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PITUITARY HORMONE-PRODUCING CELLS AFTER ESTRADIOL APPLICATION IN RAT MODELS OF MENOPAUSE

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HORMON-PRODUKUJUĆE ĆELIJE HIPOFIZE NAKON PRIMENE ESTRADIOLA U ANIMALNOM MODELU MENOPAUZE

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ABSTRACT

Female ageing represents the biological process of structural and functional changes in endocrine cells and tissues, as well as in pituitary hormone-producing cells. In addition to the hypothalamic releasing hormones, estradiol plays a significant role in the regulation of the synthesis/secretion of pituitary hormones and is still used therapeutically for menopausal symptoms. The effects of ageing or ovariectomy and synthetic estradiol application under these circumstances were evaluated in pituitary hormone-producing cells of female rats (animal models of menopause); i.e., the following cells were observed: gonadotropes (FSH and LH), thyrotropes (TSH), somatotropes (GH), mammotropes (PRL) and corticotropes (ACTH). The cells were immunostained and histologically analysed. The ELISA method was used for hormonal analyses. Ageing was found to cause diverse, commonly reductive changes regarding the volume, number and secretion of menopausal rat pituitary hormone-producing cells, except for PRL cells that exhibit significantly increased numbers and intensified secretion. After the treatment of middle-aged female rats with estradiol, the absolute and relative pituitary weights significantly increased in comparison with the control females. Histological parameters such as the cell and volume density of PRL and ACTH cells were significantly increased compared with the control values. The mentioned parameters of FSH, LH, GH, and occasionally TSH cells after estradiol treatment significantly decreased in comparison with the controls. The corresponding hormone levels followed the changes in the histological parameters. These data indicate that the application of estradiol to menopausal females may specifically, in two directions, modify the histological characteristics and secretory activities of different pituitary-hormone producing cells.

Key words: *female rat, middle age, pituitary cells, estradiol.*

SAŽETAK

Starenje kod ženskog pola je biološki proces tokom kojeg dolazi do promena u strukturi i funkciji endokrinih ćelija i tkiva, a posebnu osetljivost ispoljavaju hipofizne hormon-produkujuće ćelije. Pored oslobađajućih hormona hipotalamusa i estradiol ostvaruje zapaženu ulogu u regulaciji sinteze i sekrecije hipofiznih hormona, a još uvek se koristi u terapiji menopauzalnih tegoba. Efekti starenja ili ovarijektomije, kao i primene sintetskog estradiola pod tim okolnostima su ispitivani na hormon-produkujućim ćelijama hipofize ženki pacova srednjeg doba (animalni modeli menopauze) i to: gonadotropima (FSH i LH), tirotropima (TSH), somatotropima (GH), mamotropima (PRL) i kortikotropima (ACTH). Sve ćelije su imunohistohemijski bojene i histološki analizirane, dok je za određivanje nivoa hormona u krvi korišćena ELISA metoda. Utvrđeno je da starenje prouzrokuje različite, uglavnom reduktivne promene volumena, broja i sekrecije hormon-produkujućih ćelija hipofize u našim animalnim modelima menopauze, izuzev na primeru PRL ćelija čiji je broj značajno povećan, a sekrecija intenzivirana. Posle tretmana ženki pacova srednjeg doba estradiolom apsolutna i relativna masa hipofize je značajno povećana u poređenju sa kontrolnim ženkama. Histološki parametri poput volumena ćelija i volumenske gustine PRL i ACTH ćelija su značajno povećani u poređenju sa kontrolnim vrednostima. Sa druge strane, pomenuti parametri u FSH, LH i GH, a u izvesnim slučajevima i TSH ćelijama su značajno smanjeni nakon tretmana estradiolom, u poređenju sa kontrolama. Koncentracije odgovarajućih hormona u krvi su pratile navedene promene histoloških parametara. Ova zapažanja ukazuju da estradiol davan menopauzalnim jedinkama ženskog pola može na specifičan način, dvosmerno, modifikovati histološke karakteristike i sekretornu aktivnost različitih hormon-produkujućih ćelija hipofize.

Ključne reči: *ženke pacova, srednje doba, hipofizne ćelije, estradiol*

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INTRODUCTION

Endocrine function realisation is achieved through the multi-level systemic organisation, implying the participation of the hypothalamus, pituitary gland, immune system and various target endocrine organs and tissues. Therefore, the pituitary gland represents the entity of special interest because it orchestrates the immuno-neuro-endocrine interactions by the means of neuronal, hormonal, paracrine, juxtacrine and autocrine communication (1-2). The anterior part of the gland consists of several types of hormone-producing cells (gonadotropes - LH and FSH, thyrotropes - TSH, somatotropes - GH, mammotropes - PRL and corticotropes - ACTH), as well as folliculo-stellate non-hormone producing cells (3). Considerable interest has permanently been generated regarding hypothalamo-pituitary age-related changes. The most common anterior pituitary morphologic lesions in ageing involve some hyperplasia or adenoma (4), which are pathologies that are strongly linked to reproductive system function decline (5). Pertinent to this decline, ovarian follicular depletion in ageing females, followed by a decrease in circulating estradiol, is a critical factor triggering the menopausal transition (6), along with the distinctive changes at the level of the hypothalamus and anterior pituitary hormone-producing cells in the same individuals (7-8). In support of the latter, it was observed that the LH response to GnRH is decreased in older females compared with young females (7). Simultaneously, alterations in some other hormone-producing cell populations were also found in ageing female rats and women (5, 9-11). Despite some risks (12), estradiol replacement represents the classical therapeutic approach in the prevention of health problems due to ovarian hormone deficiency (13, 14). Various animal models have been developed to define the space for problematising the multiple morphofunctional changes during menopause, as well as to solve the present controversies regarding the application of estradiol in humans. Our experimental work in the field implied the usage of intact middle-aged (14 months old) or ovariectomised adult (3 months old) Wistar female rats, for which the pituitary histological and hormone secreting studies had a central role (10, 11, 15-18). Herein, we aim to present the accumulated data regarding the various populations of anterior pituitary hormone-producing cells in our animal models of menopause and in related studies, as well as to elaborate the observed effects of synthetic estradiol application in these experimental studies.

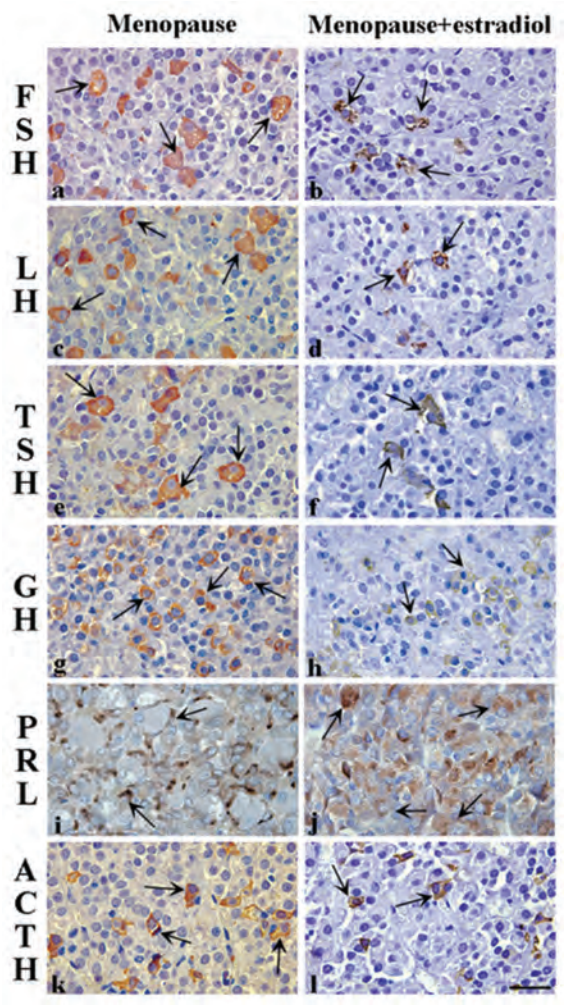

Figure 1. Immunohistochemically labelled pituitary hormone-producing cells in menopausal (a – FSH; c – LH, e – TSH, g – GH, i – PRL and k – ACTH) and estradiol-treated menopausal (b – FSH, d – LH, f – TSH, h – GH, j – PRL and l – ACTH) female rats. Peroxidase-antiperoxidase method, bar=16 μm.

Menopausal rat pituitary hormone-producing cells

Ageing is associated with a myriad of anatomical and functional changes of the endocrine glands, in the last instance as a result of programmed cell death and also as a consequence of autoimmune-mediated destruction or neoplastic transformation of the glandular tissue (19). Commonly, ageing represents the critical biological period for the control of gene expression and secretory activity in various endocrine cells and tissues, as well as in the entire hormone-producing cell pool in the pituitary gland (5, 20, 21). Female ageing has been our research focus for years, and we exploited intact or ovariectomised female rats as a good menopausal model. The absolute and relative pituitary weights in our models significantly increased by 16.2% and 14.0%, respectively, compared with those of intact adult female rats (22).

As mentioned previously, gonadotropes are subjected to substantial changes with reproductive ageing in women. Our histological analysis of FSH and LH cells in middle-aged female rats showed that they were both ovoid and polyhedral in shape, occurred individually or more frequently in clusters, and established close contact with blood vessels (Figure 1 a, c) (15). Their nuclei appeared to be round, vesicular and mostly eccentric (Figure 1 a, c) (23). In middle-aged female rats, the volume of FSH cells was 1375 μm^3, and the volume of LH cells amounted to 998 μm^3. The number of immunolabeled FSH and LH cells *per* unit area (mm^2) was 78 and 142, respectively (23). Ultrastructural investigations showed that the gonadotropes had well-developed rough endoplasmic reticula (RER) and Golgi complexes (4). Secretory granules in female gonadotropes were spherical or slightly irregular with a small

diameter (200-250 nm) (24). Plasma levels of FSH and LH in our middle-aged female rats were 3.67 mU/L and 537.30 mU/L, respectively (15). In middle-aged female rats, changes in pulsatile LH secretion were observed, whereas gonadotrophin-releasing hormone (GnRH) could not be detected in the peripheral plasma. Therefore, LH may be used as an indirect index of GnRH secretion in this age range (25).

TSH cells in middle-aged female rats from our experiments were mainly localised in the ventral or middle portion of the anterior pituitary gland (10). These cells adopted a variety of shapes (round, polyhedral or irregular) and had an intensely immunostained cytoplasm (Figure 1 e). Their volume density amounted to approximately 15% (10). Specifically, the number of TSH cells in middle-aged female rats *per* unit area (mm^2) was 110 (10), and their volume decreased with age (26). The observed reduction of TSH activity in middle-aged female rats is associated with its large molecular form accumulating in the pituitary gland and in blood (27).

Immunohistochemical studies identified GH cells in middle-aged female rat pituitary glands having ovoid to pyramidal shapes with a spherical, centrally located nucleus. These cells were uniformly distributed throughout the gland (Figure 1 g) (18, 28). In our model of menopause, GH cells were usually found situated along the sinusoid capillaries (18). In terms of absolute values, the cellular and nuclear volumes of the GH cells, and their relative volume density were 1215 μm^3, 61 μm^3 and 28%, respectively (29). Morphological analysis of the GH cells revealed that their heterogeneity rises in aged females (30), whereas the number of type II and III of GH cells increased with ageing (31). Namely, the type II GH cells contained large and small secretory granules, whereas the type III GH cells contained only small secretory granules (28). At the ultrastructural level, the RER were found disposed in parallel arrays, and the Golgi complex was moderately developed. Lysosomes that were occasionally observed in GH cells of acyclic female rats (4, 28, 32) demonstrated that the GH secretion declines with ageing and the GH-mRNA content decreases due to diminished transcriptional activity of the adequate genes (33). The serum GH level decreased in middle-aged female rats, which is explained by the increased release of somatostatin and the decreased release of growth hormone-releasing hormone (GHRH) from the hypothalamus (34), as well as with a reduced pituitary responsiveness to GHRH stimulation (35). In young female rats, the blood GH levels were significantly higher than those of middle-aged females, whereas the amplitude of GH pulsatile release was lower in the middle-aged rats compared with the young rats (34). In our model of ovariectomised female rats, the GH levels significantly decreased, by 75.8%, compared with the intact controls (22).

In the middle-aged female rat pituitary glands we examined, immunohistochemically labelled PRL cells were polygonal, elongated in shape, had a spherical eccentrically located nucleus and and were sporadically distributed in the anterior-ventral portion of the gland (Figure 1 i) (11). We observed that the cellular and nuclear volumes of the PRL cells, and their relative volume density in this animal model of menopause were 1180 μm^3, 124 μm^3 and 35.8%, respectively (29). Immuno-electron-microscopical studies showed that the PRL cells have abundant RER and a prominent Golgi complex, which contains ovoid or markedly pleomorphic electron-dense secretory granules (4). Importantly, rat PRL cells are divided into three subtypes based on the size of the granules (31). Type I cells contain large, irregularly shaped secretory granules (300-700 nm), type II have spherical granules (150-250 nm), and type III contain some small round granules (of 100 nm) (31). A significant increase in the number of PRL cells and their storage secretion was observed in middle-aged female rats by Takahashi et al. (1984) and Itoh at al. (2001), who investigated their pituitary morphophysiology (36, 37). Takahashi and Kawashima (1987) observed a significant age-related increase of the DNA content in PRL cells in rats of both sexes; however, this increase was more conspicuous in the females than in the males (38). Interestingly, age-related differences were significant in the relative proportion of each subtype of PRL cells (28). Namely, with age, the number of PRL cells with large secretory granules (type I) (31) and the concentration of PRL in the blood of female rats increased (5). Ovariectomy decreased the volume density of type I PRL cells by 32% but increased the same parameter of type II PRL cells by 52% and of type III by 16% (28). In ovariectomised female rats, the PRL blood levels remain elevated (28).

In our menopausal female rats, immunohistochemically labelled pituitary ACTH cells were stellate in shape, possessed cytoplasmatic processes amongst neighbouring cells and resided between the capillaries (Figure 1 k) (17). The nuclei followed the cell shape (Figure 1 k) (17). Compared to the same cell phenotype in adult female rats, their localisation and shape did not significantly change (39). In other studies of female rat ageing, electron microscopy analyses of ACTH cells demonstrated that the secretory granules are usually numerous, spherical or irregularly shaped (40) and have small secretory granules of (150-200 nm) distributed mainly at the periphery of the cytoplasm (4). Additionally, the RER, Golgi complex and mitochondria appear to be near the nucleus (41). Histological and ultrastructural changes annotated at the level of ACTH cells during ageing generally decrease the sensitivity of the hypothalamo-pituitary system to glucocorticoid negative feedback (42).

The effects of estradiol application on pituitary hormone-producing cells in menopausal rats

The synthesis and secretion of pituitary hormones is centrally regulated by hypothalamic-releasing hormones; however, they are also secreted by some other peripheral hormones. Among other things, estradiol is considerably involved in the control of various pituitary hormone-producing cells in female rats (22, 23, 28, 43). Namely, the presence of estradiol in the pituitary *pars distalis* was observed in the nuclei of all of the hormone producing cells, including acidophils, basophils and chromophobes (44). We have demonstrated previously that multiple estradiol doses administered to middle-aged female rats increase the number of chromophobe and PRL cells

(35, 45). Finally, estradiol replacement represents the classical therapeutic approach in the prevention of menopausal problems, although manifesting some risks (12-14). We used the above-mentioned menopausal rat models to critically evaluate the effects of synthetic estradiol application, and the following pituitary hormone-producing cell changes were of special interest. Primarily, the absolute and relative pituitary weights in estradiol-treated middle-aged female rats significantly increased by 110% and 123%, respectively, in comparison with the control female rats (11).

The extensive analysis showed that the volume and volume density of FSH cells, and the FSH blood levels in middle-aged female rats treated with estradiol-dipropionate (EDP) significantly decreased by 35%, 65% and 46%, respectively, in comparison with the control values (15, 46). In this animal model, the above-mentioned parameters of LH cells significantly decreased by 13%, 54% and 55%, respectively, compared with the corresponding control values (15, 46). After multiple EDP treatments, the FSH and LH cells of middle-aged females were smaller and pycnotic and exhibited darker immunohistochemical staining in comparison with the untreated controls; however, their shape and distribution were not significantly altered (Figure 1 b, d) (15, 23). The numbers of FSH and LH cells per mm^2 significantly decreased after treatment with EDP in the same model by 54% and 55%, respectively, compared with the control females (23). In ovariectomised rats treated with EDP, the number of FSH cells *per* mm^2 was 6-fold, and the LH cells exhibited a 4-fold decrease in comparison with the control values (15). These reduced parameters of the gonadotropes, reflecting, *inter alia,* their decreased content of stored FSH and LH, suggest the suppression of their synthetic activity after EDP treatment (45).

In middle-aged female rats treated with EDP, the immunopositive TSH cells were pycnotic and darkly stained (Figure 1 f) (10). In the animal model of menopause we used, the volume and volume density of the TSH cells significantly decreased after treatment with EDP by 44% and 67%, respectively, in comparison with the control females (10). In contrast, the relative cellular and nuclear volumes of TSH cells increased (by 7% and 30%, respectively) in ovariectomised female rats treated with EDP compared with the control intact females (47). The observed differences in the immunohistomorphometric characteristics of TSH cells in intact and ovariectomised female rats treated with EDP may be the result of different administered estradiol doses.

In our EDP-treated menopausal rats, the immunohistochemically stained pituitary GH cells were smaller and irregularly shaped and possessed a strong cytoplasmatic immuno-signal compared with the controls (Figure 1 h) (18). In these animals, the cellular and nuclear volumes, and the relative volume density of the GH cells significantly decreased 72%, 11% and 86%, respectively, compared with the control menopausal females (29). We also observed that estradiol inhibits GH secretion up to 65% (28, 29), which reduces sensitivity to GHRH (35). Mooradian (1993) suggested an age-related decrease in the number of pituitary oestrogen receptors (19). Takahashi (1992) demonstrated that estradiol increases the percentage of type II and III GH cells (31). These cell types contain small secretory granules, a pronounced characteristic of the older rat population (31), which may support our results. Additionally, the GH blood levels after EDP treatment in ovariectomised female rats increased by 45% compared with intact female rats (21), indicating the significance of the removal of the ovaries the outcome of GH secretion.

An increase in the pituitary weight after estradiol treatment was previously observed and is the consequence of PRL cell proliferation (48). Murai and Ben-Jonathan (1990) postulated that estradiol plays a crucial role in the release of PRL (49). The pituitary PRL cells in middle-aged female rats after multiple treatments with the EDP are larger in size, irregularly shaped and more intensely immunostained compared with the controls (Figure 1 j) (11). The treatment of female middle-aged rats with EDP significantly increases the immunohistomorphometric parameters of their PRL cells, especially their volume density (11, 28, 29). Furthermore, estradiol application increased the percentage of PRL cells with large secretory granules (type I) and decreased the percentages of type II and III cells (31). The observed increase was consistent with higher serum (11, 29) and pituitary PRL levels (50) in rats, which is the biochemical data also found in menopausal women (51). The described changes were attributed to the reduction of hypothalamic dopamine activity (4). Treatment of ovariectomised, middle-aged female rats with 17β-estradiol significantly increased the volume density of the PRL cells, of which the most numerous were type I cells, by 160% compared with the control females (28).

