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CUPRINS

VITAMIN D IMPROVES VASCULAR FUNCTION IN EXPERIMENTAL DIABETES

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ABSTRACT

Controversy exists over the role of the active form of vitamin D, 1,25-dihydroxycholecalciferol, in the cardiovascular system in the presence of diabetes mellitus. The present study were purported to investigate the protective effect of 1,25-vitamin D3 on endothelial function in diabetic rats. To this aim, male Sprague-Dawley rats (8 weeks old) were injected with streptozotocin (STZ, 50 mg/kg, single injection intraperitoneally) to induce diabetes. After one month of hyperglycemia, thoracic aorta was removed and used for organ bath experiments. Endothelial function was evaluated in aortic rings isolated from diabetic and control animals, after 24 hours incubation in the absence or presence of 1,25-cholecalciferol (0.1μM). Endothelial-dependent relaxation to acetylcholine was significantly reduced in diabetic as compared with normal rats. Vitamin D significantly improved vascular function in diabetic rat aortic segments. In conclusion, *in vitro* administration of vitamin D alleviated diabetes-induced endothelial dysfunction in experimental setting.

Keywords: vitamin D, endothelial dysfunction, diabetes mellitus

INTRODUCTION

Cardiovascular disease is the major cause of morbidity and mortality in patients with diabetes. During the pathophysiological process of vascular damage, endothelial dysfunction is an early stage of disease development [1]. There is a large body of evidence suggesting that conditions associated with hyperglycemia can lead to different perturbations in endothelial function via activation of multiple oxidative stress pathways [2-5]. In diabetes, the evolution of atherosclerosis starting from endothelial dysfunction and ending up with the complicated plaques (prone to rupture) is accelerated [6-9]. Several cardiovascular risk factors have been recognized as contributors to the atherosclerotic process. Among them, vitamin D deficiency is emerging as a new one, being a highly investigated issue [10-12]. The active form of vitamin D, 1,25-dihydroxy-vitamin D3 (calcitriol) which functions via binding to vitamin D receptors. An increasing body of evidence suggests that vitamin D has also cardiovascular effects besides the regulation of calcium-phosphate homeostasis, and calcitriol and its analogs may play a role in modulating cardiovascular function [13-16]. Ecological studies revealed the fact that the incidence of cardiovascular disease augments with increasing distance from the Equator, suggesting an association with vitamin D insufficiency in regions with less sun exposure [17,18]. In diabetic patients, serum 25-hydroxyvitamin D (25OHD) concentration is inversely correlated with prevalence of cardiovascular disease [19], but the mechanism of vascular improvement induced by vitamin D is not very well understood. Vitamin D exerts a variety of favorable effects on endothelial dysfunction, VSMC proliferation [20,21], inflammatory process of atherosclerosis [22-25], insulin resistance, β-cell dysfunction [26,27], dyslipidemia [28], renin-angiotensin-aldosterone-system [22,29], therefore suggesting a potential therapeutic role, but the information regarding the mechanisms are missing.

Progression of endothelial dysfunction in diabetes involves the following general mechanism: high glucose levels increase reactive oxygen species (ROS) generation in vascular tissues with subsequent development and progression of endothelial dysfunction

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[30]. Several experimental and clinical studies revealed the fact that NADPH oxidase and the uncoupling of eNOS are the most important enzymatic systems of ROS generation [31,36]. In this regard it was showed that vitamin D down-regulates subunits p47 and p22 of NADPH oxidase [37] with a significant reduction in ROS production.

The present study was purported to assess the effect of 1,25-dihydroxy-vitamin D_3, the major metabolite of vitamin D, on vascular reactivity in aortic segments harvested from diabetic rats.

MATERIAL AND METHODS

Animal model and tissue preparation

Male Wistar male rats were purchased from the Cantacuzino Institute (Bucharest, Romania) and were acclimated for 2 weeks prior to the study. At the age of 8 weeks, diabetes was induced by a single injection of streptozotocin (50 mg/kg STZ, IP). Age-matched control rats received an equal volume of vehicle (0.01 M citrate buffer, pH 4.5). Two days after the injection, a blood sample was collected from the tail vein to measure the blood glucose. Rats with blood glucose over 200 mg/dl were considered diabetic. Animals were housed under standard conditions (constant temperature and humidity of 22.5 ± 2° C and 55 + 5%, 12-h light/dark cycle). The duration of the diabetes evolution was 8 weeks and blood glucose and body weight were systematically monitored. Twenty-four hours prior to the experiment solid food was withdrawn with no limitation in water supply.

All experimental procedures used in this study were conducted in accordance with the Directive 2010/63/EU and the Romanian Law nr. 43/May 2014 concerning the protection of animals used for scientific purposes. The experimental protocol was approved by the Committee for Research Ethics of "Victor Babes" University for Medicine and Pharmacy of Timişoara, Romania.

All reagents used were purchased from Sigma Aldrich, Invitrogen, Applichem and Abcam.

Organ culture

Rat aortic segments were dissected under sterile conditions, cleaned, and incubated for the times indicated at 37°C in EBM culture medium containing 0.1% BSA, in the presence or absence of 1,25-cholecalciferol (0.1µM, Sigma-Aldrich). Subsequently, the tissue was studied in organ chambers.

Organ bath studies

The rat aortic rings were suspended in organ chambers that contained 5 ml of Krebs solution (37°C) aerated with 95% O_2-5% CO_2 gas mixture (pH 7.4). Each ring was connected to a force transducer for isometric force recording. The rings were stretched to an optimal tension of 2 g and allowed to equilibrate for 60 min. They were then exposed to 80 mMKCl to obtain a submaximal contraction. Organ bath experiments were performed in the presence of diclofenac (10 µmol/L). The concentration of phenylephrine, used for pre-constriction, was adjusted to obtain a pre-constriction level of 80% of the contraction elicited by KCl(80 mmol/L). Subsequently, was recorded the endothelium-dependent relaxation to cumulative concentrations of acetylcholine (Ach) and contractility to endothelial nitric oxide synthase (eNOS) inhibitor L-NAME (10µM).

Statistics

Data are presented as mean + SEM and were analyzed using a one-way ANOVA or student t-test where appropriate. Post-hoc comparison among the groups was performed using the Tukey's test. Data analysis of the dose-effect response curves was performed using the ANOVA F-test (comparisons of bottom and top values, EC50 and the Hill slope). Values of $p<0.05$ were considered statistically significant.

RESULTS

Animals

Body weight and blood glucose from the day of experiment are shown in Table I. Four weeks after STZ administration, body weight was decreased in STZ rats than in normal rats. Blood glucose levels in STZ rats were significantly higher *vs.* controls.

Table I. Body weight and blood glucose at 4 weeks after STZ administration.

	CONTROL	STZ
n	8	8
Body weight (g)	421.6 ± 24.8	282.5 ± 49.5*
Blood glucose (mg/dL)	104.4 ± 10.6	490 ± 38.4*

*$p < 0.05$ *vs.* CONTROL

Vitamin D improves vascular contractility in diabetic aortic segments

The role of Vitamin D in improving vascular function was studied here in rats with experimental diabetes induced with STZ. After 1 month of constant hyperglycemia, thoracic aorta were isolated, incubated for 24 h with 1,25-cholecalciferol (0.1 µM) and studied in organ bath setup. The vascular contractility in diabetic segments was significantly increased in response to cumulative doses of phenylephrine and incubation with vitamin D partially normalized the contractility response (Figure 1).

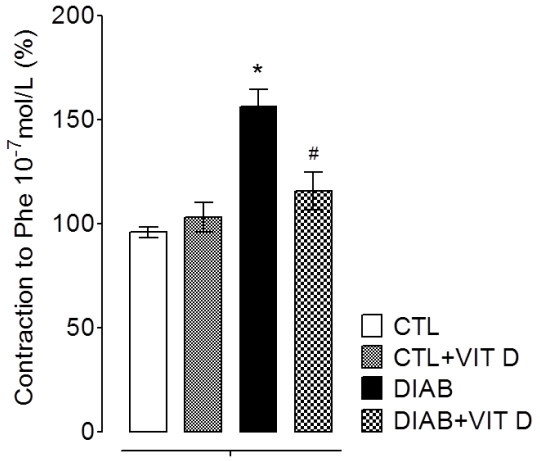

Fig. 1.The effects of vitamin D3 incubation on vascular contractility in diabetic aortas. Aortic segments from STZ-diabetic rats and controls were treated in vitro 24 h with 1,25-cholecalciferol (organ culture, 0.1 μM) and evaluated in organ bath system. Phenylephrine-induced contractions, n=10, *p<0.05 with and without diabetes (DIAB), #p<0.05 with and without vitamin D.

