus intensity 6–10 V) and (B) Long-term potentiation (LTP) (normalized values). HFFD, high-fat-fructose diet; HFFD8, HTG rats fed 8 weeks with HFFD; HFFD5+3, HTG rats fed 5 weeks with HFFD and then 3 weeks with standard diet; HFFD8+RA, HTG rats fed 8 weeks with HFFD and during last 3 weeks of this diet treated with rosmarinic acid; HFFD5+3+RA, HTG rats fed 5 weeks with HFFD and then 3 weeks with standard diet and treated with rosmarinic acid. Data are expressed as means ± SEM; for (a) n = 17–24 hippocampal slices/10 rats/group; and for (b) n = 10 hippocampal slices/10 rats/group. ###p<0.001 versus HFFD5+3.

mechanisms. Our animal model was a combination of genetic- and diet-induced MetS-like conditions. Metabolic changes of MetS can lead to the development of cardiovascular and cerebrovascular diseases, and it is, therefore, necessary to look for an appropriate method of intervention. In the present work, we tested the effect of pharmacological and non-pharmacological treatments and their combination on individual risk factors. Changing diet to standard caused decrease in total cholesterol levels almost to their original levels. HDL-cholesterol tended to rise again as a result of both pharmacological and non-pharmacological therapies. LDL-cholesterol levels were increased because of HFFD and decreased after caloric restriction. Changing the diet to standard reduced lipid peroxidation, and RA has multiplied this effect, but RA alone had no effect. No change in the oxidative stress marker MDA was found in rat brain cortex. Neuronal transmission was slightly improved in hippocampus by dietary change as well as LTP was retained in this group, but RA alone or its combination with caloric restriction did not cause any improvement in hippocampal function.

In our study, we used three ways to ameliorate MetS components, that is, caloric restriction induced by changing HFFD to standard diet, pharmacological treatment with RA, and combination of both approaches. Caloric restriction is an effective therapeutic approach to improve metabolic state in the body. Caloric restriction associated weight loss decreases the accumulation of triacylglycerols (TAG) in tissues. It also causes decrease in adipose mass, oxidative stress, and inflammation, leading normalization of glucose homeostasis and lipid metabolism (Nikhra, 2018). RA has several biological effects, such as antiviral, antibacterial, anti-inflammatory, antioxidant, and anticancer effects (Pettersen and Simmons, 2003). RA promotes good endothelial and blood cell status and is also used to combat skin carcinogenicity as it can be absorbed through and stored in the skin, muscle, and bones (Osakabe et al., 2004; Ueda et al., 2003). It is also absorbed from the gastrointestinal tract; thus we administered it orally (Al-Sereiti, 1999). Unconjugated RA remains in the bloodstream long enough to reach the brain (Fale et al., 2011) and it crosses the blood–brain barrier via transporters (Kuo and Rajesh, 2017). On the basis of the literary data, effect of RA has not been studied in combination with caloric restriction so far.

We found that lipid peroxidation in blood serum increased after consumption of HFFD. The combination of pharmacological and non-pharmacological therapies caused a significant decrease in lipid peroxidation, but the values were still elevated compared to the original ones. A change in diet or RA alone did not cause a significant change. No change in lipid peroxidation was observed in the cortex. Matsuo and coauthors (1993) found that caloric restriction reduces the rate of accumulation of oxidatively damaged molecules and inhibits the increase in lipid peroxidation. It has been repeatedly reported that caloric restriction is capable of inducing mechanisms to protect against stress, especially those involved in the detoxification of reactive oxygen species (ROS) (Ristow and Schmeisser, 2014). RA has the ability to penetrate lipid bilayer, which alters membrane fluidity and protects cell membrane against chain-breaking free radicals (Fadel et al., 2011).