Immunohistochemical analysis of ACTH cells in our middle-aged female rats treated with EDP showed that neither their shape nor their localisation significantly changed in comparison with the controls; however, the ACTH cells appeared less intensely immunostained (Figure 1 l) (17). In parallel, EDP application in the same model significantly increased the volume and volume density of ACTH cells by 10% and 43%, respectively, in comparison with the control females (17).

CONCLUSIONS

Our observations of various histological and hormone secreting changes in the various hormone-producing cells in the ageing or ovariectomised female pituitary gland, as well as after synthetic estradiol application, are based on the exploitation of the adequate animal models of menopause. We have found that ageing causes diverse, commonly reductive changes regarding the volume, number and secretion of menopausal rat pituitary hormone-producing cells, except for PRL cells that manifest increased number and intensified secretion. As expected, after the treatment of middle-aged female rats with estradiol, the absolute and relative pituitary weights significantly increased in comparison with the control females. Histological parameters such as the cell and volume density of PRL and ACTH cells significantly increased compared with control values. In contrast, the

mentioned parameters of FSH, LH, GH, and occasionally TSH cells after estradiol treatment significantly decreased in comparison with the controls. Notably, ovariectomy and estradiol treatment in adult female rats caused the increase of the TSH cell histological parameters. In general, the corresponding hormone level alterations followed the observed changes of histological parameters in our models. These data indicate that the application of estradiol to menopausal females may specifically, in two directions, modify the histological characteristics and secretory activities of various pituitary-hormone producing cells.

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UNFOLDED PROTEIN RESPONSE IS ACTIVATED IN THE HEARTS OF CATECHOLAMINERGIC POLYMORPHIC VENTRICULAR TACHYCARDIA (CPVT) MICE

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ODGOVOR NESAVIJENIH PROTEINA JE AKTIVIRAN U SRCU MIŠEVA SA GENETSKI IZAZVANOM KATEHOLAMINERGIČKOM POLIMORFNOM VENTRIKULARNOM TAHIKARDIJOM

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ABSTRACT

Isoform 2 of calsequestrin (CSQ2) is the main calcium-binding protein of the sarcoplasmic reticulum (SR) and is expressed in both cardiac and skeletal muscle. CSQ2 acts as an SR calcium (Ca^{2+}) sensor and regulates SR Ca^{2+} release via interactions with triadin, junctin, and the ryanodine receptor. Various mutations of the csq2 gene lead to altered Ca^{2+} release and contractile dysfunction and contribute to the development of arrhythmias and sudden cardiac death in young individuals affected by CPVT . Transgenic mice carrying one of the identified CSQ2 point mutations (R33Q) associated with CPVT were developed, and a drastic reduction in the mutated protein was observed. Following a biomolecular approach, several analyses were performed using different antibody treatments to identify when the reduction of CSQ2 begins, to unveil the mechanism involved in the reduction of CSQ2 and to verify whether other proteins are affected by the presence of the mutated protein. The results of this study showed that mutated CSQ2 levels decreased soon after birth, in conjunction with decreased levels of other important junctional SR proteins, including triadin (TD). The up-regulation of proteins associated with the unfolded protein response (UPR) was also observed, and the ATF6-dependent pathway was activated by the UPR. The presence of the R33Q mutation induced the decrease of CSQ2 via UPR activation and subsequent proteasomal degradation.

Keywords: *Calsequestrin, CPVT, ERAD, UPR, Triadin, Chaperones.*

SAŽETAK

Izoforma 2 kalsekvestrina (CSQ2) je glavni kalcijum-vezujući protein sarkoplazmatskog retikuluma (SR) i nalazi se kako u srčanom tako i u skeletnom mišiću. CSQ2 deluje kao kalcijumski receptor koji reguliše oslobađanje Ca^{2+} jona iz SR, putem interakcije sa triadinom, junktinom i rianodinskim receptorom. Različite mutacije csq2 gena mogu da izazovu poremećaje u oslobađanju Ca^{2+} i time kontraktilne funkcije, čime doprinose ravoju aritmija i iznenadnoj srčanoj smrti mladih osoba koje boluju od kateholaminergičke polimorfne ventrikularne tahikardije (CPVT). Razvojem transgenetskih miševa sa CSG2 point mutacijom (R33Q) i CPVT-om, primećen je drastičan pad nivoa mutiranog proteina. Prateći biomolekularni pristup, nekoliko analiza je izvedeno, koristeći tretman različitim antitelima, sa ciljem da se otkrije kada počinje smanjenje nivoa CSQ2, rasvetli mehanizam uključen u redukciju CSQ2 i ispita da li prisustvo mutiranih proteina utiče i na druge proteine. Rezultati ove studije su pokazali da se nivoi mutiranih CSQ2 smanjuju ubrzo nakon rodjenja, što je udruženo sa smanjenim nivoom ostalih značajnih proteina SR, uključujući triadin (TD). Takođe je primećeno da odgovor nesavijenih proteina može biti povezan sa ushodnom regulacijom proteina i aktivacijom ATF-6 zavisnog signalnog puta. Prisustvo R33Q mutacije je izazvalo smanjenje nivoa CSQ2 putem aktivacije odgovora nesavijenih proteina i posledične proteozomalne degradacije.

Ključne reči: *Kalsekvestrin, CPVT, ERAD, UPR, triadin, haperoni*

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INTRODUCTION

Sudden cardiac death causes approximately 300,000 deaths each year in Europe and the U.S.A.; ventricular fibrillation is the underlying mechanism of most diseases that lead to sudden cardiac death. The identification of mechanisms causing life-threatening arrhythmias is a major priority in the biomedical field (1, 2). Given the crucial role of Ca^{2+} in the generation of focal arrhythmias, the mechanisms responsible for Ca^{2+} homeostasis in the SR of cardiomyocytes are of great importance in cardiac pathophysiology. The discovery of SR proteins, such as ryanodine receptor (RyR) and CSQ, that play key roles in the mechanisms behindunderlying inherited arrhythmia syndromes such as CPVT emphasises the link between changes in intracellular Ca^{2+} homeostasis and arrhythmogenesis (3, 4). CPVT occurs at a young age as a result of exercise or emotional stress, is characterised by recurrent syncope, convulsions, and cardiovascular collapse and can lead to sudden death (4). Homozygous CSQ2 mutations (5) explain 3% of all CPVT cases, while approximately 50% of patients have mutations in RyR2 (6). Song and colleagues (7) have demonstrated the existence of common pathophysiological mechanisms caused by alterations in RyR2 and/or CSQ2 that relate to CPVT.

To close the gap between experimental and clinical data, it is extremely important to develop experimental animal models that phenotypically copy the clinical disease. This "challenge" is particularly demanding in arrhythmogenic diseases because of the large differences between humans and mice in ion channel profiles and action potential durations. CPVT has proven to be an exception in comparison with other hereditary diseases, represented by transgenic mice that have a low level of analogy with the phenotype of human patients . Knock-in (KI) RyR2-R4497 (8) and CASQ2-R33Q (9) mouse models currently exist as autosomal dominant and recessive CPVT models, respectively. The delicate balance that regulates the flow of calcium through the membrane of the SR in cardiomyocytes and, consequently, the balance between cytoplasmic and extracellular Ca^{2+} play critical roles in ensuring cell viability, preserving normal contractile function and providing heart rate stability. The SR, an important cell organelle, and the two SR proteins RyR and CSQ play crucial roles in these processes. In fact, the mechanism associated with depolarisation of the muscle fibre plasma membrane followed by release of Ca^{2+} from the SR, called coupling excitation-contraction (EC), depends on the plasma membrane calcium channel L type receptor dihydropyridine (DHPR) and the RyR receptor present on the membrane of SR terminal cisternae; both are Ca^{2+} release units (CRUs) (10). Calsequestrin is the major Ca^{2+}-binding protein present in the junctional SR of skeletal and cardiac muscle fibres. In mammals, there are two CSQ isoforms encoded by different genes: the skeletal isoform CSQ1 is expressed in fast- and slow-twitch skeletal muscle, while the cardiac isoform CSQ2 is expressed in both cardiac and skeletal muscle. Some CSQ2 mutations seem to affect protein synthesis, leading to the reduction or absence of CSQ2 in the heart (9), while other mutations seem to induce the expression of defective proteins, which show altered abilities to bind Ca^{2+} and/or to perform their proper function (9). All experimental evidence has implicated mechanisms of post-transcriptional regulation. Among the possible mechanisms involved in this regulation is endoplasmic reticulum (ER)-associated protein degradation (ERAD), which is responsible for the degradation of proteins synthesised at the ER lumen via the proteasome following activation of an ER stress response pathway.

Various factors can activate an ER stress response pathway. One such factor, the presence of incorrectly folded proteins, activates the unfolded protein response (UPR). To reach a possible explanation for the drastic reduction of CSQ2-R33Q in 8-week- old adult mice (9), the first objective was to determine at what age the CASQ2 reduction began. The other objectives were to determine whether any other proteins were affected by the reduced levels of CSQ2 and to reveal the mechanisms involved in the reduction of CSQ2-R33Q.

MATERIALS AND METHODS

Molecular analyses were performed on total heart homogenates of 4 wt/wt and 4 knock-in CSQ2-R33Q/R33Q mice, respectively. All experimental protocols were approved by the Institutional Ethical Committees of the Universities of Padova and Chieti. Mice were kept in accredited animal facilities and sacrificed by anaesthetic euthanasia. Four hearts were used for each genotype, and only male mice were selected for the analyses. The samples were homogenised, and the corresponding proteins were quantified using the Lowry biochemical method.

Western Blotting was used for protein level determination. For each sample, 15–50 μg of protein was loaded onto 7.5–10% SDS-polyacrylamide gels, depending on the protein being investigated. The following dilutions and primary antibodies were used: polyclonal anti-CASQ1/CASQ2 (1:1,000), monoclonal anti-TR (1:1,000), polyclonal anti-BiP (GRP78) (1:500), and polyclonal anti-CRT (1:1000), all from Affinity Bioreagents (Affinity Bioreagents, Golden, CO); polyclonal anti-GRP94 (1:1,000) from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA); and monoclonal anti-ATF6 from Thermo Scientific (Thermo Fisher Scientific Inc., Waltham, MA). Secondary anti-mouse or anti-rabbit (1:5,000) alkaline phosphatase-conjugated antibodies were from Sigma (Milan, Italy). After electroblotting on nitrocellulose paper, the equivalence and homogeneity of the loaded samples were verified by staining the nitrocellulose membrane with Ponceau Red. The immunodecorated bands were visualised with a ready-to-use precipitating substrate system for alkaline phosphatase (BCIP/NBT liquid substrate system; Sigma). Images were obtained with an HP Scanjet scanner and Adobe Photoshop

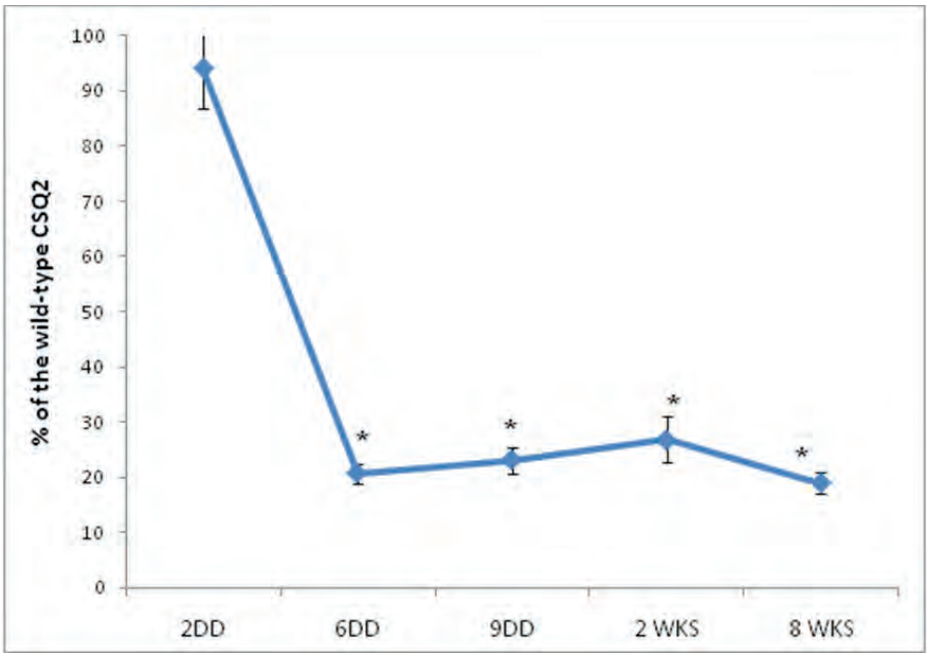

Figure 1: CSQ2 protein levels compared between CSQ2-wt/wt and knock-in CSQ2-R33Q/R33Q mice of different ages (2DD – 2 days, 6DD – 6 days, 9DD – 9 days, 2 WKS – 2 weeks, 8 WKS – 8 weeks). CSQ2 expression levels of homozygous knock-in mice are expressed as the percentage of that of wt mice CSQ2 levels. (*) P<0.05 same age comparison between knock-in CSQ2-R33Q/R33Q and CSQ2-wt/wt protein levels.

CS2, version 9.0. Densitometry was performed with Scion Image Software without modification of the images (raw images) to quantify protein band intensities.

Data are expressed as the means ± SE. Statistical analyses were performed using Origin 8.5 software. Student's *t*-test was used for comparisons between data from wt/wt and knock-in CSQ2-R33Q/R33Q mice. Statistical significance was set at $P< 0.05$.

RESULTS

Western blot data showed that the amount of mutated protein was reduced early after birth, with a drastic reduction at 6 days (Figure 1). Subsequently, increased protein levels were observed at 9 days and 2 weeks. After 2 weeks, decreased levels of CSQ2-R33Q were observed, and protein levels reached a minimum at 8 weeks. Thus, the lowest levels of CSQ2-R33Q expression were observed at 6 days and 8 weeks (20% of the CSQ2 in wt/wt mice).

Triadin levels were also reduced in 8-week- old knock-in CSQ2-R33Q/R33Q mice. TD32 is the main cardiac isoform of triadin and is one of the proteins that anchor CSQ2 in proximity to RyR2. In control hearts, the TD32 content increased markedly and quickly after birth (Figure 2A). After 6 days, TD32 expression levels were 5-fold higher than those in 2-day-old wt/wt mice. However, after 6 days, TD32

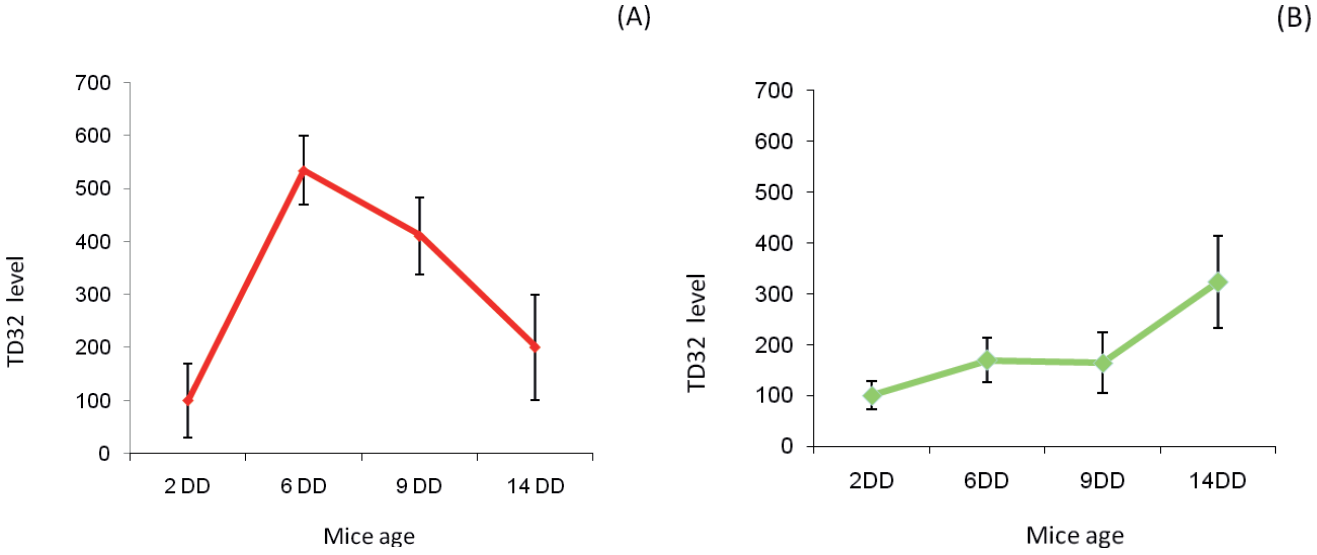

Figure 2: TD32 time course in CSQ2-wt/wt (A) and CSQ2-R33Q/R33Q (B) mice of different ages (2DD – 2 days, 6DD – 6 days, 9DD – 9 days, 14DD – 2 weeks).

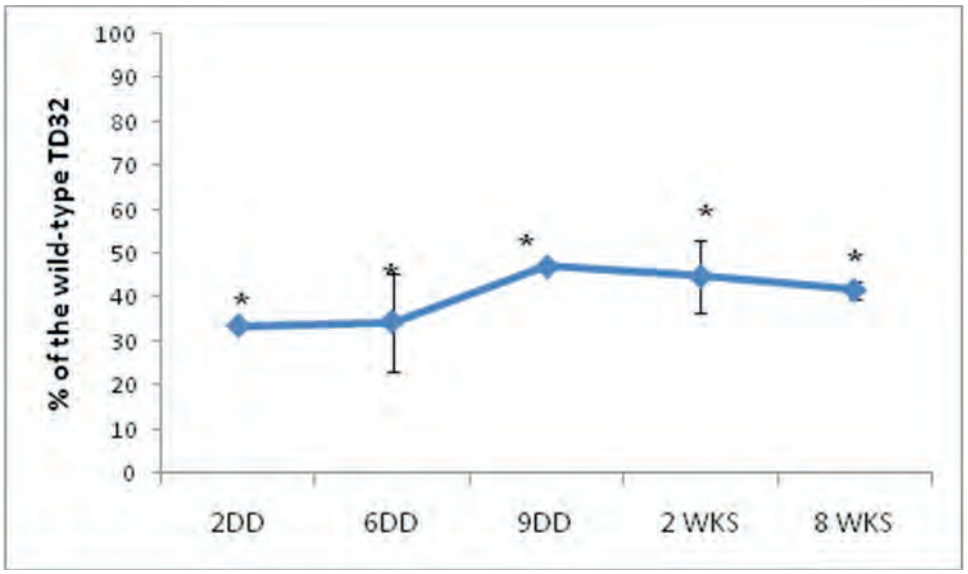

Figure 3: TD32 protein levels compared between wt/wt and knock-in CSQ2-R33Q/R33Q mice of different ages (2DD – 2 days, 6DD – 6 days, 9DD – 9 days, 2 WKS – 2 weeks, 8 WKS – 8 weeks). TD32 expression levels of homozygous knock-in mice are expressed as a percentage of the TD32 levels of wt mice. (*) P<0.05 same age comparison between knock-in CSQ2-R33Q/R33Q and CSQ2-wt/wt protein levels.

levels began to quickly decrease in wt/wt mice. In knock-in mouse hearts, TD32 showed a different expression pattern, increasing slowly over time (Figure 2B). When TD32 levels in knock-in hearts were compared with controls at different ages, a drastic reduction in the protein levels early after birth (~50%) was observed, and this reduction remained constant at all ages (Figure 3).