Vitamin D restores the endothelial-dependent relaxation in aortas from diabetic rats

Also, endothelium-dependent relaxation, after 4 weeks of hyperglycemia was significantly attenuated and 24 h incubation; again, vitamin D partially restored the relaxation response (Figure 2).

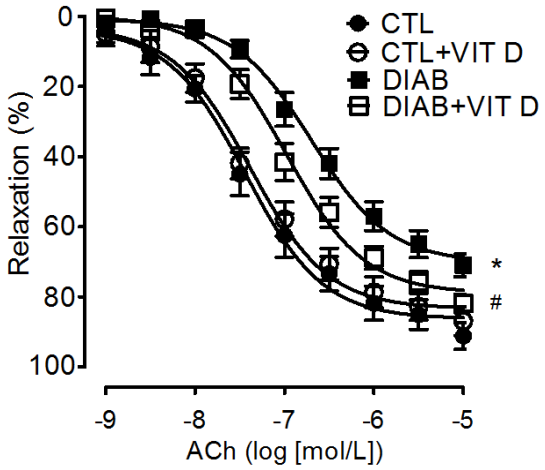

Fig. 2.The effects of vitamin D3 incubation on endothelial-dependent relaxation in diabetic aortas. Aortic segments from STZ-diabetic rats and controls were treated in vitro 24 h with 1,25-cholecalciferol (organ culture, 0.1 μM) and evaluated in organ bath system. Acetylcholine-induced endothelium-dependent relaxation. n=6, *p<0.05 with and without diabetes (DIAB), #p<0.05 with and without vitamin D.

Vitamin D improves NO bioavailability in aortas form diabetes rats

In diseased vessels the contractility to L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride, 10 μM) was increased and was significantly attenuated after incubation with vitamin D overnight, observation suggestive for the implication of NO in the mechanism of vitamin D-dependent vascular function improvement.

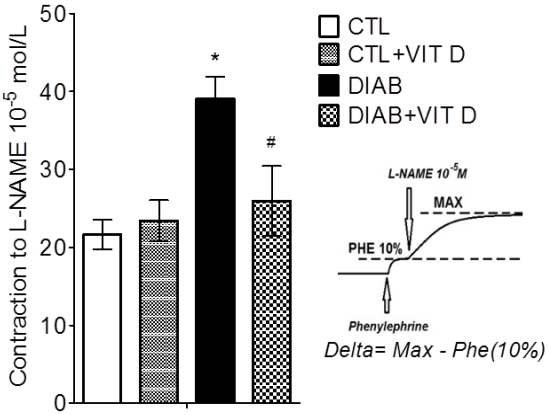

Fig. 3.The effects of vitamin D incubation on NO bioavailability in diabetic aortas. Aortic segments from STZ-diabetic rats and controls were treated in vitro 24 h with 1,25-cholecalciferol (organ culture, 0.1 μM) and evaluated in organ bath system. Contraction to L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride, 10μM), DELTA= Max.– 10% Phe contr. (Max.= maximal contraction to L-NAME, 10% Phe.contr.= 10% from contraction to 80 mMKCl induced by Phe), n=6, *p<0.05 with and without diabetes (DIAB), #p<0.05 with and without vitamin D.

DISCUSSION

Atherosclerosis represents the most important cause of cardiovascular diseases; its pathogenesis has complex mechanisms involving several cells - endothelial, vascular smooth muscle and immune cells, and also different mediators of inflammation [18]. The process of endothelial dysfunction is extremely accelerated in the presence of diabetes mellitus, the most severe metabolic disease.

An increasing body of evidence links vitamin D deficiency with atherosclerosis and cardiovascular disease [18]. Vitamin D, in addition to its role in phosphate and calcium metabolism, has been recognized to be an important factor in the cardiovascular system. The active metabolite of vitamin D, calcitriol (1,25-dihydroxicholecalciferol), has been revealed to exert protective effects by several mechanisms: inhibition of platelets' adhesion and aggregation, inhibition of the

release of pro-inflammatory mediators and adhesion molecules (intercellular adhesion molecule ICAM-1, vascular cell adhesion molecule - VCAM-1) [38,39].

The present study demonstrates an important vascular effect of the main active metabolite of vitamin D. The major finding of the study is that 1,25-cholecalciferol modulate vascular tone by reducing the contraction and endothelial-dependent relaxation in vasculature in conditions associated with hyperglycemia.

In organ bath experiments, we report a significant reduction in the endothelium-dependent relaxation of vascular segments in diabetic animals vs. controls. Incubation with the vitamin D3 partially restored the endothelium-dependent relaxation, suggesting a beneficial effect, at least in this in vitro model. Obviously, endothelial dysfunction in diabetes is attributable to vascular ROS production. Several clinical and experimental studies has shown that among the many enzymatic systems capable of producing ROS, NADPH oxidase and uncoupled eNOS are the main sources in the vascular wall in diabetic patients [40,41]. Besides these classical sources of ROS generation, monoamine oxidase (MAO), with 2 isoforms, at the outer mitochondrial membrane has emerged as a novel source of oxidative stress in the cardiovascular system [42,43]. Moreover, in a very recent study we observed an upregulation of MAO-A in the aortic rings and the hearts harvested from STZ-diabetic rats [44].

To date it has been demonstrated that vitamin D can modulate the activity and expression of NADPH oxidase [18,37], classically the most important source of ROS. However, modulation of other sources of oxyradicals has not been investigated so far.

Endothelial dysfunction in diabetes can be a consequence of hyperglycemia, inflammation, hypertension [39,45,46] and vitamin D can play an important role in modulation of each of these pathogenic mechanisms: future studies are warranted to investigate these directions.

CONCLUSIONS

In summary, the present study demonstrates that the major active metabolite of vitamin D, 1,25-dihydroxy-cholecalciferol, reduces endothelial dysfunction by decreasing the vascular contractility and by improving the endothelial-dependent relaxation. These results may help to explain the link between the lower levels of vitamin D and vascular complications in diabetes. Elucidating the signal transduction of the vascular effects of vitamin D in pathological conditions, including diabetes, might bring novel therapeutic alternatives in the management of diabetes complications.