We found out that total cholesterol levels were increased after HFFD. Thus caloric restriction in combination with changing HFFD to standard diet plus RA treatment caused a decrease in these levels. HDL-cholesterol levels decreased after the consumption of food rich in fat and fructose. Significant improvement in HDL-cholesterol levels was achieved by 3-week caloric restriction applied after HFFD. LDL-cholesterol levels increased after HFFD and decreased after caloric restriction and also in the combined HFFD5+3+RA group. LDL-cholesterol is now considered as MetS sign, and it is still the most common marker of cardiovascular diseases (CVD) risk and atherogenic dyslipidemia. The results of a clinical study by Al-Sarraj and co-workers (2009) showed a significant decrease in small LDL-cholesterol particles in individuals who consumed less calories, in particular less saccharides. Fat reduction in the diet has minimal effect on HDL-cholesterol but carbohydrate restriction had a significant effect (Al Sarraj et al., 2009). The mechanism that may be responsible for the improvement in the lipid profile after caloric restriction, especially carbohydrate restriction, is that high insulin suppresses lipolysis and increases de novo lipogenesis. Caloric limitation simultaneously leads to a decrease in the concentration of malonyl-coenzyme A and dis-inhibition of carnitine acyltransferase, which caused an increase in β-oxidation of fatty acids (Volek et al., 2009). Our results are in accordance with these results and also with the results of Govindaraj and Pillai (2015), who reported elevated levels of free fatty acids, total cholesterol, and TAG in the rats with HFD. Rats fed with HFD have hypertriacylglycerolemia and hypercholesterolemia, the condition which is reversed after oral administration of RA. These results show the beneficial effects of RA in preventing metabolic complications.

In our work, we anticipated an improvement in the electrophysiological responses of the hippocampus. In the electrophysiological response of the hippocampus, we observed that diet change alone caused a slight increase in the EPSP amplitude. No improvement in neurotransmission was observed in RA-treated group and in combination of non-pharmacological and pharmacological treatments. LTP retained only in the group with caloric restriction. Recently, it was shown that caloric restriction induces a neuroendocrine response such as an increase in neuropeptide Y (NPY) (Minor et al., 2009; Bi et al., 2003). Activation of NPY receptors has neuroprotective effects in various regions of the brain and results in delayed neurodegenerative damage (Decressac and Barker, 2012). In addition to NPY, the production of ghrelin, which has many physiological functions throughout the body and in the central nervous system, is also increasing

by caloric restriction (Ferrini et al., 2009). Ghrelin is also involved in memory and learning and has a neuroprotective effect in neurodegenerative diseases and in ischemic brain damage (Spencer et al., 2014). Previous studies, as well as our work, suggest that cognitive decline occurs with MetS, which deepens over time. Among the factors connecting MetS with cognitive decline, the brain-derived neurotrophic factor (BDNF) in the hippocampus is reduced. After 28 day of caloric restriction, body weight, insulin and fasting glucose levels, adiponectin, systolic blood pressure, and oxidative stress in the hippocampus were significantly reduced and BDNF expression in the hippocampus was significantly higher (Kishi et al., 2015). We assume that in mechanism of slightly improved response in the hippocampus on electrical stimulation induced by caloric restriction, an increase in BDNF expression and its ability to affect neurotransmitter release might be involved (Sasaki et al., 2013; Yan and Yan, 2006). Concerning RA effect, it was reported that glutamate receptor-2 (GluR-2) is enhanced by RA treatment, which has an important impact on synaptic plasticity because GluR-2 has an effect on intracellular translocation and folding of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunits (Cull-Candy et al., 2006). RA also acts at the level of *N*-Methyl-d-aspartate (NMDA) receptors (Morris, 1989). The reason or mechanism why RA did not act in our study on improvement of hippocampal neurotransmission as well as LTP is unclear. There is a possibility that administration of RA was not long enough or there was some underlying mechanism that inhibited passing of RA through blood–brain barrier in our experimental design. On the other hand, the best reduction of blood serum MDA level was achieved by the combination of RA treatment and caloric restriction. As a conclusion, we can assume that caloric restriction, RA acid treatment, and their combination could be promising in the management of MetS-related disorders; therefore, further detailed studies are needed.

ACKNOWLEDGMENTS

The study was supported by the Slovak Grant Agency for Science with grants VEGA 2/0054/15 (2015 -2018) and VEGA 2/0120/19 (2019-2022).

ABBREVIATIONS

ACSF – artificial cerebrospinal fluid
AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA – analysis of variance
BDNF – brain-derived neurotrophic factor
CVD – cardiovascular diseases
EPSP – excitatory postsynaptic potential
GluR 2 – glutamate receptor 2
HDL-cholesterol – high-density lipoprotein cholesterol
HFD – high-fat diet
HFFD – high-fat-fructose diet
HTG – hypertriacylglycerolemic
IEPT CEM SAS – Institute of Experimental Pharmacology and Toxicology, Centre of Experimental Medicine, Slovak Academy of Sciences
LDL-cholesterol – low-density lipoprotein cholesterol
MDA – malondialdehyde
MetS – metabolic syndrome
n – number
NMDA – *N*-Methyl-d-aspartate
NPY – neuropeptide Y
RA – rosmarinic acid
ROS – reactive oxygen species
SEM – standard error of the mean
TAG – triacylglycerols

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