This research was also directed towards the molecular analysis of 3 unfolded protein response (UPR) proximal effectors. The protein expression of one of these proximal effectors, transcriptional factor activating transcription factor 6 (ATF6), was analysed in different age groups of wt/wt and knock-in CSQ2-R33Q/R33Q mice. These analyses showed the presence of increased levels of the active form of ATF6 in 2-week-old knock-in CSQ2-R33Q/R33Q mice (Figure 4). The transcriptional activation of UPR target genes, including those that function as part of the ER protein-folding machinery (ER chaperones) such as calreticulin (CRT), glucose-regulate protein-78 (GRP78) and glucose-regulate protein-94 (GRP94), was also analysed. The results showed that all three analysed ER chaperones were up-regulated, with peak expression levels observed in the hearts of 2-week-old knock-in CSQ2-R33Q/R33Q mice (Figure 5). For example, GRP78 levels were five times higher in 2-week- old knock-in CSQ2-R33Q/R33Q mice than in wt/wt mice (Figure 5B). Unlike GRP78, GRP94 protein levels remained high in knock-in CSQ2-R33Q/R33Q mice even after 2 weeks (Figure 5C).

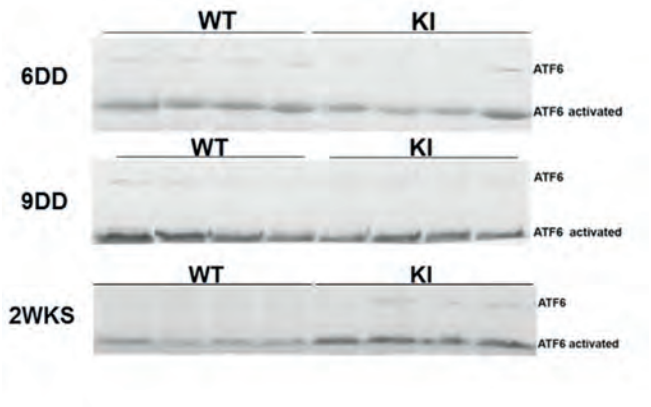

Figure 4: WT and knock-in CSQ2-R33Q/R33Q blots incubated with monoclonal anti-ATF6 antibody, which binds the full-length and active/cleaved forms of ATF6. Heart proteins from 2 day-, 9 day- and 2 week-old mice were used in these experiments.

DISCUSSION

Calsequestrin is an acid glycoprotein with a moderate affinity (Kd = 1 mM) and high ability for bindingto Ca^{2+}; it brings the total amount of Ca^{2+} within the SR to 20 mM and maintains the concentration of free Ca^{2+} at 1 mM (11). CSQ has a greater ability to bind Ca^{2+} in its polymeric form in the presence of 1 mM Ca^{2+}. The C-terminus of CSQ contains the highest density of amino acids and is forced to form a Ca^{2+}- binding pocket in polymeric form (12). CSQ plays an important role as a Ca^{2+} buffer and regulates the activities of RyR in relation to the Ca^{2+} concentration in the SR lumen (13). Furthermore, CSQ, together with triadin and junctin, was proposed to play an important role in SR junction development (14). As proposed by Beard (11), CSQ may also act as a chaperone through its kinase activity to ensure the correct folding of proteins present in the SR lumen and most likely plays an important role in removing toxic substances from the SR

lumen. CSQ is present in the lumen of the SR terminal cisternae as a linear polymer and is anchored to RyR via two transmembrane proteins, triadin and junctin, forming the Ca2+ release channel complex (11).

Presently, 8 recessive mutations in the *csq2* gene have been linked to CPVT. Four of them are non-sense mutations that generate premature stop codons, while the other four are point mutations (15-17). The unique characteristic of each mutation can alter the clinical phenotype of the disease (18). In these analyses, we chose specific criteria to obtain complete information regarding postnatal development; thus, we have a detailed picture of the events triggered by the presence of the CSQ2 R33Q point mutation. The first age used was as close to birth as possible (2 days); then, 1 week after birth was used. Nine-day-old mice were chosen for molecular analysis because this age represented an intermediate time point between the first and second weeks. Two weeks after birth was considered a critical time point for the cardiomyocyte based on the results of previous studies (10) that showed the appearance of optimal morphological cardiomyocyte characteristics at this point, which were responsible for the correct functioning of the heart muscle in adulthood. Finally, as reported by Flucher and Franzini-Armstrong, 8 weeks was considered a good point to analyse adult hearts (10). The mutated protein levels were analysed first, using the wild- type protein levels as a control. CSQ2-R33Q is CSQ2 with a mutated amino acid in position 33; this mutation did not seem to affect the secondary or tertiary conformation of the overall protein (29). The CSQ2 mutation knock-in mouse (9) was characterised *in vitro* and *in vivo* and was demonstrated to be an identical copy of the patient phenotype. CSQ2 and triadin protein levels were reduced, while the corresponding mRNAs levels remained unchanged (9). The CSQ2-R33Q protein levels found in this study confirmed the results of Rizzi and colleagues (9), who reported that CSQ2-R33Q proteins levels were significantly lower than wild-type protein levels. Comparison of the results reported in Figures 2A and 2B suggested that TD32 levels decreased due to the lack of functional CSQ2 from birth in the CSQ2-R33Q/R33Q mice. Results from a previous study (19) also showed that CSQ2 protein levels were significantly reduced in adult Trdn -/- mice. The mechanism of the post-transcriptional control of unfolded proteins can explain the reduction of CSQ2-R33Q protein levels without any effect on mRNA content.

ER stress is defined as an imbalance between the load of unfolded proteins that enter the ER and the capacity of the cellular machinery to handle this load; ER stress results in a series of different responses. In eukaryotic cells, the majority of secreted and trans-membrane proteins are synthesised and matured in the ER lumen. Proteins enter the ER as unfolded polypeptide chains. The incorrect folding of nascent proteins may occur in response to many environmental stresses or as a result of mutations that disrupt the protein structure. An imbalance between the load of unfolded proteins entering the ER and the ability of the cell machinery to handle this load is defined as ER stress, which triggers a

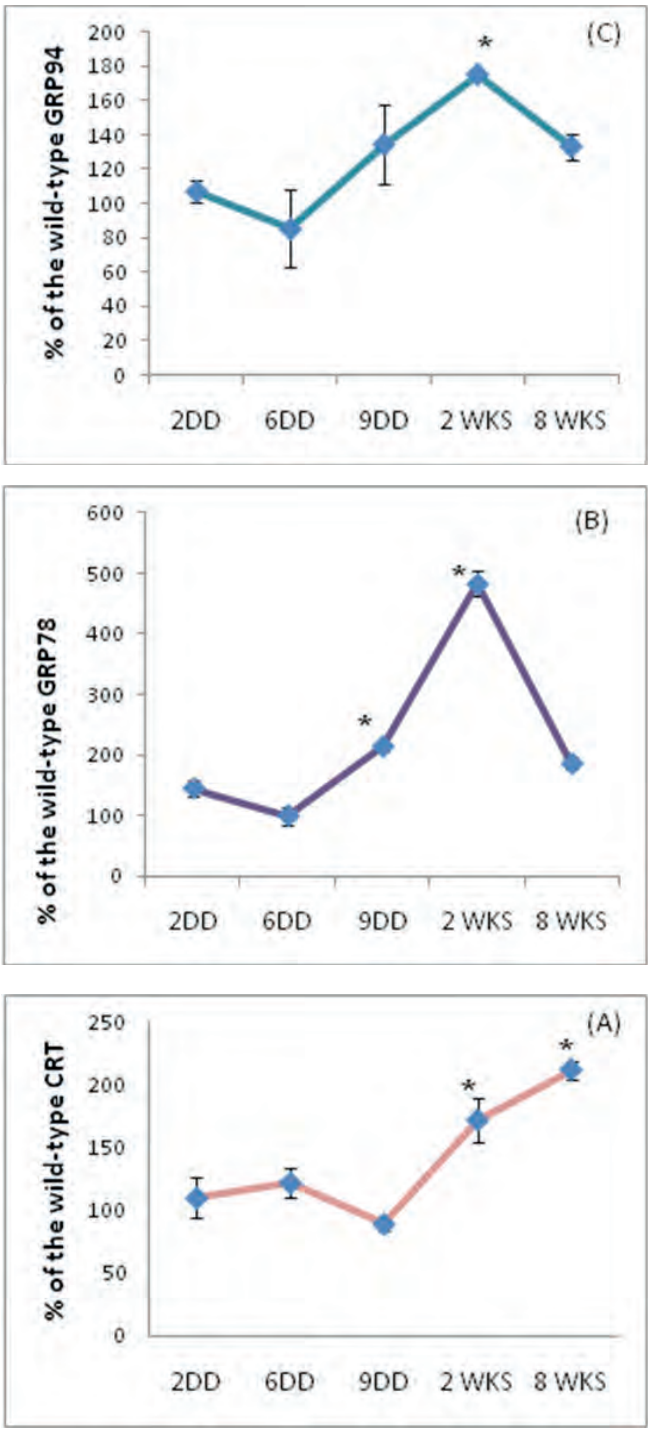

Figure 5: CRT (A), GRP78 (B) and GRP94 (C) protein levels comparisons between wt/wt and knock-in CSQ2-R33Q/R33Q mice of different ages (2DD – 2 days, 6DD – 6 days, 9DD – 9 days, 2 WKS – 2 weeks, 8 WKS – 8 weeks). All analysed chaperone expression levels of homozygous knock-in mice are expressed as the percentage of those of relative protein levels of wt mice. (*) P<0.05 same age comparison between knock-in CSQ2-R33Q/R33Q and CSQ2-wt/wt protein levels.

series of responses known as the UPR (20). When ER stress occurs, three ER transmembrane sensors are activated to initiate adaptive responses. These sensors include protein kinase-like ER kinase (PERK), inositol-requiring kinase 1 (IRE1), and ATF6. The N-termini of these transmembrane

UPR sensors are located inside of the ER lumen and the C-termini are within the cytosol, thereby connecting the ER lumen to the cytosol. All three sensors are maintained in an inactive state through the interaction of their N-termini with GRP78. When unfolded proteins accumulate in the ER, GRP78 releases these sensors, allowing for their oligomerisation and initiation of the UPR. Under these conditions, GRP78 translocates into the ER lumen, binds proteins and facilitates their folding. At the same time, IRE-1, ATF6, and PERK are activated and induce the transcription of ER stress response genes (21). To analyse the signal transduction cascade involving PERK, attention has focused on the expression of ribosomal protein eIF2α, a translation initiation factor. Phosphorylation of eIF2α by PERK leads to the decrease of most mRNA translation (22). This first response to ER stress reduces the load of proteins that must enter the ER. This is a transient form of adaptation that is realised by slowing the synthesis and translocation of protein into the ER lumen. Preliminary results have shown an up-regulation of phosphorylated eIF2α expression in 2-week old knock-in CS2R33Q/R33Q mice. The inactive form, eIF2α-P, lowers the levels of newly synthesised proteins. Subsequently, the capacity of the ER to handle unfolded proteins is increased, representing a more long-term adaptation that entails transcriptional activation of UPR target genes, including those that function as ER chaperones such as CRT, GRP78 and GRP94.

The activated form of ATF6 plays an important role in the synthesis of chaperones such as GRP78 and CRT (23). This was confirmed by experimental data showing that in 2-week-old mice, increased ATF6 levels corresponded to the highest, statistically significant expression levels of GRP78 (Figure 5B) and CRT (Figure 5A). Results in the literature show that ERAD is activated if it is not possible to restore the balance. ERAD provides 1) the recognition of incorrectly folded proteins, 2) translocation of such proteins into the cytoplasm, where they are bound to numerous ubiquitin molecules and 3) degradation via the proteasome (24). GRP94 is thought to play an important role in the response to incorrectly folded proteins, participating in their translocation from the ER lumen to the cytosol (25). Unlike GRP78, GRP94 protein levels remained high in knock-in CSQ2-R33Q/R33Q mice even after 2 weeks (Figure 5C). This indicated the possible activation of ERAD. Therefore, in accordance with these experimental data, it was speculated that the R33Q mutation induced structural and/or functional changes to CSQ2 that led to UPR activation and subsequent CSQ2 degradation. Thus, the reduction of CSQ2-R33Q might be due to its degradation via ERAD. Proteins with serious folding errors and protein aggregates are degraded by autophagy. In fact, many of the components that mediate autophagy are UPR target genes (26). If ER stress is permanent and irreparable, the process of apoptosis is activated and leads to cell death (27). Thus, it is of extreme interest to extend analyses to markers of autophagy and apoptosis such as CHOP, an ER stress-associated proapoptotic transcription factor (28).

CONCLUSIONS

These study results showed that mutated CSQ2 levels decreased soon after birth in conjunction with the decreased levels of other important junctional SR proteins such as TD. The up-regulation of proteins associated with the UPR was also observed; this led to ATF6-dependent pathway activation by the UPR. The R33Q mutation induced the decrease of CSQ2 levels through UPR activation and subsequent proteasomal degradation. Further studies are necessary to confirm these results.

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BEHAVIOURAL EFFECTS OF SHORT-TERM TOTAL FOOD RESTRICTION IN RATS

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UTICAJ KRATKOTRAJNOG POTPUNOG PREKIDA UNOSA HRANE NA PONAŠANJE PACOVA

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ABSTRACT

*Reducing food intake can decrease anxiety indices in rats. The aim of this study was to evaluate the influence of short-term (48 hours) total food restriction on the behavioural characteristics, the motor coordination and balance, of rats. Three-month-old male Wistar albino rats (n=20) weighing 350-400 g were divided into a control group (food and water intake **ad libitum**) and an experimental group (total food restriction 48 hours before testing). Behavioural studies were performed using the open field, elevated plus maze, Barnes maze, beam walking, evoked beam walking and linear locomotor tests. The total distance moved, the velocity, the movement and the frequency in the centre zone of the open field were significantly higher in the treated group. The cumulative duration in the centre zone of the open field did not significantly increase in the treated group. The number of entries into the open arms, the total time spent in the open arms and the total distance moved in the elevated plus maze significantly increased with no change in the velocity in the food-restricted animals. The 48 hours of total food restriction did not affect the Barnes maze test parameters or the parameters of the linear locomotor test. The velocity recorded during the beam walking test was not affected by the food restriction, but the velocity recorded during the evoked beam walking test significantly decreased in the treated group. In summary, short-term total food restriction did not produce significant changes in the physical performance of rats but did result in anxiolytic-like behaviour accompanied by food-seeking behaviour due to enhanced motivation to forage for food.*

Keywords: *food restriction, behaviour, anxiety, rat*

SAŽETAK

*Redukcija unosa hrane može dovesti do smanjenja anksioznosti kod pacova. Cilj ovog istraživanja je bio da se ispita uticaj kratkotrajnog (48 h), potpunog prekida unosa hrane na ponašanje, kao i motoričku koordinaciju i ravnotežu kod pacova. Tri meseca stari, muški Wistar albino pacovi (n=20), težine 350-400 g su podeljeni u kontrolnu grupu (unos hrane i vode **ad libitum**) i eksperimentalnu grupu – potpuni prekid unosa hrane 48 h pre testiranja. Bihejvioralna istraživanja su sprovedena uz korišćenje testova: „otvorenog polja", „uzdignutog krstastog lavirinta", Barnsovog lavirinta, „hodanja po gredi", „izazvanog hodanja po gredi " i linearnog lokomotornog testa. Ukupno pređeno rastojanje, brzina, kretanje i frekvencija ulazaka u centralnu zonu „otvorenog polja" su bili značajno veći u tretiranoj grupi. Ukupno vreme provedeno u centralnoj zoni „otvorenog polja" se nije značajno povećalo u tretiranoj grupi. Broj ulazaka u otvorene krake, ukupno vreme provedeno u otvorenim kracima i ukupno pređeno rastojanje u „uzdignutom krstastom lavirintu" su bili značajno povećani, dok se brzina nije menjala kod životinja bez unosa hrane. 48 h potpunog prekida unosa hrane nije imalo uticaja na parametre testa Barnsovog lavirinta, kao ni na parametre linearnog lokomotornog testa. Brzina registrovana kod testa „hodanja po gredi" se nije menjala usled restrikcije unosa hrane, ali se brzina registrovana kod testa „ izazvanog hodanja po gredi" značajno smanjila u grupi tretiranih životinja. Sumarno, kratkotrajni potpuni prekid unosa hrane nije izazvao značajne promene fizičke sposobnosti pacova, ali je na ponašanje ispoljio efekte koji podsećaju na anksiolitičko dejstvo, uz obrasce koji podsećaju na traženje hrane usled povećane motivacije da se obezbedi hrana.*

Ključne reči: *ograničenje unosa hrane, ponašanje, anksioznost, pacov*

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INTRODUCTION

In a variety of animal species, calorie restriction is a well-known factor in and the most effective and reproducible intervention for increasing lifespan (1, 2), and it is a cancer-prevention regimen in experimental carcinogenesis models (3). Restricted food intake can also be beneficial via its control of weight gain, as well as the improvement in insulin sensitivity in type 2 diabetes (4). Additionally, calorie restriction can prevent cardiovascular diseases (5, 6). Some behavioural tests have indicated that reducing food intake can decrease indices of anxiety in animals (7).

There are different regimens for calorie restriction in animal models. During a chronic calorie restriction diet, the nutrient composition, that is, the vitamins, minerals and proteins, must be maintained to prevent undernutrition or malnutrition (8).

A study by Ross showed that rats fed a high-protein/high-carbohydrate *ad libitum* diet had lower mortality rates at younger ages than those fed a nutrient-poor, low-protein/low-carbohydrate *ad libitum* diet. However, the animals with the poor nutrition diet had lower mortality rates than the animals with the rich nutrition diet. This finding implies that a high caloric intake is appropriate for growth, development and reproduction, but it does not provide a protective influence against age-related diseases (9).

Regarding chronic food restriction, it is known that maintaining the body weight at 80% led to behavioural changes and physiological stress (corticosterone level), but water restriction did not have that effect (10).

A whole battery of tests is now available to evaluate the behavioural characteristics of laboratory animals. Some of the tests are better suited for estimating the physical performance of the animals (eg, beam walking test, rotarod, and grip test), while other tests are often used to evaluate emotionality/anxiety (eg, open field test and the elevated plus maze test) or memory and learning (eg, the Barnes test and the Morris water maze test).