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REFERENCES

1. Seals DR, Kaplon RE, Gioscia-Ryan RA, *et al*. You're only as old as your arteries: translational strategies for preserving vascular endothelial function with aging. *Physiology* (Bethesda, Md). 2014; 29(4): 250-64.
2. Heitzer T, Schlinzig T, Krohn K, *et al*. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Circulation.* 2001; 104(22): 2673-8.
3. Manganaro A, Ciraci L, Andre L, *et al*. Endothelial dysfunction in patients with coronary artery disease: insights from a flow-mediated dilation study. *Clin Appl Thromb Hemost.* 2014; 20(6): 583-8.
4. Bertoluci MC, Ce GV, da Silva AM, *et al*. Endothelial dysfunction as a predictor of cardiovascular disease in type 1 diabetes. *World J Diabetes.* 2015;6(5):679-92.
5. Gleissner CA. The vulnerable vessel. Vascular disease in diabetes mellitus. *Hamostaseologie.* 2015; 35(3).
6. Flammer AJ, Anderson T, Celermajer DS, *et al*. The assessment of endothelial function: from research into clinical practice. *Circulation.* 2012; 126(6): 753-67.
7. Lafont A. Basic aspects of plaque vulnerability. *Heart*. 2003; 89(10): 1262-7.
8. Rohde LE, Lee RT. Pathophysiology of atherosclerotic plaque development and rupture: an overview. *Semin Vasc Med.* 2003; 3(4): 347-54.
9. Burgmaier M, Hellmich M, Marx N, *et al*. A score to quantify coronary plaque vulnerability in high-risk patients with type 2 diabetes: an optical coherence tomography study. *Cardiovasc Diabetol.* 2014; 13: 117.
10. Muscogiuri G, Nuzzo V, Gatti A, *et al*. Hypovitaminosis D: a novel risk factor for coronary heart disease in type 2 diabetes? *Endocrine.* 2015.
11. Voipio AJ, Pahkala KA, Viikari JS, *et al*. Determinants of serum 25(OH)D concentration in young and middle-aged adults. The Cardiovascular Risk in Young Finns Study. *Ann Med.* 2015; 47(3): 253-62.
12. Winckler K, Tarnow L, Lundby-Christensen L, *et al*. Vitamin D, carotid intima-media thickness and bone structure in patients with type 2 diabetes. *Endocr Connect.* 2015; 4(2): 128-35.
13. Garcia LA, Ferrini MG, Norris KC, *et al*. 1,25(OH)(2)vitamin D(3) enhances myogenic differentiation by modulating the expression of key angiogenic growth factors and angiogenic inhibitors in C(2)C(12) skeletal muscle cells. *J Steroid Biochem Mol Biol.* 2013; 133: 1-11.
14. Holick MF. Vitamin D: evolutionary, physiological and health perspectives. *Curr Drug Targets.* 2011; 12(1): 4-18.
15. Luong KV, Nguyen LT. Vitamin D and cardiovascular disease. *Curr Med Chem.* 2006; 13(20): 2443-7.

16. Wu-Wong JR. Vitamin D therapy in cardiac hypertrophy and heart failure. *Curr Pharm Des.* 2011; 17(18): 1794-807.
17. Fleck A. Latitude and ischaemic heart disease. *Lancet.* 1989; 1(8638): 613.
18. Kassi E, Adamopoulos C, Basdra EK, *et al*. Role of vitamin D in atherosclerosis. *Circulation.* 2013; 128(23): 2517-31.
19. Cigolini M, Iagulli MP, Miconi V, *et al*. Serum 25-hydroxyvitamin D3 concentrations and prevalence of cardiovascular disease among type 2 diabetic patients. *Diabetes Care.* 2006; 29(3): 722-4.
20. Chen S, Law CS, Grigsby CL, *et al*. A role for the cell cycle phosphatase Cdc25a in vitamin D-dependent inhibition of adult rat vascular smooth muscle cell proliferation. *J Steroid Biochem Mol Biol.* 2010; 122(5): 326-32.
21. Somjen D, Kulesza U, Sharon O, *et al*. New vitamin D less-calcemic analog affect human bone cell line and cultured vascular smooth muscle cells similar to other less-calcemic analogs. *J Steroid Biochem Mol Biol.* 2014; 140: 1-6.
22. Mandarino NR, Junior F, Salgado JV, *et al*. Is vitamin d deficiency a new risk factor for cardiovascular disease? *Open Cardiovasc Med J.* 2015; 9: 40-9.
23. Menezes AR, Lamb MC, Lavie CJ, *et al*. Vitamin D and atherosclerosis. *Curr Opin Cardiol.* 2014; 29(6): 571-7.
24. Singh J, Merrill ED, Sandesara PB, *et al*. Vitamin D, Low-Grade Inflammation and Cardiovascular Risk in Young Children: A Pilot Study. *Pediatr Cardiol*. 2015.
25. Wobke TK, Sorg BL, Steinhilber D. Vitamin D in inflammatory diseases. *Front Physiol*. 2014; 5: 244.
26. Challa AS, Makariou SE, Siomou EC. The relation of vitamin D status with metabolic syndrome in childhood and adolescence: an update. *J Pediatr Endocrinol.* 2015
27. Kavadar G, Demircioglu DT, Ozgonenel L, *et al*. The relationship between vitamin D status, physical activity and insulin resistance in overweight and obese subjects. *Bosn J Basic Med Sci.* 2015; 15(2): 62-6.
28. Rusconi RE, De Cosmi V, Gianluca G, *et al*. Vitamin D insufficiency in obese children and relation with lipid profile. *Int J Food Sci Nutr.* 2015; 66(2): 132-4.
29. Vaidya A, Brown JM, Williams JS. The renin-angiotensin-aldosterone system and calcium-regulatory hormones. *J Hum Hypertens*. 2015.
30. Giacco F, Brownlee M. Oxidative stress and diabetic complications. *Circ Res.* 2010; 107(9): 1058-70.
31. Huynh K, Kiriazis H, Du XJ, *et al*. Targeting the upregulation of reactive oxygen species subsequent to hyperglycemia prevents type 1 diabetic cardiomyopathy in mice. *Free Radic Biol Med.* 2013; 60: 307-17.
32. Li H, Horke S, Forstermann U. Vascular oxidative stress, nitric oxide and atherosclerosis. *Atherosclerosis.* 2014; 237(1): 208-19.
33. Patel H, Chen J, Das KC, *et al*. Hyperglycemia induces differential change in oxidative stress at gene expression and functional levels in HUVEC and HMVEC. *Cardiovasc Diabetol*. 2013; 12: 142.
34. Teshima Y, Takahashi N, Nishio S, *et al*. Production of reactive oxygen species in the diabetic heart. Roles of mitochondria and NADPH oxidase. *Circulation Journal: Official Journal of the Japanese Circulation Society.* 2014; 78(2): 300-6.
35. Brandes RP, Schroder K. Differential vascular functions of Nox family NADPH oxidases. *Curr Opin Lipidol.* 2008; 19(5): 513-8.
36. Ebrahimian TG, Heymes C, You D, *et al*. NADPH oxidase-derived overproduction of reactive oxygen species impairs postischemic neovascularization in mice with type 1 diabetes. *Am J Pathol.* 2006; 169(2): 719-28.
37. Kono K, Fujii H, Nakai K, *et al*. Anti-oxidative effect of vitamin D analog on incipient vascular lesion in non-obese type 2 diabetic rats. *Am J Nephrol.* 2013; 37(2): 167-74.
38. Hirata M, Serizawa K, Aizawa K, *et al*. 22-Oxacalcitriol prevents progression of endothelial dysfunction through antioxidative effects in rats with type 2 diabetes and early-stage nephropathy. *Nephrol Dial Transplant*. 2013; 28(5): 1166-74.
39. Howangyin KY, Silvestre JS. Diabetes mellitus and ischemic diseases: molecular mechanisms of vascular repair dysfunction. *Arterioscler Thromb Vasc Biol.* 2014; 34(6): 1126-35.
40. Sena CM, Pereira AM, Seica R. Endothelial dysfunction - a major mediator of diabetic vascular disease. *Biochim Biophys Acta.* 2013; 1832(12): 2216-31.
41. Vaquer G, Magous R, Cros G, *et al*. Short-term intravenous insulin infusion is associated with reduced expression of NADPH oxidase p47(phox) subunit in monocytes from type 2 diabetes patients. *Fundamental & Clinical Pharmacology.* 2013; 27(6): 669-71.
42. Sturza A, Leisegang MS, Babelova A, *et al*. Monoamine oxidases are mediators of endothelial dysfunction in the mouse aorta. *Hypertension.* 2013; 62(1): 140-6.
43. Kaludercic N, Mialet-Perez J, Paolocci N, *et al*. Monoamine oxidases as sources of oxidants in the heart. *J Mol Cell Cardiol.* 2014; 73: 34-42.
44. Sturza A, Duicu OM, Vaduva A, *et al*. Monoamine oxidases are novel sources of cardiovascular oxidative stress in experimental diabetes. *Can J Physiol Pharmacol.* 2015: 1-7.
45. Kaur J. A comprehensive review on metabolic syndrome. *Cardiol Res Pract.* 2014; 943162.
46. Ladeia AM, Sampaio RR, Hita MC, et al. Prognostic value of endothelial dysfunction in type 1 diabetes mellitus. *World J Diabetes.* 2014; 5(5): 601-5.

VITAMINA D ÎMBUNĂTĂȚEŞTE FUNCȚIA CARDIACĂ ÎN DIABETUL ZAHARAT EXPERIMENTAL

REZUMAT

Rolul 1,25-colecalciferolului, forma activă a vitaminei D, la nivelul sistemului cardiovascular în prezența diabetului zaharat este controversat. În orice caz, mecanismele prin care vitamina D exercita aceste efecte nu sunt pe deplin înțelese. Scopul acestui studiu a fost investigarea rolului protector al vitaminei D asupra funcției endoteliale la șobolani diabet zaharat. Pentru aceasta, am folosit un model experimental de diabet zaharat cu streptozotocină la șobolan (administrare intra-peritoneală, 50 mg/kgc, doză unică). Dupa o luna de hiperglicemie constantă, aorta toracică a fost prelevată și utilizată pentru studii de reactivitate vasculară în baia de organ. Funcția endotelială a fost evaluată pe segmente aortice, izolate de la animalele din lotul diabetic comparativ cu cel martor după 24 de ore de stimuare in vitro cu 1,25-colecalciferol (0,1 μM). La animalele diabetice relaxarea endotelial-dependentă la acetilcolină a fost semnificativ alterată. Vitamina D a îmbunătățit semnificativ funcția vasculară pe aceste segmente vasculare. În concluzie, administratrea in vitro a vitaminei D a îmbunătățit funcția vasculară în diabetul zaharat experimental.

Cuvintecheie: vitamină D, disfuncție endotelială, diabet zaharat

IN VITRO ASSESSMENT OF TUMOR-ASSOCIATED FIBROBLASTS' PROLIFERATION ABILITY AND VIABILITY

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ABSTRACT

The origin of tumor-associated fibroblasts is not fully understood. There are multiple data according to which TAFs are a subset of MSCs and are recruited from bone marrow or from tissue level during tumor development process. Tumor microenvirnment provides the conection between tumor and host, at the level of primary site and at distance site. This communication is possible through the soluble factors which are secreted by tumor cells and by cellular component of the tumor microenvironment, in which TAFs represent an essential component. TAFs plasticity and their ability to induce tumor cells development, invasion, migration and metastasis, but also their involvement in survival of tumor cells and acquirement of drug-resistance, makes exploration ofthese cells a priority for characterization and use as future therapeutic targets.

We investigated the proliferative ability and viability of two types of tmor-associated fibroblasts – breast cancer-derived TAFs (bTAF) and uterine cancer-derived TAFs (uTAF) – comparative to their physiological counterparts, the bone marrow-derived mesenchymal stem cells (MSCs). We used the *in vitro* toxicology assay (MTT-based) and flowcytometric evaluation using Annexin V/PI. The results were similar for all cellular types under investigation, with a difference for bTAFs regarding the late apoptosis stage, which was significantly lower compared to MSCs and uTAFs.

Although they present many common features with MSCs, there are still some questions to be answered regarding the distinct characteristics and functions of TAFs, as well as their specific involvement in tumor pathogenesis.

Key words: mesenchymal stem cells (MSCs), tumor-associated fibroblasts (TAFs), viability tests, MTT

INTRODUCTION

Tumor-associated fibroblasts (TAFs) represent the main cellular component of tumor stroma. They are actively involved in tumor development and progression, providing secretion of pro-tumorigenic factors, enabling connection with extracellular matrix and de novo formed vessels, thus contributing to structuring and functioning of tumor in an organ-like fashion. Intercellular interactions are very complex due to involvement of several cellular types – tumor cells, endothelial cells, immune system cells, fibroblasts, pericytes, adipocytes, etc. Secreted factors are enrolled in establishment of plurivalent signaling pathways with the role of tumor developing and acquirement of so-called cellular plasticity (acquirement of new features allowing the cells long-term survival, expansion, and malignant behavior).

Tumor-associated fibroblasts (TAFs) population is not homogenous; it is endowed with important heterogeneity, cellular plasticity allowing these cells to transform both morphologically and functionally, thus contributing to tumor pathogenicity. TAFs modulation of tumor development is both positive, and negative [1].

Heterogeneity of TAFs subpopulations within a tumor makes difficult to explore and to establish some specific markers; there are topographical differences (depending on originating tissue), and also within the same tumor [2]. Moreover, analysis of gene expression in breast cancer samples revealed specific molecular markers for multiple TAF subtypes [3], a certain TAF subtype being able to induce an inhibitory or stimulatory effect on tumor development, depending on activation or inhibition of certain factors secretion, characteristics which are still under investigation.

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However, TAFs origin is under debate, numerous data pleading for mesenchymal origin of these cells, being considered a subset of mesenchymal stem cells, which are either migrating from bone marrow [4], or are transformed locally from stromal cells.

The objective of this study was to investigate the proliferative ability and viability of tumor-associated fibroblasts (TAFs) isolated from both breast cancer and uterus cancer samples, comparative to bone marrow-derived mesenchymal stem cells (MSCs).

MATERIALS AND METHODS

1. Isolation and culture of tumor-associated fibroblasts (TAFs)

TAFs were isolated from 10 breast cancer and 5 uterus cancer surgical pieces using collagenase type IV-S from *Clostridium histolyticum* (Sigma-Aldrich Company, Ayrshire, UK) method. Breast cancer surgical pieces of approximately 5 cm^2 were obtained form 10 female patients, with the histopathological diagnosis of infiltrative ductal mammary carcinoma. Uterus cancer samples of approximately 10 cm^2 were obtained from patients with the diagnosis of endometroid adenocarcinoma. Cells isolated from tissues were washed several times with phosphate buffered saline (PBS, Sigma) solution and passed through 0.70/0.40 µm strainer filters and then plated as suspension in adherent plastic culture plates using Dulbecco's modified Eagle Medium (DMEM; Sigma), supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany) and 2% Penicillin/Streptomycin mixture (Pen/Strep, 10,000 IU/ml; PromoCell), and incubated incubation at 37°C in 5% CO_2 atmosphere. Every 3 days, medium was replaced with freshly prepared medium.

All tissue samples were obtained after signing the informed consent elaborated under an approved protocol, according to the World Medical Association Declaration of Helsinki.

2. Isolation and culture of bone marrow-derived mesenchymal stem cells (BM-MSCs)

Human mesenchymal stem cells (MSCs) were obtained from bone marrow from healthy Orthopedics patients undergoing bone surgery. Approximately 10 ml of bone marrow were placed in culture plates, and the fibroblastic-like, plastic adherent fraction, was isolated following multiple passages and used in our experiments. The MSCs were further cultured and expanded in alpha-minimum essential medium (MEM; Gibco BRL, Invitrogen), supplemented with 10% fetal calf serum (FCS; PromoCell), 10ng/mL basic fibroblast growth factor (FGF; Sigma-Aldrich Company) and 2% Penicillin/Streptomycin mixture (Pen/Strep, 10,000 IU/ml; PromoCell), by incubation at 37ºC in 5% CO_2 atmosphere. Medium was replaced every three days and when the confluence was 80-90% the cells were passed using 0.25% Trypsin-EDTA solution (Sigma-Aldrich Company) followed by centrifugation (10 minutes, 300g) and replated in T75 culture flasks at a density of 10,000 cells/cm^2 to ensure optimal proliferation.

3. Analysis of proliferation rate using MTT assays

An MTT-based *in vitro* toxicology assay kit (Tox-1, Sigma-Aldrich Company) was used to determine proliferation rate of BM-MSCs, uterine cancer TAFs (uTAFs) and breast cancer TAFs (bTAFs) at 24 and 48 hours. Trypan Blue cell counting and viability assessment were simultaneously performed at the same time intervals for better correlation of the results. Cells were passaged at confluence after treatment with 5mM EDTA; the living cells were then assayed by the addition of 20 µL of 5 mg/mL MTT solution. The intact mitochondrial reductase converted and precipitated MTT as blue crystals during a 4h contact period. The medium was then removed, and the precipitated crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO; Sigma-Aldrich Company, Ayrshire, UK). Cells were seeded at 2,000 cells/well in 96-well plates in quadruplicate, and the average value of specific medium extinction was subtracted from the samples extinction read at 570/655 nm using a benchmark PR 2100 microplate reader from Bio-Rad (Hercules, CA, USA).