In 1966, a direct observation of rat activity during food deprivation confirmed that food-restricted animals showed improved physical performance, as indicated by increased locomotor activity and a lower frequency of resting; this physical change was more distinct in younger animals (11).

Various tests have been used to evaluate the effects of food restriction on memory. Depending on the protocol of the trials, the results have demonstrated that a short-term restriction of food intake had a beneficial effect on the spatial memory in male rats, with the opposite effect in female rats (12). However, two weeks of 40% calorie restriction did not influence the spatial memory during a Y maze test (13), whereas a lifelong hypocaloric diet, which started in young animals, was able to prevent the further decline in the memory test (Morris water maze test) that occurs between middle age and old age (14).

Epidemiological studies indicate that anxiety disorders are the most common psychiatric disorders (15). Anxiety can be defined as "a physiological, psychological, and behavioural state induced in animals and humans by a threat to well-being or survival, either actual or potential" (16). The behaviour patterns of anxiety vary from ongoing behaviours, such as explorative activity and feeding, to defensive patterns (eg, escape). Numerous behavioural tests allow us to estimate the ability of animals to cope with unexpected situations and adverse environments. It is well known that the calorie restriction state in animals may provide a model for investigating the neurobiology of anxiety.

The open field and elevated plus maze tests are two widely used models of emotionality/anxiety. The open field test is a useful animal model of anxiety-like behaviour. This test has been used to estimate the "emotional reactions" of rats since 1936 (17)is and is currently one of the most popular procedures in animal psychology. The open field consists of a novel large arena containing an aversive central area (18). The animal (mainly a rodent) is usually placed in the centre of the arena to encounter a completely new environment. Anxiety-like behaviour is characterised in the open field as walking close to the walls (cumulative duration in border zone), a behaviour called thigmotaxis. Additionally, a few additional parameters are usually considered to be indicators for anxiety-like behaviour in the open field test, such as decreases in the cumulative duration in the centre zone and the number of entries in centre zone. In contrast, an increase in the total distance moved in the central zone or in the time spent in the central part of the device without an alteration of the total locomotor or vertical exploration can be interpreted as an anxiolytic-like effect; the opposite effect, that is, a decrease in these variables, is associated with anxiogenic effects (19). Food-restricted rats showed enhanced activity in the centre zone of the open field (10). Anxiolytic drugs can increase the total locomotor and vertical exploration activity in the open field (19).

The elevated plus maze test, a well-known test for examining anxiety, is based on the conflict displayed by rats between the tendency to explore a new environment and the fear of open elevated areas (20). Placing the rat on the central platform on the elevated plus maze can evoke both the exploratory drive and the fear drive, thus generating an approach-avoidance conflict behaviour. In the elevated plus maze, the percentage of entries and the time spent in open arm are measures of anxiety, whereas the number of total arm entries (open + closed) is an indicator of the overall activity (21). From the central zone of the elevated plus maze, rats show head-dipping exploratory activity, as well as attend/approach responses toward the open arm. It should be noted that explorative activity can be enhanced by some factors, such as food or water deprivation. Studies on the effects of anxiolytic drugs must verify that a given treatment does not act on such variables before reaching a conclusion about the possible effects on anxiety-like behaviours (19). Heiderstadt showed that male rats that received approximately 40% of the amount consumed by freely fed animals, or a calorie restriction of 60%, had increased explorative activity and ambulation in the open field test (10). Addi-

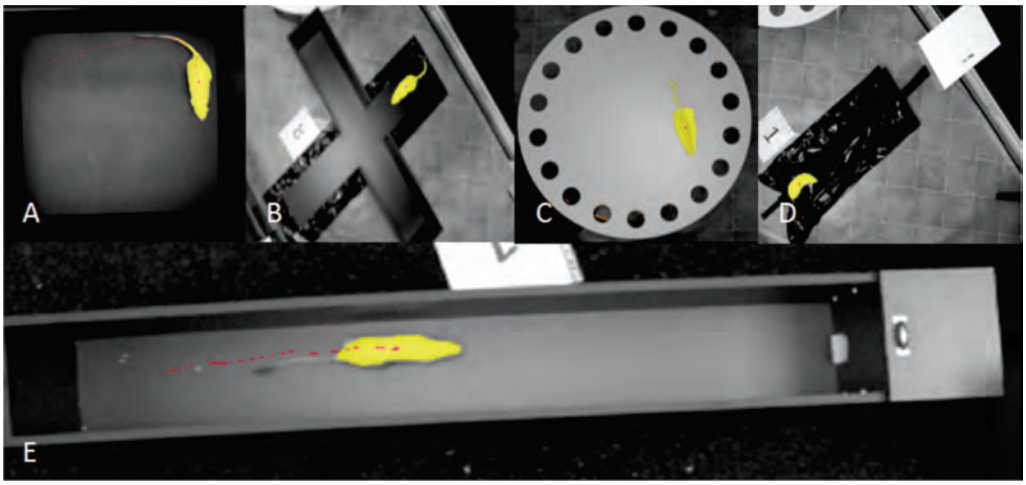

Figure 1. Open field (A), Elevated plus maze (B), Barnes maze (C), Beam walking (D), and Linear locomotor (E) tests.

tionally, male rats that maintained 85% of their body weight compared with that of control animals showed more entries into the open arms and a greater percentage of time in the open arms of the elevated plus maze (22). Three weeks of calorie restriction (75% of the usual voluntary food intake) in rats led to more entries into the anxiety-provoking (open) arms, indicating less anxiety (23).

In our research, we examined how short-term (48 hours) total food restriction influences the behavioural characteristics, as well as the motor coordination and balance, of rats.

MATERIAL AND METHODS

Animals

Three-month-old male Wistar albino rats (n=20) weighing 350-400 g were used. The animals were housed in controlled standard environmental conditions of temperature (23±1 °C) and light (12/12 h light/dark cycle), and the rats had free access to food and water until the groups were separated. The animals were divided in two groups (10 animals in each group). The control group had *ad libitum* access to food and water, and food intake of the experimental group was restricted for 48 hours before testing. The rats were placed in the testing room for 1-2 h prior to the initiation of each training and/or testing session. All research procedures were carried out in accordance with European Directive for welfare of laboratory animals N°86/609/EEC and principles of Good Laboratory Practice (GLP) approved by Ethical Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

BEHAVIOURAL STUDIES

Open field test

One of the tests that measures the general motor activity of animal is the open field test. This test may be used to evaluate potential motor deficits, and the moving pattern in the arena shows information about anxiety-like states. The time spent in the centre arena of the open field was determined as the major index for anxiety, and more ambulation towards the centre arena of the open field reflected less anxiety. The movements of the rats were recorded using a digital video camera centrally mounted 150 cm above the open field. The activity of the rats was recorded for a period of 5 minutes and then analysed. The apparatus consisted of a square arena (60 x 60 x 30 cm) made of black wood (Fig. 1A). During the trials, the experimenter was not present in the test room. At the beginning of a test, each rat was placed in the centre of arena. The following parameters were scored: *total distance moved* (TDM, cm), *velocity* (cm/s), *movement, frequency in centre zone* and *cumulative duration in centre zone*. The frequency in the centre zone and cumulative duration in the centre zone are considered indicators of an anxiolytic-like effect (10, 19). At the end of each session, the rats were removed from the open field, and the experimental chamber was thoroughly cleaned with water and ethanol (70%) to remove possible interfering scents. The behaviour of the animal in the open field was tracked by video using Ethovision software [version XT 10 base], a video tracking system that automatically records behavioural experiments [Noldus Information Technology, the Netherlands] (Fig. 1A).

Elevated plus maze

Anxiety-like behaviours were evaluated using the elevated plus maze test. This test can determine the emotional reactivity of animals by generating a conflict between the secure parts of the maze (2 enclosed arms) and the aversive parts of the maze (open arms). The elevated plus maze for rats consisted of two open (50 x 20 cm) and two enclosed arms (50 x 20 x 30 cm) and an open roof; the entire maze was elevated 100 cm from the floor (Fig. 1B). Each rat was placed in the centre of the elevated plus maze with its head facing toward the open arm and was given 5 minutes for free exploration. During the 5 minutes of the test, the *number of entries (frequency)* into the open arms and the *total time spent in open arms (cumulative duration)* of the maze, *total distance moved* (TDM, cm) and *velocity* (cm/s) were recorded. The number

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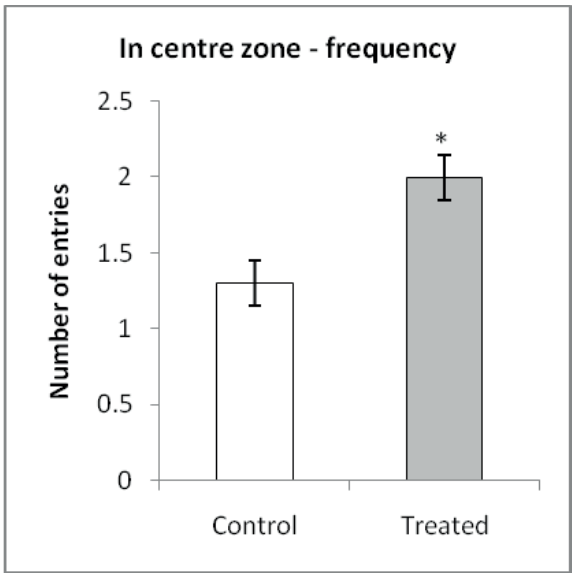

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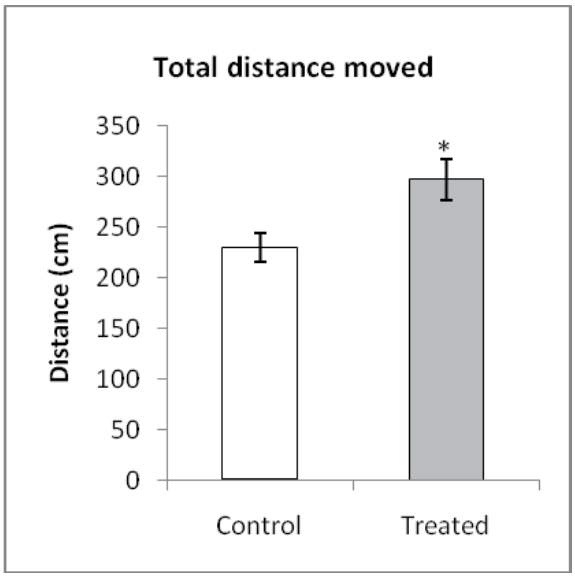

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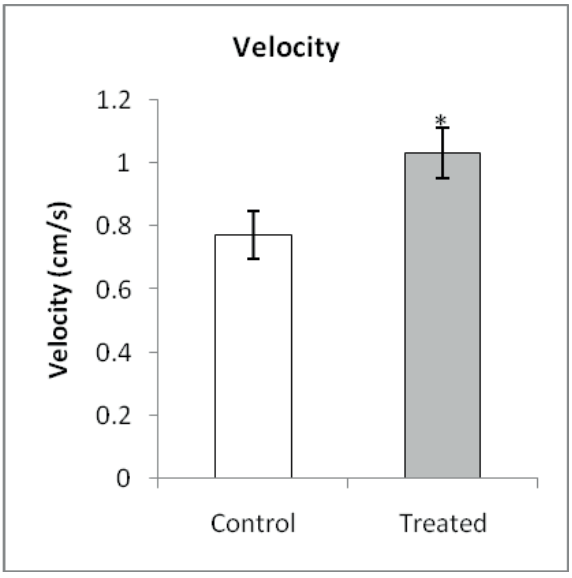

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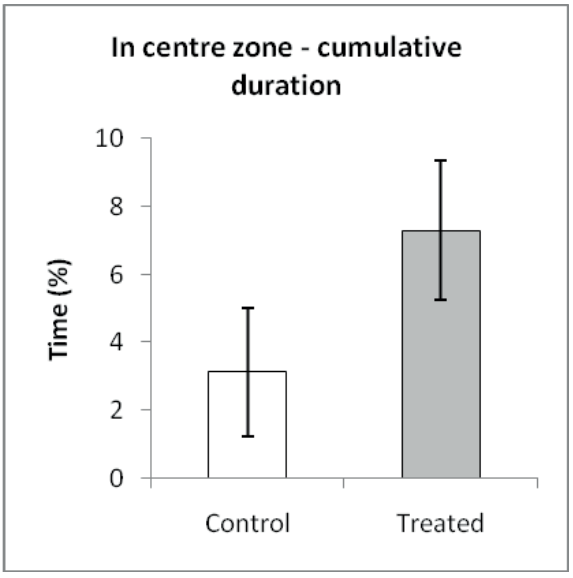

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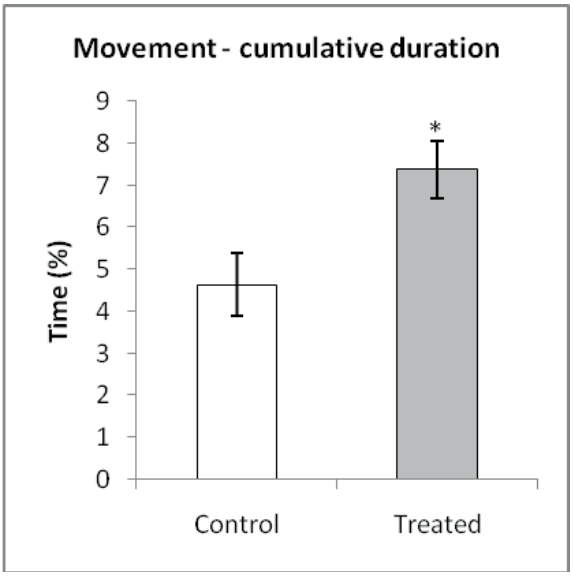

Figure 2. Parameters calculated from the open field test (Mean ± SEM, * denotes a significant difference $p < 0.05$). The food intake was completely restricted for 48 h in the treated animals.

of entries and the cumulative duration in the open arms are considered indicators of an anxiogenic effect (20, 24). The activity of the rats was recorded using a digital video camera (resolution of 30 samples per second) centrally mounted 250 cm above the elevated plus maze. The behaviour of the animal in the elevated plus maze was tracked by video using Ethovision software [version XT 10 base], a video tracking system that automatically records behavioural experiments [Noldus Information Technology, the Netherlands] (Fig. 1B).

Barnes maze

The Barnes maze is a spatial learning and memory task that requires subjects to escape from a brightly lit area and learn the location for escaping from the box under one of the holes. The task relies on the innate preference of rodents for dark, enclosed spaces over open areas. The Barnes maze (25) consists of a flat, circular disk (122,5 cm in diameter, suspended 70 cm off the ground) with 18 holes (10 cm in diameter) around its perimeter that permit the subject to exit the maze into an escape box (Fig. 1C). Animals learn the location of an escape hole using high contrast spatial cues (2 large yellow stars on the black wall) fixed on one wall of the room. The Barnes maze does not involve swimming and is therefore considered less anxiogenic than the Morris water maze (26). On the day before the formal testing began, the rats were transported in their home cages to the central room of the testing suite and were allowed to acclimate for approximately 1 h. Testing began by placing animal in the centre of a circular platform. The animal was monitored as it found its way from the centre of the platform to an escape hole. Trials were recorded using a digital video camera (resolution of 30 samples per second) mounted centrally 250 cm above the Barnes maze. The following parameters were estimated: *time to find escape box in the first run, time to find escape box in the second run, distance to find escape box in the first run, distance to find escape box in the second run, velocity of the first run, velocity of the second run, percentage of shortening of distance moved to escape box between two runs* of each rat, *percentage of shortening of time to find escape box between two runs, percentage of increase in the velocity between two runs.* The behaviour of the animal in the Barnes maze was tracked by video using Ethovision software [version XT 10 base], a video tracking system that automatically records behavioural experiments [Noldus Information Technology, the Netherlands] (Fig. 1C).

Beam walking test

The beam walking test was used to assess motor coordination, integration (27), balance performance (28) and motor skills. The beam walking apparatus consisted of a rectangularly shaped base. In this test, the ability of rats to pass through the beam to reach a goal box is evaluated. A white wooden box (20 x 20 x 20 cm) with a black hole served as a nest for motivating the animal to cross the beam. A stainless steel, squared, rubber-topped beam (100 x 3 x 2 cm) was fixed between the base of the goal box (100 cm above the floor) and a vertical stainless steel pole (60 cm above the floor). The doweled rod was graduated from 0 to 50 cm. The whole apparatus was placed above cushions, which protected the fallen animals from injury (Fig. 1D). Rats were pre-trained to cross the beam. On the day of the test, four trials were performed before recording the results. The interval between trials was 15 minutes. At the start of the trial, the rat was placed at the end of the beam opposite to the goal box. The goal was to accustom the rats to the beam and to allow them to become aware of the presence of the goal box at the end of the beam. In this test, the *number of forelimb and hind limb foot faults, number of falls from the beam* and *time to cross the beam* were recorded, and then the *velocity* (cm/s) was calculated (for defined distance). A fault was defined as any foot slip off the top of the surface of the beam or any limb use on the side of the beam. The beam walking test was conducted under the proper conditions of silence and illumination. The behaviour of the animal in the beam walking test was tracked by video using Ethovision software [version XT 10 base], a video tracking system that automatically records behavioural experiments [Noldus Information Technology, the Netherlands] (Fig. 1D).

Evoked beam walking test

This test was performed using the same apparatus as for the beam walking test. Rats were pre-trained to cross the beam using the same protocol as for beam walking test. At the start of the trial, the rat was placed at the end of the beam opposite to the goal box, while the experimenter started tapping (every 3 seconds) with a metal stick at the base of the stainless steel pole while rat traversed the beam (anxiety-provoking pattern). The tapping was performed until the rat reached the goal box. The recorded parameters in this test were the *number of forelimb and hind limb foot faults, number of falls from the beam* and time to cross the beam, and then the *velocity* (cm/s) was calculated (for defined distance). A fault was defined as any foot slip off the top of the surface of the beam or any limb use on the side of the beam. The velocity (relative to the previously performed beam walking test) was used as an indicator of anxiety. The beam walking test was conducted under proper illumination.

Linear locomotor test

A linear locomotor test was used to assess motor coordination and motor skills. The apparatus consisted of an enclosed rectangular box (100 x 20 x 30 cm) with start and goal boxes (20 x 20 x 30 cm) at the ends (Fig. 1E). In this test, the time to reach the goal box was recorded, and then the *velocity* (cm/s) was calculated (for defined distance). Using Ethovision software, the floor of the arena was virtually divided into three equal zones (centre, right and left) to evaluate the linearity of the animal locomotor while moving from the start box to the goal box using the *zone transitions frequency*. The behaviour of the animal during the linear locomotor test was tracked by video using Ethovision software [version XT 10 base], a video tracking system that automatically records behavioural experiments [Noldus Information Technology, the Netherlands] (Fig. 1E).