4. Annexin V/PI assay

Annexin V-FITC (Miltenyi Biotec, Gladbach, Germany) was used in cell death flowcytometric studies (apoptosis) combined with Propidium Iodide Staining Solution (BD Biosciences, San Jose, CA, USA) following the manufacturer protocol. Shortly, 10^6 cells were washed in 1 x Annexin V Binding Buffer (BD Pharmigen) and centrifuged at 300 x g for 10 minutes, resuspended in the same solution and incubated with 10 µl of Annexin V-FITC for 15 minutes in the dark. After washing the cells with 1 ml specific binding buffer and centrifugation, the cell pellet was resuspended in 500 µl binding buffer and 1 µg/ml of PI solution was added immediately prior to analysis by flowcytometry. Data acquisition for Annexin V/PI assay flowcytometric procedure was performed on a four-color capable FACSCalibur (Becton-Dickinson) flow cytometer, while data analysis employed Flowing Software 2.5.

5. Statistical analysis

Data were analyzed for statistical relevance using Excel Microsoft Office 2007 software (Microsoft Corporation, Redmond, WA, USA). The central

tendencies of the variables were expressed as a mean (*M*), and the dispersion ones as a standard deviation (sd). In order to perform the statistical comparisons, Student's *t* test and the variance analysis (ANOVA) were conducted for continuous variables. Differences were considered significant for $p < 0.05$.

RESULTS

In vitro toxicology assay (MTT) was performed 24 and 48 hours after plating of cells in appropriate 96-well plates. We comparatively analyzed the mean values of 5 TAFs populations obtained from both breast cancer and uterine cancer pieces and bone marrow-derived mesenchymal stem cells (MSCs). As negative control we used DMSO which was added in amount of 10 µl/well on a layer of breast cancer-derived TAFs.

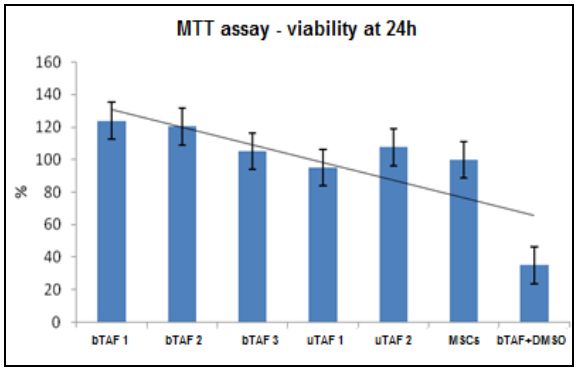

Fig. 1. Viability of TAF populations at 24h. Similar values were obtained for breast cancer-derived TAFs (bTAF), uterine tumors-derived TAFs (uTAF) comparative to MSCs viability at 24h.

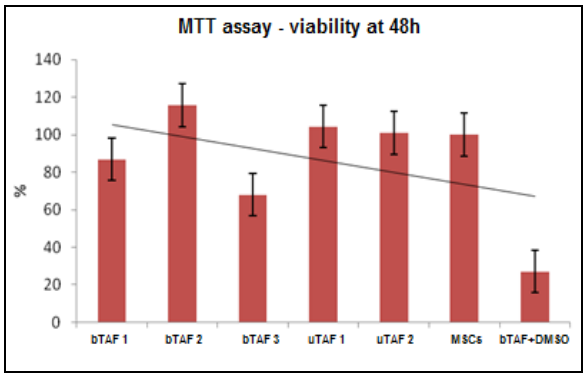

Fig. 2. Viability of TAF populations at 48h. Similar values were obtained for breast cancer-derived TAFs (bTAF), uterine tumors-derived TAFs (uTAF) comparative to MSCs viability at 48h.

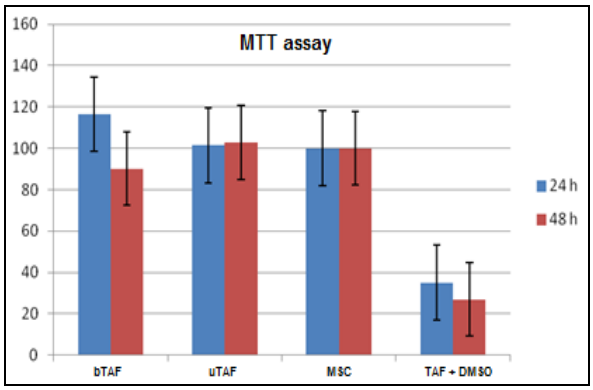

Fig. 3. Comparative viability between the three cellular populations tested at 24 and 48h. Negative control used DMSO on a layer of breast cancer-derived TAFs.

Analysis of data obtained by MTT assay revealed a similar proliferation rate and viability for both breast cancer and uterine cancer-derived TAFs, which was comparable to bone marrow-derived MSCs proliferation rate.

Annexin V/PI viability assay was performed by flowcytometric methods, and revealed the 3 stages of cellular death: early apoptosis (lower right quadrant), late apoptosis (upper right quadrant) and dead cells (upper left quadrant). There were no significant differences between the 3 cellular types analyzed for early apoptosis and dead cells ($p>0.05$). Regarding the late apoptosis stage, analysis revealed differences with statistical significance ($p<0.05$) between MSCs and breast cancer-derived TAFs.

Correlations between the MTT and Annexin V/PI assays showed that bTAFs have a different proliferative behavior compared to MSCs and uTAFs, thus demonstrating that breast cancer stromal compartment has better supportive role in tumor development. These analyses also demonstrated the heterogeneity between tumor-associated fibroblasts isolated from different tumor sites, regardless of their identical phenotype.

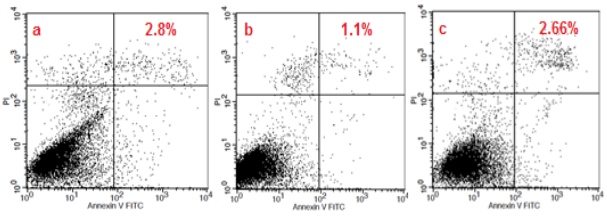

Fig.4. Annexin V/PI viability assay. Late apoptosis of a. MSCs, b. bTAFs, c. uTAFs.

CONCLUSION AND DISCUSSION

Proliferation assays performed in this study (MTT method) revealed a proliferation rate of TAFs similar to that of mesenchymal stem cells at 24 and 48 hours; the results were following the same pattern for both breast cancer and uterine tumors-isolated fibroblasts.

Cellular viability evaluated using the flowcytometric technique with Annexin-V/PI showed that bone marrow-derived mesenchymal stem cells have an increased apoptotic process compared to tumor-associated fibroblasts isolated from breast cancer and uterine tumors.

The origin of tumor-associated fibroblasts is not fully understood. There are multiple data according to which TAFs are a subset of MSCs and are recruited from bone marrow or from tissue level during tumor development process [5]. Tumor microenvirnment provides the conection between tumor and host, at the level of primary site and at distance site. This communication is possible through the soluble factors which are secreted by tumor cells and by cellular component of the tumor microenvironment, in which TAFs represent an essential component. TAFs plasticity and their ability to induce tumor cells development, invasion, migration and metastasis, but also their involvement in survival of tumor cells and acquirement of drug-resistance, makes exploration ofthese cells a priority for characterization and use as future therapeutic targets.

Apart from genomic alteration which are determinat for neoplazic potential, recent data revealed the importance of stromal component of the tumor microenvironment for initiation, progression, invasion and tumor metastasis [6,7]. The fibroblasts are cells which are responsible of production and maintenance of connective tissues. They are non-vascular, non-epithelial, and non-inflammatory cells, which are the main component of the connective tissues [8]. These cells are actively involved in regulation of inflammatory processes, whound healing, as well as differentiation of epithelial cells from adjacent areas [9].

Fibroblasts are synthesizing fibrillary proteins of the extracellular matrix (fibronectin, type I, III, and IV collagen). These cells are a major source of proteases which are further degrading the matrix (matrix metalloproteinases), involved in matrix homeostasis. Under physiological circumstances, proliferation and metabolic rate of fibroblasts are very low. During the healing phase of an injury, the fibroblasts will become activated and will acquire secretory and contractile qualities, while the proliferation rate increases [10]. In our study we proved that TAFs have an increased proliferation rate and viability, compared to mesenchymal stem cells, even under *in vitro* condition, following multiple passages, thus demonstrating that TAFs are able to provide a good functional support for long-term growing of a tumor.

Moreover, activated fibroblasts are secreting chemokines which are initiating the recruitment of blood components at the site of an injury. The activated fibroblasts will regain their quiescent status after performing their task, at the end of the injury healing process [11]. Regarding their phenotype, fibroblasts present several differences, depending on the site of harvesting (joints, skin, etc.) [12].

However, the fibroblasts possess a wide variety in topographic expression of genes involved in synthesis of extracellular matrix, cellular growth and differentiation, and cellular migration. Some authors [13] noticed the differences in size and expression of some receptors (TGF- β1 receptor II) by fibroblasts isolated from different body sites. They suggested the idea that this regional diversity could be accounted for susceptibility and different behavior of different anatomic sites during the healing process and cheloid formation. Other studies revealed an increased capacity of oral mucosa-derived fibroblasts to reorganize the collagen fibers due to high expression of MMP-2, comparative to skin-isolated fibroblasts [14]. Fibroblasts heterogeneity was also shown in studies regarding the mechanisms of tissue injury and wound healing. This last process develops along 3 phases, all of them involving the stromal component: inflammation, proliferation, maturation [15].

Tumorigenesis is always accompanied by stromagenesis. The stromal compartment functions as tumorigenic barrier. In the first stages, the tumor cells are inducing stromal development and install a feed-back process between tumor ad stroma, which will further become bidirectional, maintaining both processes [16]. Consequent alterations of initially normal stroma lead to transient changes, which further will trigger phenotypical changes with the characteristics of activated stroma [17,18]. In advanced phases, the tumor and stroma become irreversible transformed from the phenotypic point of view, presenting different degrees of reciprocal independence. An interesting aspect to be elucidated would be the identification of “point of no return”, in which there is the switch between classical stroma phenotype and activated one [19].

Tumor-associated fibroblasts can be found in variable proportions at the level of the entire spectrum of carcinomas, constituting the most abundant cellular element within the tumor stroma. The term tumor/cancer-associated fibroblasts comprises at least 2 cellular types: (1) fibroblast-like cells, which are the supportive element for epithelial cells; (2) myofibroblasts with different roles and biological characteristics compared to tissue fibroblasts. Tumor myofibroblasts and tissue fibroblasts which suffered phenotypical and functional changes have multiple roles during cancer progression: proliferation, angiogenesis, invasion, metastasis; these actions were demonstrated in animal experimental models using mice predisposed to

tumorigenesis, by injecting TAFs combined with tumor cells, and by studying genetic and pharmacological alteration of cellular functionality [20-22].

Due to secretion of various components of extracellular matrix, tumor-associated fibroblasts are involved in formation of desmoplastic stroma, which characterizes advanced forms of carcinomas. There are still some questions to be answered regarding the distinct functions of the two cellular groups of TAF and their specific involvement in tumor pathogenesis.

To summarize, several studies have confirmed that tumor-associated fibroblasts action to favor tumor progression could be based on somatic mutations at the level of tumor suppression genes. Also, somatic alterations were constantly observed with significant frequency (more than 30%) even at the level of tumor-associated fibroblasts [23]. Despite these data, other studies showed that genetic alterations are induced only at the level of epithelial tumor cells, and not at the stromal level [24], while heterozygots analysis and genetic copies performed on TAFs isolated from breast cancer and ovarian cancer samples revealed that genetic alterations are extremely rare at TAFs level. Thus, at this moment, the researchers were not able to postulate the idea that cancer progression is based on tumor-associated fibroblasts [25].

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REFERENCES

1. Martin FT, Dwyer RM, Kelly J, Khan S, Murphy JM, Curran C, Miller N, Hennessy E, Dockery P, Barry FP, O'Brien T, Kerin MJ. Potential role of mesenchymal stem cells (MSCs) in the breast tumour microenvironment: stimulation of epithelial to mesenchymal transition (EMT). *Breast Cancer Res Treat*, 2010; 24(2): 317-326.

2. Sugimoto H, Mundel TM, Kieran MW, Kalluri R. Identification of fibroblast heterogeneity in the tumor microenvironment. *Cancer Biol Ther*, 2006; 5 (12): 1640-6.

3. Tchou J, Kossenkov AV, Chang L, Satija C, Herlyn M, Showe LC, et al. Human breast cancer associated fibroblasts exhibit subtype specific gene expression profiles. *BMC Med Genomics*, 2012; 5:39.

4. Haniffa MA, Wang XN, Holtick U, *et al*. Adult human fibroblasts are potent immunoregulatory cells and functionally equivalent to mesenchymal stem cells. *J Immunol*. 2007; 179: 1595-604.

5. Studeny M, Marini FC, Champlin RE, *et al*. Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. *Cancer Res*. 2002; 62: 3603-8.

6. Qian BZ, *et al*. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature* 2011; 475: 222-225.

7. Soria G, *et al*. Concomitant expression of the chemokines RANTES and MCP-1 in human breast cancer: a basis for tumor-promoting interactions. *Cytokine* 2008; 44: 191-200.

8. Gerber PA, Hippe A, Buhren BA, Muller A, Homey B. Chemokines in tumor-associated angiogenesis. *Biol Chem.* 2009; 390: 1213-1223.

9. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer*, 2006; 6: 392-401.

10. Xouri G, Christian S. Origin and function of tumor stroma fibroblasts. *Seminars in Cell & Developmental Biology* 2010; 21 :40-46.

11. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 2002; 3: 349-363.

12. Rinn JL, Wang JK, Liu H, Montgomery K, van de Rijn M, Chang HY. A systems biology approach to anatomic diversity of skin. *J Invest Dermat*. 2008; 128: 776-782.

13. Chipev CC, Simon M. Phenotypic differences between dermal fibroblasts from different body sites determine their responses to tension and TGFbeta1. *BMC Dermatol.* 2002; 2:13.

14. Stephens P, Davies KJ, Occleston N, Pleass RD, Kon C, Daniels J, Khaw PT, Thomas DW Skin and oral fibroblasts exhibit phenotypic differences in extracellular matrix organization and matrix metalloproteinase activity. *Br J Dermatol.* 2001; 144: 229-237.

15. Clark RAF, Fitzpatrick TB, Eisen AZ, Wolff K, Freedberg IM, Austen KF (eds). Mechanisms of cutaneous wound repair; dermatology in general medicine. McGraw Hill, New York, 1993: 473-486.

16. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 1986; 315: 1650-9.

17. De Wever O, Mareel M. Role of tissue stroma in cancer cell invasion. *J Pathol*. 2003; 200: 429-47.

18. Weaver VM, Gilbert P. Watch thy neighbor: cancer is a communal affair. *J Cell Sci*. 2004; 117: 1287-90.

19. Edlund M, Sung SY, Chung LW. Modulation of prostate cancer growth in bone microenvironments. *J Cell Biochem.* 2004; 91: 686-705.

20. Dirat B, Bochet L, Escourrou G, Valet P, Muller C. Unravelingthe obesity and breast cancer links: a role for cancer-associated adipocytes? *Endocr. Dev.* 2010; 19: 45-52.

21. Pietras K, Ostman A. Hallmarks of cancer: interactions with the tumor stroma. *Exp. Cell Res.* 2010; 316: 1324-31.

22. Shimoda M, Mellody KT, Orimo A. Carcinoma-associated fibroblasts are a rate-limiting determinant for tumour progression. *Semin. Cell Dev. Biol.* 2010; 21: 19-25.