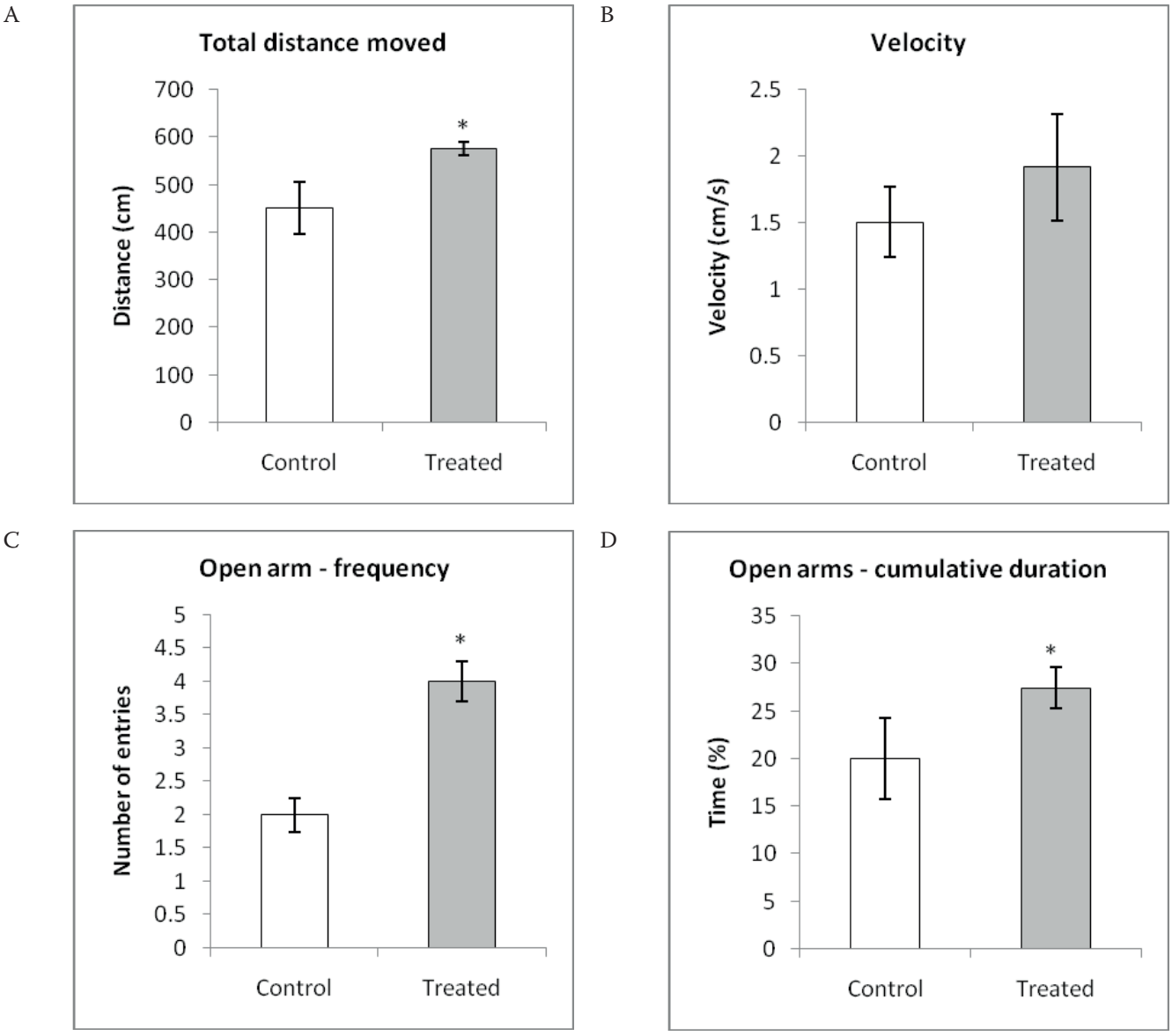

Figure 3. Parameters calculated from the elevated plus maze test (Mean ± SEM, * denotes significant difference $p < 0,05$). The food intake was completely restricted for 48 h in the treated animals.

Video recording system and analysis

All tests were recorded using a digital video camera (Practika DVC 5.10 FHD). Video files (MPEG-2 format) were analysed using Ethovision software [version XT 10 base], an integrated video tracking system for automatically recording the activity, movement and interactions of animals [Noldus Information Technology, the Netherlands] (29).

Statistical analysis

The results are expressed as the mean ± SE, and *P* values <0.05 were considered significant in all tests. When two groups of animals were compared, analysis was performed with an unpaired student *t* test. Nonparametric Mann-Whitney U tests were used for comparisons between groups that did not follow normal distribution. Variables were checked for normal distributions of the data using the Shapiro-Wilks test. A confidence level of 95% was accepted as significant. Analysis was performed using SPSS version 20.0 statistical package (IBM SPSS Statistics 20).

RESULTS

Open field test

This study showed significant effects from the 48 hours of totally restricted food intake on most of the open field test parameters in the restricted rats compared with the control rats. The total distance moved (Fig. 2A, $p<0.05$), velocity (Fig. 2B, $p<0.05$), movement (Fig. 2C, $p<0.05$) and frequency in the centre zone (Fig. 2D, $p<0.05$) were significantly higher in the treated group. The cumulative duration in the centre zone (Fig. 2E) was not significantly increased in the treated group.

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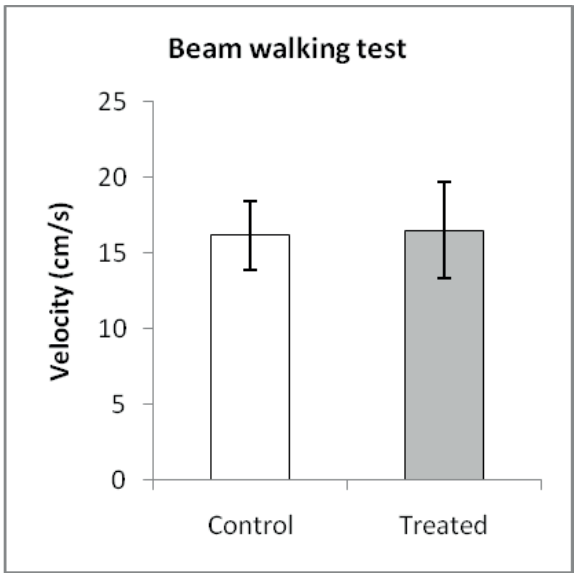

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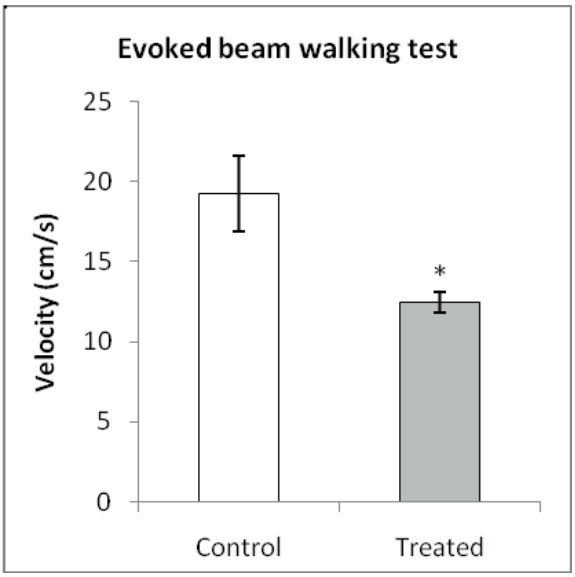

Figure 4. Parameters calculated from the beam walking and evoked beam walking tests
(Mean ± SEM, * denotes a significant difference $p < 0.05$). The food intake was completely restricted for 48 h in the treated animals.

Elevated plus maze

Most of the elevated plus maze test parameters in the rats with total food restriction showed significant changes compared with the parameters for the control rats. The number of entries (frequency) into the open arms (Fig. 3A, $p<0.05$), the total time spent in the open arms (cumulative duration) of the maze (Fig. 3B, $p<0.05$) and the total distance moved (Fig. 3C, $p<0.05$) were significantly increased in the treated group compared with the control group. The velocity (Fig. 3D) was not significantly increased in the treated group.

Barnes maze

The time to find the escape box in the first run was significantly lower than the time to find the escape box in the second run for both the treated and control groups (Tab. 1). Additionally, the distance to find the escape box in the first run was significantly lower than the distance to find escape box in the second run for both groups (Tab. 1). The velocity in the first run was not significantly different from the velocity in the second run for both groups (Tab. 1).

This study did not show significant effects on the Barnes maze test parameters when comparing the rats with 48 hours of total restriction of food intake with the control rats. The percentage of shortening of the distance moved to the escape box between two runs (36.72 ± 10.35 in the treated group and 31.72 ± 9.28 in the control group), as well as the percentage of shortening of the time to find the escape box between two runs (43.48 ± 10.54 in treated group and 42.99 ± 8.97 in control group), did not significantly change. Additionally, the percentage increase in the velocity between two runs (19.18 ± 6.21 in the treated group and 26.24 ± 11.72 in control group) did not significantly change.

Beam walking test

Our results showed that there were no forelimb and hind limb faults or falls from the beam in either of the two groups. Additionally, there was no significant change in the velocity between the food-restricted and control groups (Fig. 4A).

Evoked beam walking test

There were no forelimb and hind limb faults or falls from the beam recorded in either of the two groups. However, the velocity significantly decreased in the treated group compared with the control group (Fig. 4B, $p<0.05$).

Table 1. Parameters calculated from the Barnes maze test.

	Distance to findescape box		Time to find escape box		Velocity	
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
Control	72.15±11.54	37.76±8.47*	319.91±54.25	16.16±38.67 *	5.79±1.47	6.59±2.58
Treated	51.91±8.21	21.26±6.23 *	198.61±37.24	100.66±31.78 *	4.72±1.32	5.37±3.64

(Mean ± SEM, * denotes a significant difference $p < 0.05$). Food intake was completely restricted for 48 h in the treated animals.

Linear locomotor test

There was no significant difference in the velocity (15.52 ± 1.74 in the treated group and 15.11 ± 2.71 in the control group) and the zone transitions frequency (3.4 ± 1.2 vs. 2.8 ± 1.5, respectively) between the treated and control groups.

DISCUSSION

Food intake restriction has been reported to induce various behavioural effects in rats. However, there are substantial differences when considering the overall consequences of starvation on numerous behavioural parameters. Food restriction has been performed in various forms (acute and chronic manner, young and adult animals, different sexes, and different levels of limitation); thus, the type of protocol may be one of the reasons for the inconsistent results among numerous reports.

In the present study, the main findings are that after 48 h of total food restriction, the rats exhibited anxiolytic-like behaviour. This pattern of behaviour was recorded in a variety of behavioural tests for evaluating anxiety, such as the open field test, the elevated plus maze and the evoked beam walking test.

In the open field test, our results showed anxiolytic-like behaviour, which is confirmed by the increased time spent in the central zone, as well as the higher frequency of entry into the central zone (30). This finding, along with the higher total distance moved by the food-restricted animals and the additional movement, is consistent with the results of Levay (31), who showed that calorie restriction induces increases in the time spent in the central zone and the number of central zone entries. However, these findings in the open field test cannot be solely attributed to anxiety-like behaviour because food deprivation has been found to increase activity in male rats (10); this activity is reflected as increased motor activity, exploration and/or anxiety. The main effect of food deprivation in male rats is the changed behaviour indicated by increased exploration and reduced anxiety (22).

The results of the elevated plus maze test indicate that short-term food restriction can increase motor activity, as well as the time spent in the open arms. These data are consistent with the results of Genn, who showed that male rats have an apparent anxiolytic response in the elevated plus maze test (22). Increased open arm exploration may be considered a food-seeking behaviour. Inoue (32) also showed that the time spent in the open arms is higher in the food deprivation group, corroborating our results. A higher level of explorative activity in the open arms was also reported for the animals with food restriction (33). The increase in the time spent in the open arms might be hypothesised to result from enhanced motivation to forage for food (32). These findings do not correlate with Levay (31), whose elevated plus maze test results were opposite to both our results and previous results in the literature (33, 22, 32).

Our results for the Barnes maze test showed no effect of acute starvation on the spatial and/or short-term memory of the rats. This finding is in accordance with Mahdavi (13), who reported that food restriction for two weeks did not affect the spatial memory in the Y maze test. Moreover, a long-lasting (25 months) reduction of food intake (50% reduction of fats, 35% reduction of carbohydrates) has been reported to slow down the weakening of both the reference and working memory that accompanies with ageing (35).

The beam walking test results suggest that the total food restriction for 48 hours did not affect the motor coordination or the balance performance. Because the literature does not contain data on the influence of food restriction on beam walking test results, we can only note that the short-term starvation in our test did not produce an immediate impairment of either motor coordination or balance performance.

Although the beam walking test showed no effects of food restriction on motor coordination and balance performance, the evoked (with anxiety-provoking stimuli) beam walking test results clearly showed anxiolytic-like behaviour in the rats with total food restriction. The velocity decrease in the evoked beam walking test may be, at least partially, due to food-seeking behaviour in the tested group of animals.

The linear locomotor test results provide no evidence for an impairment of motor coordination and motor skills after total food restriction for 48 hours. The results of this test were not previously reported in the literature; thus, we can only compare these results with the results of the beam walking test (indicators of motor coordination and balance performance) and conclude that acute food restriction did not produce prompt alterations in motor coordination and motor skills.

In summary, short-term total food restriction for 48 hours did not produce significant changes in motor coordination, motor skills, balance performance and velocity. However, the food restriction resulted in anxiolytic-like behaviour accompanied by food-seeking behaviour due to enhanced motivation to forage for food.

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THE KEY ROLE OF THE LEADING EMERGING BRIC MARKETS IN THE FUTURE OF GLOBAL HEALTH CARE

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KLJUČNA ULOGA VODEĆIH RASTUĆIH BRIK TRŽIŠTA ZA BUDUĆNOST SVETSKOG ZDRAVSTVENOG SEKTORA

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ABSTRACT

The acronym 'BRIC' was coined in 2001 to describe the largest and most promising emerging markets outside the established, post-war, high-income economies. The nominal GDP growth rates of Brazil, Russia, India and China outpaced the growth rates of Western Europe, North America and Japan before, during and after the global economic crisis. This global phenomenon will have a significant impact on many branches of the economy, including the global demand for and provision of healthcare services. The key driver of this economic development is the existence of an enormous middle class in each of the BRIC countries. Both health insurance coverage and the package of services covered by health insurance plans are expanding in BRIC countries. Equally important is the overall increase in purchasing power in BRIC nations, which has been followed by the increased affordability of a vast portion of the medical goods and services that are commonly paid for out-of-pocket by ordinary citizens. When considering the changing landscape of global health care, one should also account for the slow and steady economic growth of most mature, saturated markets. This supports the notion that although consumer demand for health services remains strong in wealthy countries, the true expansion of the global market is occurring elsewhere. All major market analysis agencies have acknowledged this development and urged multinational healthcare companies to focus on emerging markets, and BRICs in particular, if they want to survive. Investment in emerging markets will remain the key to long-term profits and sustainability for pharmaceutical firms and medical equipment manufacturers across the globe for many years to come.

Key words: *BRICs, emerging markets, global, health care, demand, medical services*

SAŽETAK

Akronim BRIK je stvoren 2001 godine sa ciljem da se izdvoje ključna „rastuća tržišta" van tradicionalno bogatih i razvijenih društava. Brazil, Rusija, Indija i Kina svojim stopama rasta nominalnog bruto nacionalnog dohodka nastavljaju da prevazilaze vodeće privrede Evrozone, Severne Amerike i Japana pre, tokom i posle poslednje svetske ekonomske krize. Ovaj globalni fenomen će ozbiljno uticati na preoblikovanje tražnje i ponude medicinskih usluga kao i u drugim oblastima privrede. Ključan uzrok novonastalih promena je nastanak i narastanje masivnog srednjeg građanskog sloja u ovim zemljama. Pokrivenost stanovništva zdravstvenim osiguranjem kao i raznovrsnost usluga u okviru uobičajenih polisa se značajno proširuju. Jednako je važan rast ukupne kupovne moći praćen povećanjem prihvatljivosti onih medicinskih dobara i usluga koje se tradicionalno plaćaju iz džepa građana. Činilac koji doprinosi ovakvim promenama jesu i ustaljene stope sporog rasta u zrelim, zasićenim tržištima. Ovo se takođe ogleda u tražnji za zdravstvenim uslugama koja je stabilna u bogatim zemljama iako se istinski globalni rast zapravo događa u rastućim tržištima širom sveta. Ova činjenica je prepoznata od strane svih vodećih analitičara tržišta. Takve agencije otvoreno savetuju medicinske multinacionalne kompanije da se usredsrede na BRIK privrede ukoliko žele dugoročni opstanak na svetskom tržištu. Ciljna strategija da bi se postigle i održale profitne marže u industriji farmaceutika i medicinske opreme će ostati ulaganje u tržišta u razvoju, za dugi niz godina koje dolaze.

Ključne reči: *BRIK, rastuća tržišta, globalna zdravstvena zaštita, tražnja, medicinske usluge*

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THE ECONOMIC MIRACLE OF BRIC NATIONS

The 'BRIC' acronym was coined by US economist of Irish origin Jim O'Neill in a 2001 paper in which he posited that a particular set of countries (namely, Brazil, Russia, India and China)—the so-called major emerging markets—had become the primary drivers of global economic growth (1). This development was made possible by fundamental societal changes that transpired independently in each BRIC country several decades ago. BRIC nations share a common history of centrally planned and managed economies that were successfully transformed into market economies after policy makers in the various BRIC countries embraced this goal in their respective long-term strategies. An important aspect of this unseen prosperity was the development and growth of South-South trade among the emerging economies (2). Economic history dating back to the Colonial Age has been dominated by North-North trade and North-South investment, the latter being used by firms in the North to produce affordable goods and services using the skilled but cheap local labour that is available in the South; the goods and services produced in the South were then sold at high prices in the wealthier North markets. South-South commerce has tripled over the past several decades, primarily as a result of the abundant supply of natural resources in Russia and Brazil and the massive service and manufacturing sectors in India and China, respectively. Today, India and China are the major purchasers and consumers of the natural resources found in Russia and Brazil. The Russian Federation's unique position as the leading global supplier of fossil energy (both oil and natural gas) to China, which has an enormous and growing hunger for energy and resources, also contributes significantly to South-South trade (3).

Another key factor is that the traditional, mature, high-income markets of Western Europe, the US and Japan are now saturated and characterised by stable or steadily decreasing demand for goods and services. These wealthy countries were actually more vulnerable to the recent global economic crisis, and they have since suffered significantly from long-lasting recessions. In contrast, the BRIC countries recovered from the economic crisis relatively quickly (4) because they used the recessional downturn in foreign demand and exports as an opportunity to reorient themselves towards domestic consumption. Given the huge populations of each of the BRIC countries and the overall trend of a growing middle class with more purchasing power than ever before, this strategy proved very successful for BRIC countries, and it even drove additional domestic growth and recovery in these regions. In sum, all of these phenomena have contributed to the overall impression that most global market growth is occurring outside of the developed Western economies (5). US-based Goldman Sachs® is one of the most frequently cited sources for the prediction that the combined nominal GDP of BRICs will likely overtake the combined nominal GDP of G7 countries until 2030 (6). This development is likely to be particularly evident in the healthcare field and associated industries, as explained further below.