23. Kurose K, Gilley K, Matsumoto S, Watson PH, Zhou XP, and Eng C. Frequent somatic mutations in PTEN and TP53 are mutually exclusive in the stroma of breast carcinomas. *Nat Genet* 2002; 32: 355-357.

24. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, Porter D, Hu M, Chin L, Richardson A, Schnitt S, Sellers WR, Polyak K. Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell* 2004; 6: 17-32.

25. Qiu W, Hu M, Sridhar A, Opeskin K, Fox S, Shipitsin M, Trivett M, Thompson ER, Ramakrishna M, Gorringe KL, Polyak K, Haviv I, Campbell IG. No evidence of clonal somatic genetic alterations in cancer-associated fibroblasts. *Nat Genet.* 2008; 40(5): 650-5.

ANALIZA *IN VITRO* A CAPACITĂȚII DE PROLIFERARE ŞI A VIABILITĂȚII FIBROBLASTELOR PERI-TUMORALE

REZUMAT

Originea fibroblastelor peri-tumorale nu este pe deplin clarificată. Există date multiple conform cărora TAF sunt un subset al MSC şi sunt recrutate din măduva osoasă sau de la nivel tisular în momentul dezvoltării tumorii. Microclimatul tumoral asigură conexiunea tumoră-gazdă atât la nivelul site-ului primar, cât şi la distanţă. Această comunicare este posibilă prin intermediul factorilor solubili secretaţi de celulele tumorale şi de către componenta celulară a microclimatului tumoral, TAF reprezentând o componentă esenţială a acesteia. Plasticitatea TAF şi capacitatea acestora de a induce dezvoltarea celulelor tumorale, invazia, migrarea şi metastazarea, dar şi implicarea acestora în supravieţuirea celulelor tumorale şi dobândirea rezistenţei la chimioterapice, sunt motive bine întemeiate în a explora aceste celule în vederea caracterizării lor şi utilizării drept ţinte terapeutice.

În acest studiu am investigat capacitatea de proliferare și viabilitatea a două tipuri de fibroblaste peri-tumorale – TAF izolate din cancere mamare (bTAF) și TAF izolate din tumori uterine (uTAF) – comparativ cu celulele stem mezenchimale derivate din măduva osoasă (MSC). Am utilizat testul de viabilitate MTT și evaluarea flowcitometrică folosind Anexina V/iodura de propidiu. Rezultatele au fost similare pentru toate tipurile de celule investigate, diferențe semnificative statistic obținându-se pentru bTAF aflate în faza de apoptoză tardivă, care au prezentat valori scăzute comparativ cu MSCs și uTAF.

Cu toate că prezintă numeroase caracteristici similare cu MSCs, există încă multe necunoscute în ceea ce privește caracteristicile și funcțiile distincte ale TAF, precum și în ceea ce privește implicarea specifică a acestora în patogeneza tumorală.

Cuvinte cheie: celule stem mezenchimale (MSCs), fibroblaste peri-tumorale (TAFs), teste de viabilitate, MTT

THE RELATIONSHIP BETWEEN VITAMIN D, INFLAMMATION AND THE ACTIVITY OF SYSTEMIC LUPUS ERYTHEMATOSUS

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ABSTRACT

Objective: Systemic lupus erythematosus (SLE) is considered a chronic inflammatory autoimune disease. The role of vitamin D in the prevention or therapy of LES is still debated. The objective of our study was to determine the serum levels of vitamin D in patients with SLE and its relationship with inflammatory markers and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI).

Method: A prospective study was realised on 20 women diagnosed with SLE according to American College of Rheumatology (ACR) criteria and 12 aged and gender-matched healthy controls. We determined the serum levels of high sensitivity C reactive protein (hsCRP), erythrocyte sedimentation rate (ESR), 25-hydroxy vitamin D, total calcium, phosphorus and parathyroid hormone in all patients. The activity of SLE was evaluated through SLEDAI. The levels below 30 ng/mL of 25(OH) D were considered abnormal. The serum creatinine levels and glomerular filtration rate (GFR) were measured to assess the renal affection in these patients.

Results: The mean age of SLE patients included in this study was 44.1±11.65 years. It was observed that the levels of vitamin D were significantly lower in SLE patients then in healthy controls. The SLE patients with hypovitaminosis D had higher levels of serum creatinine, GFR, ESR and PCR. SLEDAI was significantly increased in patients with hypovitaminosis D compared to patients with normal vitamin D levels.

Conclusion: In our study, the deficiency of vitamin D was more prevalent in SLE patients. The patients with SLE and hypovitaminosis D have significantly higher values of SLEDAI than the patients with normal Vitamin D levels. Thise results support the importance of maintaining the normal levels of vitamin D to combat lupus disease activity.

Keywords: vitamin D, SLE, inflammation, SLEDAI

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by systemic inflammation and the immune system attacks various "self-tissues" throughout the body [1]. SLE predominantly affects women mainly at childbearing age, with a ratio of nine women for every man [2]. The reported prevalence of SLE is between 20 and 150 cases per 100,000 individuals [3]. The etiology of SLE is still unknown and its progression has been linked with the interaction of genetic, immunological, hormonal, and environmental factors [4]. A low vitamin D status might have an important role in SLE pathology, since hypovitaminosis D is more prevalent in SLE patients than in the general population [5]. It has been shown that vitamin D has an important role in regulating immune response especially related with B-cells and T cell homeostasis, and in tissues "self -tolerance" [6]. In SLE the balance between the "effector" / "regulatory" cells is directed in the favor of the first [9]. Moreover, the "regulatory" cells in these patients are dysfunctional [9]. Vitamin D exerts several actions at the cellular level to modify this balance [10].

It has been demonstrated that SLE patients may have various risk factors which influence the appearance of vitamin D deficiency. The patients with SLE also have a typical photosensitivity which might determine the use of sunscreen and a lower sun exposure, which blocks

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UVB radiation, and makes this population at risk for developing vitamin D deficiency. The drugs frequently used in the treatment of patients with SLE, like corticosteroids could change the metabolism of the vitamin D in the body [11]. In addition, the renal involvement, one of the determining factors in morbidity and mortality of SLE patients, can change the stage of hydroxylation of vitamin D [2,11]. In fact, deficiency of vitamin D was demonstrated to be associated with higher lupus disease activity [12,13]. Additionally, low levels of vitamin D were associated with severity of disease and nephritis in patients with SLE [14].

The purpose of this study was to evaluate the levels of vitamin D in SLE patients and to determine the relationship with inflammatory markers and the activity of disease evaluated thorough Systemic Lupus Erythematosus Disease Activity Index (SLEDAI).

METHODS

The study was performed on 20 women with SLE attending the outpatient clinic of Department of Rheumatology, Hospital Municipal Timisoara and Department of Nephrology, County Emergency Hospital, Timisoara. The women with SLE patients was diagnosed based on the criteria of the American College of Rheumatology (ACR) 1997 [15]. The exclusion criteria: pregnant women, individuals with systemic disease such as vasculitis, acute infectious diseases. In addition, 12 healthy people matched age and sex were recruited as controls.

The season in which the patients were included was considered for this study. The patients treated with vitamin D and calcium supplements renounced at this thearpy for 2 months (considered three times the half-life of supplements) before joining the study.

The inflammation status was assessed by determining the serum levels of C reactive protein (CRP) and erythrocyte sedimentation rate (ESR). The activity of lupus disease was evaluated using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI). The values of vitamin D ≥ 30 ng/ml were considered normal, while the vitamin D insufficiency was defined as a level between 15 to 29 ng/ml and vitamin deficiency at levels <15 ng/ml (16). The serum creatinine levels and glomerular filtration rate were measured to assess the renal involvement in these patients (GFR).

The statistical processing was carried out using Excel software packages, Epi Info 7 and SPSSv.17. In all cases we used the significance threshold of 0.05 (5%), corresponding to a 95% confidence level.

The local ethics committee approved the protocol, and written informed consent was obtained from all patients in accordance with the Helsinki Declaration.