THE IMPACT OF BRICS ON GLOBAL HEALTH CARE

The nominal GDP growth rates of Brazil, Russia, India and China outpaced those of Western Europe, the US and Japan before, during and after the global economic crisis (7). This global phenomenon will have a substantial impact on many branches of the economy, including the global demand for and provision of healthcare services (8). The key driver of this economic growth is the recent emergence of enormous middle classes in BRIC countries (9). Although a middle class previously existed in Russia, reaching its maturity at the height of real socialism, the Russian middle class effectively disappeared during the depths of the severe Russian recession that reached its nadir in 1998 and affected most of the Eastern European satellite economies. In comparison, the other three BRIC countries have never before successfully created this critical population of consumers with decent purchasing power. During last twenty-five years, each of the BRIC countries has undergone a painful but curative societal transformation towards increased work productivity and greater overall economic efficiency (10). Each country evolved at its own pace and overcame its own unique hurdles to lay the groundwork for long-term prosperity and an increased likelihood of achieving a welfare state (recall that it is widely believed that the US, Japan and Western Europe each achieved a welfare state during the early post-war decades (11)).

The milestones achieved by BRIC nations in health care accessibility include bold expansions of health insurance coverage for the general population and of the package of medical services provided to the insured. These expansions were made possible by increased health care expenditures in BRIC countries (12, 13).

Investment in healthcare-related research and development (R&D) by both government and private sector funds in BRIC countries is growing correspondingly. Nonetheless, with the exception of Russia, the contributions by BRIC societies, in terms of genuine, patented innovations, remain low relative to Western countries. However, this situation is likely to change soon due to the massive build-up of human resources and institutional R&D capacities in BRIC nations (14, 15).

Even more important is the overall growth of purchasing power in BRIC countries, which has been followed by the increased affordability of a vast portion of the medical goods and services that are commonly paid for out-of-pocket by ordinary citizens (16). When assessing the changing landscape of global healthcare, one should consider the slow, steady (and even decreasing, during recessions) economic growth rates of most mature, saturated markets. This trend supports the notion that although consumer demand for health services remains strong in

wealthy countries, the expansion of the global healthcare market is occurring elsewhere, namely, in the emerging regions of the globe. Most major market analysis agencies have recognised this development (17) and thus have urged multinational healthcare companies based in the West to focus on emerging markets, and BRICs in particular, if they want to survive. Moreover, emerging markets will remain the key to sustainability and long-term profits for pharmaceutical firms and medical equipment manufacturers across the globe for many years to come (18). Note that this forewarning applies not only to pharmaceutical companies but also to firms involved in laboratory assays, diagnostic imaging, implants, surgical equipment, orthopaedics and dental products (19). Chinese companies already have near monopolies in medical equipment markets in Third World regions, due to the relatively low cost of Chinese products, and have made significant inroads in developed markets (20, 21).

The rare exception to the market trends described above is that the pharmaceutical markets in the US and Japan are likely to remain the first- and second-largest pharmaceutical markets in the world, respectively, for many years to come, particularly if market value is measured based on sales of branded drugs (22). However, this prime example of an old market-hierarchy resistant to change is already being gradually eroded by the aggressive marketing of generic drug manufacturers based primarily in India. Low-income and most middle-income regions across the globe are unable to afford branded drugs; most of these regions are also unable to afford the relatively expensive "branded generics" marketed by multinational firms such as Swiss firm Novartis® and Israeli firm Teva®. Therefore, poor countries were the first target markets for inexpensive generic drugs coming from India and, to a lesser extent, from China. The powerful Indian generic drug manufacturing sector is globally competitive and has, thus far, adapted to over 200 different national markets across the globe, including the highly regulated drug markets of Japan and the major Western economies (22). If market value is measured based on sales of branded drugs, the dominance of prominent commercial companies remains undisputed. However, if consumption and sales are measured in terms of defined daily doses (DDD), large Indian companies such as Ranbaxy® have already overtaken in many markets that are likely to grow substantially in the future. As the standards of living and GDPs of many minor emerging countries increase, the values and global market shares of their respective pharmaceutical markets will also increase, as will the revenues of their BRIC-based suppliers (23).

One of the vulnerabilities of such dynamic and sudden development is the continuing rapid urbanisation of BRICs' respective populations, particularly China and India (24). Urbanisation entails an ambitious build-up of infrastructure, including improved networks of all types of healthcare facilities, in remote regions. The Semashko healthcare system developed in Russia during the Soviet era has left a vast hospital-based system with high bed availability and large physician numbers across most of Eastern Europe (25). This was not the case for the three other BRIC countries; thus, they needed extensive "de novo" development (26). The massive expansion of healthcare institutions into rural areas of China, India and, to a lesser extent, Brazil, will have a significant effect on dominant trends in other branches of medicine, which in turn will provide a large portion of the world population—a portion of the population that is currently rather isolated—access to modern day healthcare technology and medicine. This access will increase standards of living in these communities by increasing life expectancy, quality of life and access to modern medical care (27). Cutting edge medical technologies were previously reserved almost exclusively for wealthy societies and the rather small 'elite' segments of low- and middle-income communities across the globe. Based on all of the developments discussed above, multiple additional waves of consumer demand for medical services are likely to occur throughout BRIC countries in the future (28).

THE LONG-TERM IMPACT OF GROWTH IN EMERGING MARKETS ON GLOBAL HEALTHCARE

Leading multinational healthcare companies have been watching the development in BRIC countries closely for a number of years. Many of these firms have implemented complex and extensive strategies to increase their presence in BRIC regions and thereby secure long-term competitive success. However, the enthusiasm of multinational firms has been dampened by the recent adoption of protectionist national policies by BRIC governments (29). BRIC countries are aware of their newly acquired geopolitical reach and significance and have implemented certain economic policies to improve the global competitiveness of domestic companies. For example, the Russian Federation has decided to support market domination by locally produced medicines. Brazil has imposed additional taxes on imported goods and services to distinguish them from less expensive domestic options. China is currently introducing a fast-track pharmaceutical approval process that is likely to discriminate against manufacturers that submit evidence from clinical trials conducted outside of mainland China. India is developing a pricing system that will limit and/or decrease the prices of imported drugs and medical equipment, such as implants, laboratory assays and diagnostic imaging consumables, to make these products more affordable to the vast portion of India's population that remains below the poverty line (30).

Finally, there are other emerging countries that should be mentioned for their obviously strong long-term economic prospects and their potential reach in the global healthcare market. These countries are frequently identified as the "Next Eleven" or other monikers, and they are, in decreasing order of importance: Indonesia, South Africa, Vietnam, Mexico, Turkey, Argentina, Thailand, Chile,

South Korea, Malaysia, Egypt, Nigeria, Columbia, Saudi Arabia and Poland, as well as several others (31). However, despite their undisputed growth and bright future prospects, most of these countries lag substantially behind even the weakest BRIC economy in terms of natural resources, population size and real development potential (32).

The national economic growth and overall development of the People's Republic of China clearly dominates the BRICs. The long-term growth of China's GDP and its contribution to the global health care market is likely to far exceed not only those of the other BRIC nations but also those of most G7 economies (33). During this very painful and delicate transitional period, health policy authorities in emerging countries must be aware of the key weaknesses in the provision of medical services to the general population. The decisiveness of governmental authorities, and their ability to deliver solutions, will determine the extent to which healthcare developments will be manifested through better clinical outcomes, improved longevity and better quality of life (34).

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XENOBIOTIC INDUCED MODEL OF PRIMARY BILIARY CIRRHOSIS

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PRIMARNA BILIJARNA CIROZA INDUKOVANA KSENOBIOTIKOM: EKSPERIMENTALNI MODEL

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ABSTRACT

Primary biliary cirrhosis (PBC) is an autoimmune disease of the liver that is, characterised by destruction of the intrahepatic bile ducts and the presence of antimitochondrial antibodies (AMAs). Several murine models of PBC, with similar serological, biochemical, and histological features to human PBC, have been developed in recent years. These animal models enable investigators to study the etiology and pathophysiologic mechanism of PBC. Immune response in PBC is directed towards E2 components of the 2-oxo-acid dehydrogenase family of enzymes, which is in located in mitochondria and is an immunodominant epitope (a lipoylated peptide sequence shared by enzymes). Immunisation of mice with 2-octynoic acid coupled to bovine serum albumin (2-OA-BSA) (which is an antigen that is structurally related to the E2 subunit of the pyruvate dehydrogenase complex [PDC-E2]) produces histologic features similar to those found in human PBC. This model of xenobiotic induced PBC is suitable for studying the early events in PBC pathogenesis and for developing new therapeutics in PBC.

Key words: *PBC, xenobiotic, 2OA-BSA, C57BL/6 mice*

SAŽETAK

Primarna bilijarna ciroza (PBC) je autoimunska bolest jetre koju karakteriše destrukcija intrahepatičnih žučnih kanalića i prisistvo antimitohondrijalnih antitela (AMAs). Poslednjih godina je razvijeno nekoliko mišjih modela PBC koji imaju slične serološke, biohemijske i histološke karakteristike kao i humana PBC. Ovi animalni modeli su omogućili ispitivanje etiologije i mehanizama uključenih u patogenezu PBC. U PBC imunski odgovor je usmeren na E2 komponentu 2-okso-kiseline dehidrogenaza familije enzima koji su locirani u mitohondrijama, a imunodominantni epitop je peptidna sekvenca sa lipidima koja je zajednička za ove enzime. Imunizacija miševa 2-oktinoičnom kisleinom vezanom za goveđi serumski albumin (2-OA-BSA), antigenom koji je strukturno sličan E2 subjedinici kompleksa piruvat dehidrogenaze (PDC-E2), omogućava razvoj histoloških promena koje karakterišu PBC kod ljudi. Ovaj model PBC indukovan ksenobiotikom je pogodan za ispitivanje početnih događaja u patogenezi PBC i za razvoj novih lekova za PBC.

Ključne reči: *PBC, ksenobiotik, 2OA-BSA, C57BL/6 miševi*

INTRODUCTION

Primary biliary cirrhosis (PBC) is a liver-specific autoimmune disease (1). PBC has a long latency period, which is followed by the development of common symptoms: fatigue, pruritus hyperpigmentation, and (in the terminal stages) bleeding varices, and ascites (2). PBC is characterised by a multilineage humoral and cellular adaptive response against biliary epithelial cells (BECs) and destruction of small bile ducts by mechanisms that include innate immune responses (3; 4). Bile duct destruction leads to cholestasis, fibrosis, and ultimately liver cirrhosis (4). The typical characteristic of the disease is the presence of antimitochondrial autoantibodies (AMA), which are present in high amounts. The autoantigens to which the immune responese is directed in PBC has been identified as the E2 subunits of the 2-oxo-acid dehydrogenase complexes (2OADC-E2), including the E2 subunits of the pyruvate dehydrogenase complex (PDC-E2), branched chain 2-oxo acid dehydrogenase complex (BCOADC-E2), and 2-oxoglutarate dehydrogenase complex (OGDC-E2) (5). The immunodominant autoantigen within this group is PDC-E2 (6; 7). A multi-faceted immune response to the immunodominant mitochondrial autoantigen PDC-E2 in PBC

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suggests that a loss of tolerance to PDC-E2 is the initiating event in the development of PBC; there is no significant evidence of epitope spreading, which is present in other autoimmune diseases (8).

ETIOPATHOGENESIS OF PBC

The etiology of PBC, including the loss of tolerance, is still unknown. However, as in all autoimmune diseases, it is likely that genetic susceptibility and environmental factors play a role in the pathogenesis (9). Environmental factors, including xenobiotics or microorganisms, modify the autoantigen and facilitate the breakdown of tolerance (10).

Molecular mimicry: Cross-reactivity between sera of PBC patients and *E. coli* has been shown, but stronger reactivity (1,000-fold stronger than with E. coli) has been demonstrated with the xenobiotic-metabolising gram-negative bacterium *Novosphingobium aromaticivorans* (a bacterium present in human fecal specimens) (11). *N. aromaticivorans* contains two proteins highly homologous to the immunodominant epitope of PDC-E2 and serum autoantibodies. Importantly, mice infected with *N. aromaticivorans* deveope PBC-like liver lesions (12).

Xenobiotics: Because the liver plays a key role in the metabolism of toxins, the hepatocytes and BECs are continuously exposed to chemical by-products. Associations between PBC and the frequent use of nail polish support a xenobiotic pathogenesis hypothesis. 2-Octynoic acid is a food additive and xenobiotic that is present in cosmetic products, such as nail polish. The *in vitro* and *in vivo* data strongly support a potential role of 2-octynoic acid in PBC. Reactivity of 2-octynoic acid with AMAs and lipoic acid has been shown (13). Congenic nonobese diabetic NOD.1101 (NOD.B6 Idd10 Idd18r2) C57BL/6 mice, immunised with 2-octynoic acid conjugated with bovine serum albumin, develop histological features of autoimmune cholangitis (portal infiltrates enriched in CD8+ cells and liver granulomas); these mice demonstrated high titers of AMAs (14-17). This model provides convincing evidence that xenobiotics are causally related to the development of PBC.

Biliary epithelial cells

The most intriguing aspect of the pathogenesis of PBC remains the specific immune response directed at the small intrahepatic bile ducts, as all nucleated cells have mitochondria with 2-oxo-acid dehydrogenase complexes. These small biliary ducts are lined with biliary epithelial cells, BECs (i.e., cholangiocytes) and are destroyed by the immune response, mediated by specific CD4+ and CD8+ T cells (18; 19). This selective destruction indicates there are unique immunopathological characteristics of BECs. It is known that BECs are not passive bystanders in primary biliary cirrhosis; these cells can increase the expression of adhesion molecules and production of TNF-α, IFN-γ, and IL-1 upon stimulation with proinflammatory cytokines (20). Through the variable expression of adhesion molecules and proinflamamtory cytokines, BECs can modulate the degree and localizisation of the inflammatory process. Additionally, BECs have properties of antigen presenting cells by expressing HLA class II and costimulatory molecules CD80 and CD86. Based on these characteristics of BECs, it can be hypothesizised that their interactions with T cells may be responsible for bile duct damage.

BECs of small bile ducts are very susceptible to apoptosis, more than epithelial cells of larger ducts (due to a lack of production of specific protease-resistant peptides, trefoils) (21). Moreover, unique characteristics of apoptosis in BECs indicate that this process most likely plays a part in the immunopathogenesis of PBC. Autoreactive lymphocytes may be activated with neo-antigens arise from apoptotic BECs (22). When BECs undergo apoptosis, the major mitochondrial autoantigen, PDC-E2, remains immunologically intact, whereas other cells following apoptosis present a form of PDC-E2 that cannot be detected by AMAs (23; 24). Persistent exposure to PDC-E2, as derived from BECs, is caused by a failure to covalently link PDC-E2 to glutathione during the course of apoptosis in these cells. Another important observation regarding the role of apoptotic BECs in the pathogenesis of PBC is the high degree of proinflammatory cytokine production in monocyte-derived macrophages that was found in PBC patients who were incubated with apoptotic bodies from BECs (in the presence of AMAs) (25). It is important to note that the BECs used in these experiments were derived from two normal donors, which implies that there is no phenotype of biliary epithelial cells specific for PBC; this could explain the recurrence of PBC following transplantation (26).

Immunostaining of PBC biliary tract with monoclonal antibodies against mitochondrial autoantigens demonstrated a high degree of expression of PDC-E2 at the apical surface of the small bile duct cells lining the bile duct lumen (27; 28). Cholangiocytes play a role in the transport of IgA antibodies in bile duct lumen. PDC-E2-specific IgA enters the BECs via a polyimmunoglobulin receptor and forms a complex with PDC-E2; it may thereby contribute to the exposure of PDC-E2 at the apical surface of BECs. Additionally, during transcytosis through cells expressing polyimmunoglobulin receptors, dimeric IgA can initiate the activation of caspases (29). The levels of anti-PDC-E2 IgA antibodies in PBC sera directly correlate with the level of caspase activation.

IMMUNE RESPONSE IN PRIMARY BILIARY CIRRHOSIS

The mechanism of biliary destruction has not been completely determined, but the specificity of pathological changes in the bile ducts, the presence of lymphoid infiltration in the portal tracts, and the presence of major-histocompatibility-complex class II antigens on the biliary epithelium indicate that an intense immune response is directed against the biliary epithelial cells. There are data suggesting that the destruction of biliary cells is mediated by liver-infiltrating autoreactive T cells (19; 30). CD4+ and

CD8+ T cells can be detected in the portal tracts of PBC patients (19; 31-33). An increased serum level of autoantibodies specific for PDC-E2 is accompanied by a 100 times higher frequency of antigen-specific CD4+ T cells and a 10 times higher frequency of antigen-specific CD8+ T cells in liver as compared towith draining lymph nodes. Two important T helper cell subpopulations shown to have a role in the pathogenesis of PBC are Th17 and Treg cells (34). Significantly lower levels of CD4+ CD25high are detected in the peripheral blood of PBC patients and their family members. In addition, FoxP3+ Treg cells can be detected in the lymphoid infiltrates found in the portal tracts (35). Th17 cells have a pathogenic role in PBC: an increased frequency of IL-17-positive lymphocytes was found in liver tissues from patients with PBC, and in the IL-2Rα KO mouse model of autoimmune biliary disease (36).

There is granulomatous inflammation in the liver of PBC patients that is accompanied by an increased production of polyclonal IgM antibodies. Cultured human BECs express toll like receptors (TLRs), lipopolysaccharide, and lipoteichoic acids, which are present in bile. The eExpression of TLRs in BECs is mediated through biliary injury via the NF-κB pathway (37). In response to TLR stimulation, BECs may produce proinflammatory cytokines IL-6 and TNF-α and chemokines IL-8 and CX3CL1. CX3CL1 is a chemoattractant for cells expressing its receptor, CX3CR1. In PBC patients, CX3CR1 expressing CD8+ and CD4+ T cells can be found in the portal tracts and within the biliary epithelial layer of injured bile ducts (38). Another cell type known to be involved in PBC pathogenesis are NKT cells. There is a higher frequency of CD1d-restricted NKTs in PBC patients. These cells are more frequently found in the liver than in the peripheral blood. An increased number of CD1d-restricted NKT cells was found in the liver of the dnTGF-βRII mouse model (39). These CD1d-restricted NKT cells in the liver had increased IFN-γ production following exposure to α-galactosylceramide; this was accompanied by a decrease in hepatic lymphoid cell infiltration and less cholangitis when compared towith the controls.

ANIMAL MODELS OF PBC

Over the past several years, several animal models for PBC have been developed. Severe combined immunodeficient (SCID) mice develop lymphocytic infiltration around small bile ducts and present with the anti-PDC-E2 antibodies following the transfer of lymphocytes from peripheral blood of PBC patients (40). Congenic NOD.c3c4 mice are obtained by replacing the diabetes-susceptibility genes on chromosomes 3 and 4 with the diabetes-resistance genes of B6 and B10 mice (41). Overall, 50% to 60% of these mice develop autoimmune cholangitis and demonstrate AMA antibodies in their serum. Histologically, however, the cyst-like dilatation of the affected bile duct, which is characteristic of these mice, is not observed in PBC patients; when the dilatation becomes severe, the biliary epithelium of NOD.c3c4 mice frequently exfoliates, which can trigger neutrophil infiltration that is not characteristic of human disease. Another mouse model for PBC is the dnTGF-βRII mouse. These mice over express the dominant-negative form of TGF-β receptor II under the control of the CD4 promoter. A deficiency of TGF-β signalling causes various immunological abnormalities, including colitis. dnTGF-βRII mice exhibit major serological and histological characteristics of human PBC (42), indicating an important role of the TGF-β signalling pathway in the pathogenesis of PBC. Serologically, specific AMA production occurs in all mice, and histologic hepatic lesions typical of PBC (lymphocytic infiltration, interlobular bile duct destruction, and granuloma formation in the portal tract) appear at an increased frequency. Immune infiltrating cells (including B cells, plasmacytoid dendritic cells, natural killer (NK) cells, and macrophages), CD4+ cells, and CD8+ T cells are found in the portal tracts. In IL-2Rα−/− mice (43), the IL-2 signal, which is important for controlling the fate of mature T cells, is functionally blocked. These mice develop an inflammatory bowel disease and an autoimmune lymphoproliferative disease. Anti-PDC-E2 antibodies are present in the sera of all IL-2Rα−/− mice. There is increased lymphocytic infiltration in the portal tract and damage of the interlobular bile duct. CD8+ T cells are predominant among the infiltrating lymphocytes, and there is an increase in the number of CD4+ T and B cells. In addition, a small number of granulomas is formed. Increased levels of inflammatory cytokines, including TNF-α, IFN-γ, IL-12p40, and IL-6, are also observed. Using a model produced by crossing IL-2Rα−/− mice with CD4 KO and CD8 KO mice, Hsu et al. (44) showed that CD8+ T cells participate in the pathogenesis of PBC.

XENOBIOTIC INDUCED PBC

The autoantigens of the E2 enzymes have a common structure consisting of a single N-terminal catalytic domain, containing two binding sites for the covalently attached lipoic acid cofactor. These lipoyl binding domains are the epitopes that are recognizised most often by AMAs (8), suggesting an essential role of the lipoic acid domain in the etiology of PBC. The immune reactivity of AMAs are directed against a conformational epitope that is susceptible to chemical modification. This finding indicates that self-tolerance may be interrupted by chemical modification of the lipoyl domain of PDC-E2 by xenobiotics. It has been demonstrated that the modified lipoyl domain of PDC-E2 specifically binds antibodies in PBC sera, often at levels higher than the native PDC-E2 molecule (45-47). These mimicking effects are found in compounds that are widely used in the environment (including perfumes, lipstick, and many common food flavorings) (45). Studies have shown that animals immunised with selected AMA-positive xenobiotics resulted in AMAs; these animals developed liver pathology similar to PBC (48; 49).

It has been reported that B6 and NOD.1101 (NOD. B6 Idd10 Idd18r2) mice immunised with 2-octynoic acid

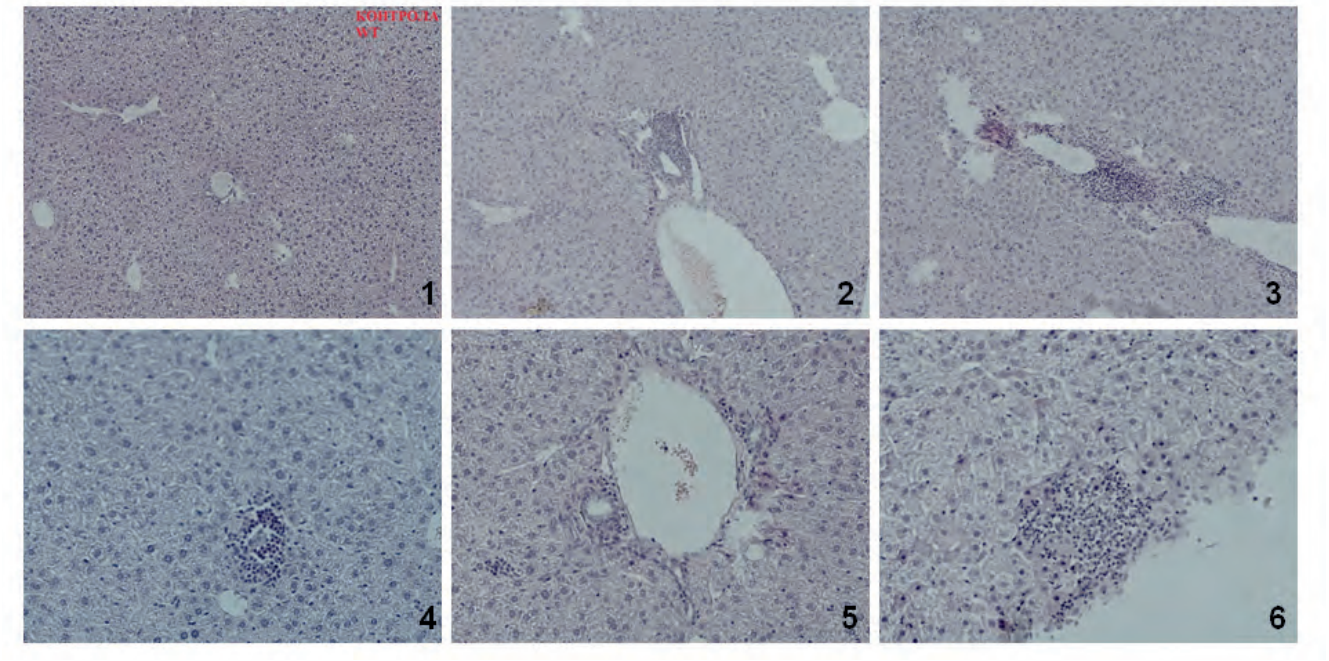

Figure 1. Histological features of the C57BL/6 mice 8 weeks after immunization with 2OA-BSA and control mice.
Different degrees of lymphocytic infiltration: 1) untreated mice; 2) portal infiltration with granuloma formation; 3) parenchymal infiltration; 4) parenchymal granuloma; 5) moderate portal infiltration; 6) subcapsular abscess (H&E staining).

(2-OA), coupled to BSA, had high AMA titers, portal inflammation, and cholangitis similar to human PBC (14).

We used this xenobiotic induced PBC model to explore, in detail, the histological characteristics of the liver.

EXPERIMENTAL PROTOCOL

Female C57BL/6 mice were maintained at the animal facilities of the Faculty of Medical Sciences University of Kragujevac. All animal procedures were approved by the ethical committee of the Faculty of Medical Sciences, University of Kragujevac.

Primary biliary cirrhosis was induced as previously described (14). Briefly, a mixture of BSA conjugated 2-octynoic acid (2OA-BSA; 100 μg/100 μL in PBS) was injected intraperitoneally with Complete Freund's Adjuvant (CFA; Sigma-Aldrich, St. Louis, MO), containing 1 mg/mL of Mycobacterium tuberculosis (strain H37 RA; Difco Laboratories, Detroit, MI). This was subsequently boosted every two weeks with 2OA-BSA in Incomplete Freund's Adjuvant (IFA; Sigma-Aldrich, St. Louis, MO). Additionally, mice intraperitoneally received 100 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) at the time of initial immunisation with 2OA-BSA in Complete Freund's Adjuvant.

Immediately following sacrifice, liver tissue was harvested, fixed in 10% buffered formalin, embedded in paraffin, and cut into 4-μm sections for routine hematoxylin and eosin (H&E) staining. Evaluation under light microscopy and scoring of liver inflammation and bile duct damage was performed on coded H&E-stained sections in a

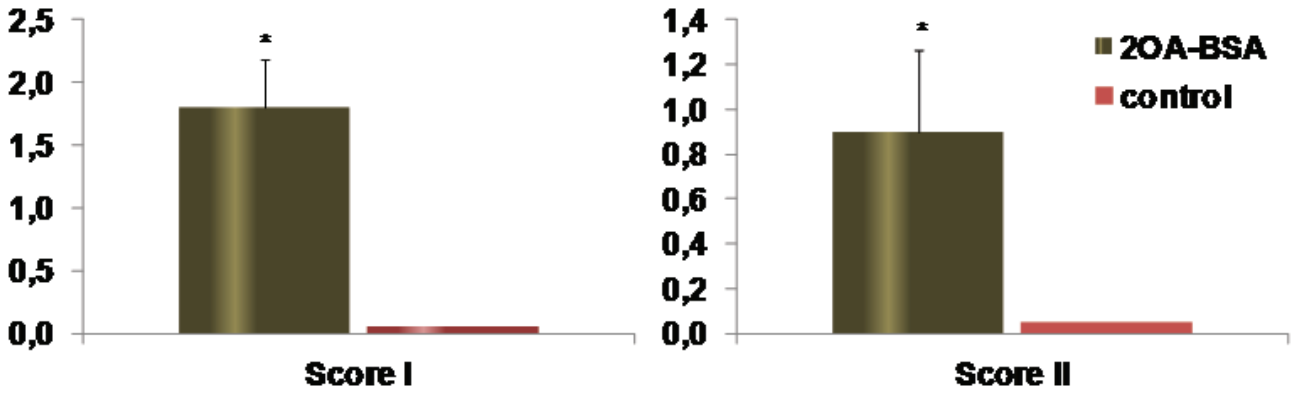

Figure 2. C57BL/6 mice immunized with 2OA-BSA develop significant infiltrates in the liver. Mean values + SD for histopathological scores I and II per group (2OA-BSA immunised and control) are presented. 0 = no significant change, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe pathology * $p<0.05$.

blinded fashion. The images were captured with a light microscope (Olympus) equipped with a digital camera.

Sections were evaluated for periportal inflammation, infiltration of bile ducts without damage, infiltration and damage of bile ducts, and subcapsular infiltrates. Based on the level of pathology, the indices were scored as 0, no; 1, mild; 2, moderate; 3, severe; or 4, very severe pathology. Score I was calculated as the mean value of each scored index. Granulomas, and fibrosis were scored as 0, no; 1, mild; 2, moderate; or 3, severe pathology; ,based on these values, score II was calculated.

All mice immunised with 2OA-BSA (9/9) developed histological findings typical of PBC (Figure 1). Our histological scoring clearly demonstrates the disease in the group of 2OA-BSA immunised mice (Figure 2).

The autoimmune cholangitis induced by 2OA-BSA immunisation recapitulates the histological features of human PBC: portal-tract inflammation with destruction bile ducts, focal-duct obliteration with granuloma formation, periportal extension of inflammation, and fibrosis. Importantly this model of autoimmune cholangitis gives us the opportunity to study the early events of PBC pathogenesis and to explore the possibility of new PBC therapeutics.

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CUTANEOUS EFFECTS OF SEA BUCKTHORN OIL EMULSION

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PERKUTANI EFEKAT EMULZIJE SA ULJEM PASJEG TRNA

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ABSTRACT

Sea buckthorn oil (Hippophae rhamnoides L.) is medically used both externally and internally, but the external application is unsuitable due to its liquid, lipophilic and highly coloured nature. These difficulties could be overcome by a formulation of semisolid emulsion with sea buckthorn oil. Previous research on this formulation showed that it has higher wound healing potential than sea buckthorn oil, possessing an enhanced structure of liquid crystals, stability and suitability for topical use.

The aim of this investigation was to completely characterizise a proposed emulsion by testing skin effects, such as moisturising potential, skin pH and potential to cause skin irritation.

The emulsion was prepared by standard emulsifying techniques using a combination of surfactants that form an enhanced structure of liquid crystals. Approximately 40% of sea buckthorn oil was incorporated. The moisturising potential and skin pH were tested on the healthy skin of volunteers. Skin tolerance was tested on a rabbit skin model and evaluated by the Draize test.

The tested emulsion containing sea buckthorn oil did not cause a significant change in skin pH, while it significantly increased skin hydration. There was an absence of edema or erythema type of irritation after 2 h, 24 h, 48 h, 72 h and 7 days of application of the emulsion with sea buckthorn oil.

The tested formulation shows good moisturizising effects and does not cause human or animal skin irritation. The study confirms that the combination of the proposed ingredients in a sea buckthorn oil emulsion is adequate and could be safe for skin application.

Key words: *sea buckthorn oil, emulsion, topical use, irritation*

SAŽETAK

Ulje pasjeg trna (Hippophae rhamnoides L.) se u medicinske svrhe upotrebljava kako za eksternu tako i za internu primenu, pri čemu je eksterna primena nepogodna usled njegove tečne konzistencije, lipofilne prirode i intenzivne obojenosti. Navedeni nedostaci bi se mogli prevazići formulacijom polučvrstih emulzija sa uljem pasjeg trna. Prethodna ispitivanja ove formulacije su pokazala da poseduje znatno veći potencijal za zarastanje rana u odnosu na ulje pasjeg trna, unapređenu strukturu tečnih kristala, stabilnost i pogodnost za lokalnu primenu.

Sprovedeno istraživanje imalo je za cilj da upotpuni karakterizaciju predložene formulacije, testirajući efekte na koži- hidrirajući potencijal, pH kože i potencijal za izazivanje kožnih iritacija.

Emulzija je pripremljena standardnim tehnikama emulgovanja, korišćenjem kombinacije surfaktanata kojima se formira poboljšana struktura tečnih kristala pri čemu je ulje pasjeg trna je bilo inkorporirano u količini od 40%. Hidrirajući potencijal i pH kože su bili testirani na koži zdravih volontera. Tolerancija kože je testirana na modelu zečje kože i procenjivana pomoću Draize-ovog testa.

Ispitivana emulzija sa uljem pasjeg trna nije pokazala značajne promene pH kože, dok je pokazan značajan hidrirajući efekat. Nisu se javile kožne iritacije, tipa edema ili eritema, nakon 2h, 24h, 48h, 72h i 7 dana od aplikacije emulzije sa uljem pasjeg trna.

Evaluirana formulacija ne izaziva iritaciju ni humane ni životinjske kože i pokazuje dobar hidrirajući efekat. Studija potvrđuje da je kombinacija preloženih sastojaka u emulziji sa uljem pasjeg trna adekvatna i može se bezbedno primenjivati na koži.

Ključne reči: *ulje pasjeg trna, emulzija, lokalna primena, iritacija*

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INTRODUCTION

Hippophae rhamnoides L. (sea buckthorn) is a bushy tree growing both in Asia and Europe (1). European northern habitats are located in Germany, while the southern habitats are located near the Black Sea. *Hippophae rhamnoides* L. grows in the Caucasus, Alps and Carpathians and in the Danube delta, and it exists as a cultivated species in Germany and Russia (2). In the past, it was also identified in Serbia near the Danube (3, 4).

Although different parts of sea buckthorn have been studied (e.g., fruits, leaves), the research on its fruits is the most abundant. The fruits are round and fleshy and are predominantly of an orange colour (4). The pulp has a mild smell and is oily due to considerable fatty oil content. The fruits are collected from September to late December and are considered a high source of vitamin C and fatty oil, the chemical composition of which depends on harvesting time (5-10).

Medical research on sea buckthorn increased at the end of XX century. Analysis of the chemical content of the fruit pulp and oil revealed a rather unusual combination of sea buckthorn oil constituents - saturated and unsaturated fatty acids (palmitic, palmitoleic, oleic, linoleic, linolenic, myristic, stearic), vitamin A, vitamin E, beta carotene, sterols, etc. (10-12). Positive pharmacologic effects of sea buckthorn oil on human health are linked to both external and internal application (7). When externally applied, tissue-regenerative, anti-inflammatory, anti-oxidant and anti-bacterial effects were observed in wounds, burns, and atopic dermatitis. Topical use of sea buckthorn oil applied to burns stimulates the proliferation of fibroblasts, collagen synthesis, the expression of specific matrix metalloproteinases, and angiogenesis, which has been connected to a high content of unsaturated omega-3 and omega-6 fatty acids, carotenoids and tocopherols in oil (6, 7, 13, 14).

The sea buckthorn oil used in this investigation was obtained from fruits from spontaneous flora (12). It was an orange, lipophilic liquidand and was difficult to apply externally in a reproducible manner. A semisolid topical formulation of an emulsion was proposed to overcome obstacles in application (dripping, leaking, and difficult absorption). Previous pharmaceutical and pharmacological research on this formulation showed that it enhances wound healing and possesses the following characteristics – an oil/water type, an acceptable pH value and organoleptic properties for skin application (15, 16). The aim of this study was to complete previous studies of the proposed formulation by evaluating the following effects on human and animal skin: moisturising potential, change of skin pH after application, and potential to cause skin irritation.

Table 1. Formulation of semisolid emulsion with sea buckthorn oil

	Components	*% (m/m)*
Inner/oily phase:	Lanette® 16	2.0
	Lanette® 18	2.0
	Brij® 72	2.5
	Brij® 721P	2.5
	Arlamol E	4.00
	Hippophae oleum	40.00
Outer/water phase:	Nipagin®	0.10
	Propylene glycol	3.00
	Aqua purificata	43.90

MATERIALS AND METHODS

For the preparation of the test emulsion, we used sea buckthorn oil obtained from plant material *ex tempore* (4). The substances, separated as an inner or outer phase based on lipophilic and hydrophilic affinity, were merged together (Table 1.). The emulsion was prepared by standard emulsifying techniques, and a sample with 40% sea buckthorn oil was prepared (15, 16). Standard laboratory equipment was used, including a digital balance (Chyo, MP-3000, Japan), water bath (Sutjeska, Belgrade, Republic of Serbia) and laboratory mixer (Velp, EU). Samples of the emulsion with sea buckthorn oil (SB) were packed in tubes and sealed immediately after the preparation.

Skin moisture and skin pH were tested on 12 healthy women, with an average age of 45.5 years. The samples were applied twice daily for 28 days, after which there was a pause of one week with no application. The last measurement was done on the 35th day. The volunteers were aware that 3 days before the application of the examined preparation, they should not apply any dermatological or cosmetic products on the place of the application (the inner under-elbow surface of the skin). The volunteers were placed in a room with constant conditions (temperature 22±1°C, humidity 55±5%) 20 min prior to the testing. The measurements of the skin moisture were done on days 1, 3, 5, 7, 14, 21, 28 and 35 by a CorneometerCM 820 (Courage+Khazaka Elektronic, Germany). Skin pH was measured by a Skin-pH-meter PH900 (Courage+Khazaka Elektronic, Germany) on days 1-9. A 2 mg/cm^2 dose of the sample was applied on the insides of forearms (9 cm^2). The Student's t-test ($p<0.05$) and ANOVA were used to evaluate the statistical significance of the measured differences.

To evaluate the potential of the proposed formulation to cause irritation, an animal model was used. The experiment was conducted on male rabbits, with a body weight of 2.0 – 2.5 kg. Rabbits were accommodated, one per cage, under conditions of controlled temperature and lighting, with food and water provided *ad libitum*. To adapt to the environment, the animals were kept in the cages at room temperature. A circadian regime, light/dark ratio of 12/12, was used during seven days before the experiment. The temperature was 22 - 26°C, and the relative humidity was 30 - 70%. The food and water provided were standard laboratory food for rabbits (Veterinarski Zavod Subotica,

Table 2. Intensity level of skin changes of rabbit skin (Draize test)

Parameters		Level
Edema (Ed)	**Erythema (Er)**	
No edema	No erythema	0
Slightly visible edema	Mild, slightly visible erythema	1
Edema with visible border	Well expanded erythema	2*
Moderate edema - ≤ 1 mm	Moderate to strong erythema	3*
Heavy edema - ≥ 1 mm	Heavy erythema and crust	4*

*positive findings

Serbia) and did not contain substances that could have influenced their health. The study protocol was based on the Guidelines for Animal Study No. 282-12/2002 of the Military Medical Academy Ethics Committee, Belgrade, Serbia.

The animals were randomly divided into 3 groups of 3 animals and were treated in the following way: the 1st group was the control group and received saline solution 0.9% NaCl, the 2nd group received only sea buckthorn oil, and the 3rd group received samples of the emulsion containing sea buckthorn oil. Rabbit use is approved for testing skin tolerance and irritation potential during external application (17, 18).

Before application of the test substance, a dorsolateral skin surface of ~ 20 cm^2 was depilated on both sides. The test substance was applied once daily in the quantity of 5 ml on the prepared area of the skin. After sample application, the depilated area was covered by a sterile cotton cloth and fixed by a non-irritative bandage for 4 h. Skin changes were observed after 2, 24, 48 and 72 h and 7 days after application of the test substance, and intensity was graded according to the Draize test (Table 2.) (19, 20).

RESULTS

The test sample of the emulsion containing sea buckthorn oil was semisolid and homogeneous, and it did not cause any allergic reactions or other side effects during the testing.

There was significant change in skin moisture that started on day 3 compared to the baseline value, registered on the first day after sample application, and continued until day 28 inclusive. The highest value for skin moisture was on day 3 (increase of 10.31%). A pause of one week influenced the change in skin moisture, and a 1.64% decrease from the baseline value was noted . The results are shown in Fig. 1.

External application of the tested emulsion did not significantly change the pH of the healthy skin. The results are shown in Fig. 2.

The results of the emulsion sample application on rabbit skin are shown in Table 3. There was no evidence of edema or erythema in any of the 3 animal groups during the experiment.

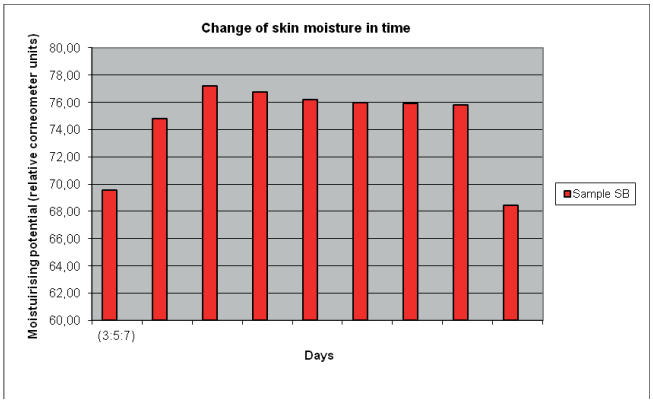

Figure 1. Skin moisturising effect of the emulsion with sea buckthorn oil

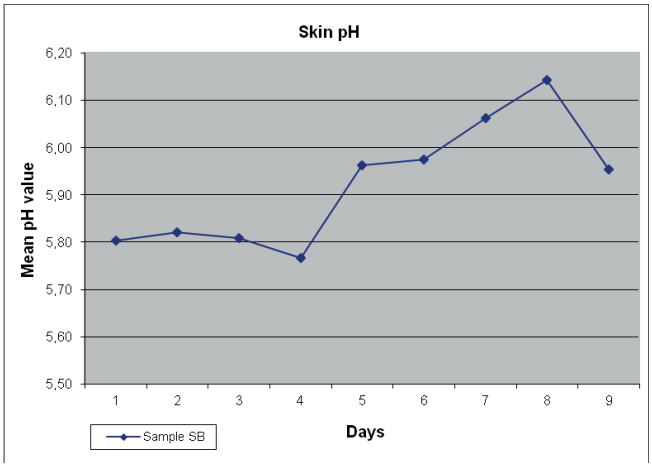

Figure 2. The mean skin pH value of healthy volunteers

Treatment	Level of skin change									
	2 h		24 h		48 h		72 h		7 days	
	Ed	*Er*	*Ed*	*Er*	*Ed*	*Er*	*Ed*	*Er*	*Ed*	*Er*
Sol. 0.9% NaCl	0	0	0	0	0	0	0	0	0	0
Sea buck. oil	0	0	0	0	0	0	0	0	0	0
Emulsion	0	0	0	0	0	0	0	0	0	0

Table 3. Results of testing the irritation-causing potential of the emulsion with sea buckthorn oil on rabbit skin

DISCUSSION

Sea buckthorn oil has anti-inflammatory and epithelisation stimulating properties (7, 15). As a lipophilic liquid, sea buckthorn oil can have the main or adjuvant role in the formulation of a semisolid topical emulsion. Previous research on this formulation showed that it's application resulted in better wound healing than sea buckthorn oil, which may be attributed to the structure of the emulsion (liquid crystals) and the synergetic activity of sea buckthorn oil and other ingredients (17). Testing additional skin effects of the proposed emulsion (skin moisturising effects, pH change on healthy human skin, and potential to cause irritation on rabbit skin) completed the evaluation of the proposed emulsion for external use, because the enhanced structure of the liquid crystals should provide proper skin hydration (21, 22). Surfactants in emulsion are used for formulation stability but, at the same time, may often cause skin irritation as an undesirable side effect. The modern approach in emulsion formulation is to choose a combination of surfactants not only to achieve a better stabilisation effect, but also to minimise the potential for skin irritation. Surfactants may disturb metabolism of lipids, which are an integrative part of the *stratum corneum.* Direct contact of surfactants with epidermal layer of keratinocytes may cause inflammation or cytotoxicity due to the release of proinflammatory cytokines or protein denaturation, leadin g to swelling of the *stratum corneum* and breaking the natural barrier function of the skin (23, 24). The effects surfactants may present on the skin depend on their type and concentration, as well as on their interactions with other ingredients used in the formulation. A successful formulation should provide both emulsion stability and good skin tolerance (25, 26). The proposed formulation is well stabilised by liquid crystals and has good skin characteristics (17, 27). Testing cutaneous effects represents an integral part of the evaluation for topical formulations and completes findings about this formulation. Because the application of the proposed emulsion with sea buckthorn oil did not cause any irritation, it seems that all ingredients used in this formulation are adequate for skin application. Thus, we can conclude that this formulation is suitable for external application.

CONCLUSION

This work completely characterizises information about the proposed formulation studied earlier. There was good skin tolerance of the proposed emulsion on both human and animal skin. It did not significantly change the skin pH and has good moisturising potential for human skin. It did not cause irritation on human or rabbit skin after topical application. This research shows that the quantities and type of components used in the proposed emulsion with sea buckthorn oil are adequate for safe external application.

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ANTI–GBM RAPIDLY PROGRESSIVE GLOMERULONEPHRITIS (SYNDROMA GOODPASTURE): A CASE REPORT

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ANTI-GBM RAPIDNO PROGRESIVNI GLOMERULONEFRITIS (SYNDROMA GOODPASTURE): PRIKAZ SLUČAJA

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ABSTRACT

Goodpasture syndrome is a severe illness caused by the formation of antibodies to the glomerular basement membrane and alveolus with consequential damage to renal and pulmonary function. With current therapy, long-term survival is more than 50%. Before, the mortality was higher than 90%.

In our patient, the disease began as dysuria, continued as anaemic syndrome, and ended with the development of end-stage renal failure.

Immunosuppressive therapy with pulse doses of methylprednisolone and cyclophosphamide has put the disease into remission, but the permanent impairment of renal function remained.

Early diagnosis of Goodpasture syndrome helps preserve renal function and improves patients' survival. In patients who achieve remission, a kidney transplant can be considered. Currently, our patient is awaiting transplantation.

Key words: *Goodpasture syndrome; collagen α3 (IV) chain; anti-GBM antibodies; crescentic forms.*

SAŽETAK

Goodpasture-ov sindrom je teško oboljenje uzrokovano stvaranjem antitela na bazalnu membranu glomerula i alveola sa posledičnim oštećenjem bubrežne i plućne funkcije. Sa sadašnjom terapijom dugogodišnje preživljavanje je veće od 50%, a pre toga smrtnost usledovog oboljenja je bila veća od 90%.

Početak bolesti kod našeg pacijenta bile su dizurične tegobe, potom anemijski sindrom, da bi se razvila bubrežna slabost završnog stadijuma.

Imunosupresivna terapija pulsnim dozama Methylprednisolona i Cyclophosphamida, uvela je ovog bolesnika u remisiju bolesti, ali je ostalo trajno oštećenje bubrežne funkcije.

Rano postavljanje dijagnoze Goodpastureovog sindroma omogućava očuvanje funkcije bubrega i povoljno utiče na preživljavanje bolesnika. Kod pacijenata kod kojih je postignuta remisija bolesti može se razmatrati i transplantacija bubrega, pa je i naš pacijent u procesu pripreme za transplantaciju.

Ključne reči: *Syndroma Goodpasture; α3 lanac kolagena tip IV; Anti-GBM antitijela; polumjesečaste formacije.*

INTRODUCTION

Anti-GBM (glomerular basal membrane) rapidly progressive glomerulonephritis (GN) is a renal disease characterised by damage to the glomerulus that has a progressive and rapid flow, caused by the formation of antibodies on the glomerular basement membrane. As a part of Goodpasture Syndrome, antibodies to the basement membrane of the alveoli could also be generated. Anti-GBM GN as a stand-alone entity occurs in approximately 20-40% of patients, while in the part Goodpasture syndrome occurs in approximately 60-70% of the cases.

The etiology is not fully known. The annual incidence in Europe is 0.5-1 case per million population (1). Although some authors suggest increasing incidence in spring and early summer (Rossert,2002), Fisher and Lager in their study, which encompassed a series of 80 renal biopsy samples, found no statistically significant difference in the incidence of Goodpasture syndrome related to the month or season (2) . Most authors agree that the disease is more common among men at a young age but is more common among women later in life. Glomerular crescents

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fractions (obtained by biopsy of the kidney) are correlated with the level of serum creatinine but not to the level of the titer anti-GBM antibodies (2).

After binding anti-GBM antibodies (which are the most common IgG class) to self-antigens, the target antigen is α3 chain of type IV collagen basement membrane (3, 4), which attracts leukocytes and causes an immune response. This leads to glomerular capillary damage, with consequent proteinuria, hematuria and renal impairment. It also damages the alveolar capillaries, resulting in laboredlaboured breathing and the coughing up of blood.

More than 90% of patients survive the acute phase of the illness. Death commonly occurs as a result of infections and pulmonary haemorrhage. In the period prior to immunosuppression and plasmapheresis, mortality of patients with Goodpasture syndrome was more than 90%, while the current therapy long-term survival is greater than 50% (5).

CASE REPORT

Our subject was a 44-year-old man who became significantly ill for the first time in January 2011. Because of complaints of dysuria, he was under the constant supervision of a urologist. (His labs were as follows: urea 13.1 mmol/l, creatinine 168 μmol/l, erythrocyte sedimentation rate 60/90, protein in the urine +, and in urine sediment approximately 30 leucocytes and 4-6 erythrocytes.) He was treated by urologists with antibiotics and uroantiseptics. At the end of March 2011, the laboratory findings returned to normal. In a control analysis in December 2011, he had advanced renal failure (urea 23 μmol/l, creatinine 686 μmol/l, proteinuria + + +, many leucocytes and erythrocytes in urine sediment), followed by anaemia (Hgb 85 g/l), radiologically enlarged heart shadow at the expense of the left ventricle, hilum extended and he was sent for treatment at our clinic. Upon admission, his main symptoms were headache, occasional dizziness, and frequent, difficult and painful urination. He was afebrile. His skin and mucosa had poor circulation. Heart and lung auscultation obtained normal findings.

With hypoalbuminemia and signs of an inflammatory syndrome, progression of renal failure occurs to the terminal level (creatinine 1.016 μmol/l, creatinine clearance of 4.8 ml/min, and diuresis < 200 ml). Treatment started with hemodialysis, three to four hours each week. Immediately, we obtained a blood sample for immunological examination and a kidney biopsy, which confirmed that it was rapidly progressive GN. (Fig. 1). Immunofluorescence findings showed linear deposits of immunoglobulin G (IgG) along the glomerular basement membrane (Fig. 2), and were foundpositive Anti-GBM, perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) and antinuclear antibodies (ANA) were found using immunological tests (which came later and were why the treatment with plasmapheresis was not immediately begun).

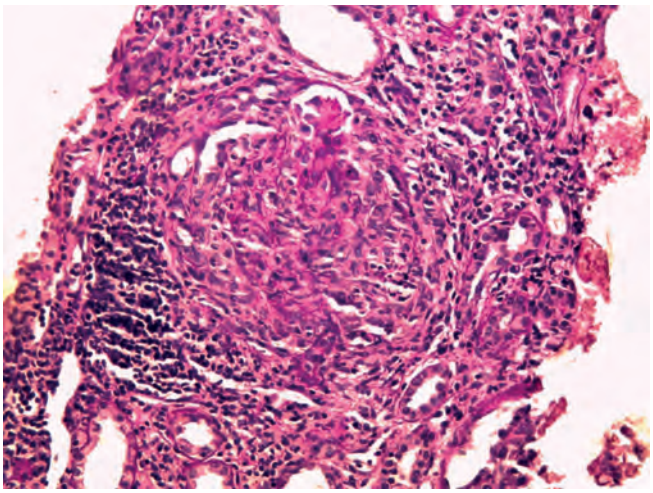

Figure 1. Optical microscopy: cellular crescent presses glomerular vascular bundle, thickening of the GBM

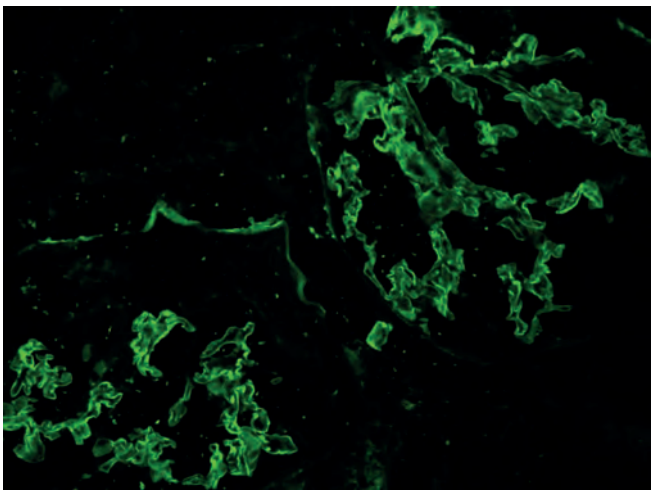

Figure 2. Immunofluorescence: IgG ++/+++ linearly along the GBM, C3 +/- linearly along the GBM and focal granular in mesangium

At the end of December 2011, the patient received the first pulse of methylprednisolone and cyclophosphamide. The treatment then was continued with prednisone tablets. Then, he developed the clinical picture of unspecified pneumonia, with radiological infiltrative changes that could suit also the primary disease. For this, the patient was treated with three antibiotics (ciprofloxacin, cephalosporin and meropenem), after which there was clinical and laboratory improvement. A complication, pseudomembranous colitis, arose, which was detected by colonoscopy. After the treatment with vancomycin, metronidazole, antifungal and mesalazine, the patient has a formed stool. Then, the treatment was continued with pulse doses of methylprednisolone and cyclophosphamide, followed by prednisone tablets orally starting with 50 mg per day. While receiving two pulses of cyclophosphamide, the patient had a relapse of pseudomembranous colitis, which was treated with metronidazole. The dose of prednisone was reduced gradually to 10 mg per day. After the fifth pulse of cyclophosphamide, there was a reduction in anti-GBM antibodies from the initial level of 698 U/ml to levels of 6 U/ml on the last control.

In addition to pulmonary and renal manifestations of the disease, a vision impairment can occur as part of Goodpasture syndrome. In our patient, bilateral retinal haemorrhage was found. Since the treatment options progressed and the life of this patient was extended, then it makes sense to include ocular manifestations of the disease in therapy (6).

CONCLUSION

In our patient, the disease initially manifested itself in the form of dysuric complaints. In less than a year, there was a decline in renal function. Our patient was positive for anti-GBM, pANCA and ANA using immune tests; thus, ich-Goodpasture syndrome was suspected. Immunochemical properties of autoantibodies do not affect the survival of patients with Goodpasture syndrome, but could be a factor of survival if detected in patients who have not had serious damage to the kidney (7).

If the immunosuppressive therapy is introduced early in the course of the disease, it can prevent or recover renal failure and may result in the cessation of pulmonary haemorrhage in the majority of patients with Goodpasture syndrome (8). Plasmapheresis, as an additional form of treatment, leads to a reduction of complement and fibrinogen, mediators that likely contribute to the damage that is incurred by the influence of autoantibodies (9). In our case, the treatment of plasmapheresis is not applied because there was a significant decrease in anti-GBM antibodies as a response to immunosuppressive therapy.

Patients for whom hemodialysis is immediately indicated have less chance of recovery of renal function (10), as was the case in our patient.

Although Goodpasture syndrome is a rare disease, it is necessary to bear it in mind, considering the weight of the disease. Additionally, it is possible to diagnose the disease at an early stage, due to the availability of immunoassays (such as anti-GBM antibody) and kidney biopsies. Only timely diagnosis (within the first few weeks of the onset of illness) makes a cure possibly. Unfortunately, diagnosis of this syndrome is usually made only when there are signs of renal failure, and then patients are almost always on dialysis (11).

Additionally, kidney transplantation is taken into account as a form of treatment of Goodpasture syndrome, but patients must be negative for anti-GBM antibodies for six months because there is a lower probability of disease transition on the transplanted kidney (less than 5%). Our patient, thanks to achieved remission, is awaiting a kidney transplantation.

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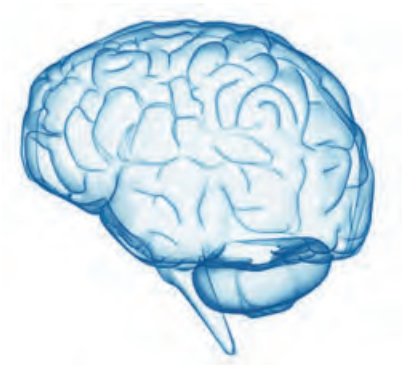

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