RESULTS

This study included 32 individuals, 20 SLE patients (SLE group) and 12 healthy patients (Control group), all female. The characteristics of the study groups showed that there were no significant age differences among groups. The age of SLE patients was between 19 and 67 years, with a mean of 44.1±11.65 years, and the age of heathy controls was between 23 and 68 years, with a mean of 42.2±12.73. The disease duration ranged from 1 to 7 years with a mean of 2.7±1.69 years. The analysis of SLEDAI score has revealed a mean of 9.5±7.34. (Table I)

The SLE patients were diagnosed according to American College of Rheumatology (ACR) criteria published in 1982 and revised in 1997 [15]. The analysis of the types of treatment applied to the study group found that chloroquine was used in a proportion of 70 % (n = 14), azathioprine in 40 % (n = 8), prednisone in 65% (n=13) and methylprednisolone at a rate of 15% (n = 3). All of SLE patients had received therapy with steroids (Table I)

The mean levels of serum creatinine in SLE patients was 1.1±0.44 (0.62-2.53) mg/dL and the mean glomerular filtration rate was 81.7±34.51(19-139) mL/min/1.73 m^2. In heathly controls, the serum levels of creatinine were 0.8±0.21 (0.41-1.13) and the mean glomerular filtration rate was 94.3±26.84 (53-146) mL/min/1.73 m^2 (Table I).

The results of inflammatory markers showed that the mean values of ESR were 44.3±23.04 mm/h in SLE patients and 7.4±3.26 in healthy control. The mean levels of CRP were 7.7±8.52 mg/dL in SLE patients and 0.7±1.78 mg/dL in healthy controls (Table I).

Table I. Characteristics of SLE patients (n=20) and healthy controls (n=12)

Characteristics	SLE patients (n=20)	Healthy Control (n=12)
Age (years) (mean± SD)	44.1±11.65 (19-67)	42.2±12.73 (23-68)
Female (%)	100 %	100%
ESR 1st hour (mm) (mean± SD)	44.3±23.04 (5-85)	7.4±3.26 (3-13)
CRP (mg/dL) (mean± SD)	7.7±8.52(0-32)	0.7±1.78 (0-6)
Total calcium (mg/dL) (mean± SD)	8.9±0.48(8.1-10.0)	9.2±0.37(8.8-9.9)
Phosphorus (mg/dL) (mean± SD)	4.4±0.75 (3.1-6.3)	4.7±0.77 (3.1-6.1)
Intact PTH (pg/mL) (mean± SD)	44±16.2 (15.67- 70.11)	45.5±11.37(15.98-57.65)
25(OH) D(ng/mL) (mean± SD)	25.5±7.95 (12.31-40.02)	33.3±6.01(22.08-42.34)
Creatinine (mg/dL) (mean± SD)	1.1±0.44(0.62-2.53)	0.8±0.21 (0.41-1.13)
GFR (mL/min/1.73 m^2) (mean± SD)	81.7±34.51(19-139)	94.3±26.84(53-146)
Duration of illness (years) (mean± SD)	2.7±1.69 (1-7)	
SLEDAI score (mean± SD)	9.5±7.34 (0-24)	
Treatment		
• Azathioprin	40 %	
• Chloroquin	70 %	
• Prednisone	65%	
• Methylprednisolone	15 %	

The analysis of vitamin D levels in the studied groups

The mean levels of vitamin D in SLE patients were 25.5±7.95 ng/mL and in healthy control were 33.3±6.01 ng/mL. All patients were considered to have deficiency of vitamin D when the levels were under 15 ng/mL, vitamin D insufficiency when the levels were between 15 and 29 ng/mL, and normal levels of vitamin D ≥ 30 ng/mL (Table I)

Table II. The analysis of 25 (OH) D levels in SLE group (n = 20)

Vitamin D	SLE patients
Deficiency < 15 ng/mL	3/20 (15%)
Insufficiency: 15-30 ng/mL	9/20 (45%)
Normal > 30 ng/mL	8/20 (40%)

The percentage distribution of vitamin D insufficiency in SLE patients was found in 9 patients in a proportion of 45 % and deficiency in 3 patients in a proportion of 15 %. Only 8 patients with SLE had normal levels of vitamin D (Table II).

In healthy controls, the vitamin D insufficiency was found in 3 patients (25 %) and the rest had normal levels (n=9). The levels of vitamin D were significantly lower in SLE patients compared to healthy controls (Mann-Whitney test, p = 0.006).

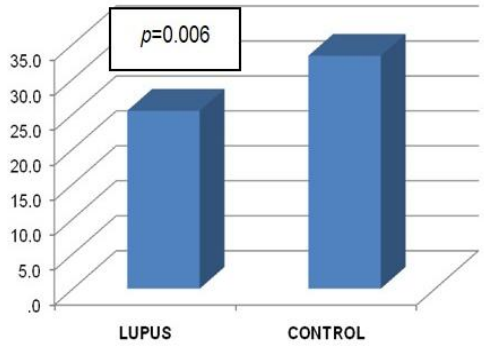

Fig.1. The graphical representation of vitamin D levels between SLE patients (n=20) and healthy control (n=12)

The correlation between vitamin D and serum creatinine is significant (p <0.001, α = 0.001), reverse and strong (Pearson coefficient r = -0.735), in SLE patients with vitamin D levels under 30 ng/mL (Figure 2).

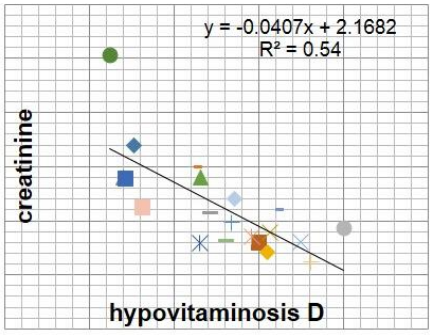

Fig. 2. Correlation of levels of vitamin D and serum creatinine in SLE patients (n=12) with hypovitaminosis D

The correlation between vitamin D levels, SLEDAI scores, ESR, PCR and renal involvement

The correlative study in SLE patient with vitamin D levels less than 30 ng/mL (n=12) and SLE patients with normal levels (n=8) with respect to vitamin D levels and SLEDAI scores, ESR, PCR and renal involvement showed that:

- SLEDAI scores were significantly higher in patients with hypovitaminosis D (12.8 ± 6.82) than in patients with normal levels (3.4 ± 1.4) - Mann-Whitney test, p = 0.003, α = 0.01.
- ESR values were significantly higher for patients with hypovitaminosis D (51.3 ± 24.59) than in patients with normal levels (31.1 ± 12.78) - Mann-Whitney test, p = 0.046, α = 0.05.
- PCR values ere not significantly increased in patients with hypovitaminosis D (9.9 ± 8.43) than in patients with normal vitamin D evels (3.7 ± 2.82) - Mann-Whitney test, p = 0.115, α = 0.05.
- The renal impairment, quantified by serum creatinine and glomerular filtration rate (GFR) was significantly increased (Z test, p = 0.04, α = 0.05) in patients with hypovitaminosis D.

DISCUSSION

This study showed that low vitamin D levels are common in patients with LES - 60% had levels <30 ng / ml and 10% had <15 ng/ml, despite that this population live in a country with many sunny days. These results are related to other studies from similar geographical areas [11]. This means that vitamin D deficiency is more prevalent in patients with SLE than in healthy controls; also in healthy control vitamin D insufficiency was found in 3 patients (25 %), explained by Fragoso *et al.* [17] to be a result from the modern life activities, which make people to avoid sun exposure, and consequently reduce vitamin D synthesis.

It is noteworthy that therapy with calcium and vitamin D did not protect completely against the deficit of 25(OH)D; from 20 patients with SLE included, seven was receiving treatment with calcium and vitamin D pills, and 4 of them (57%) had levels <30 ng/mL of 25(OH)D.

In this study, the mean serum creatinine is significantly increased for the group with SLE compared with healthy control (p <0.012). Higher values of creatinine have been observed in this study in patients with SLE and hypovitaminosis D ((1.3 ± 0.47) mg / dL)) than patients with normal levels of vitamin D levels (0.9 ± 0.16mg / dl)(p = 0.06, α = 0.01). The value of GFR was slightly lower in SLE patients compared with healthy controls (p = 0.288). Most of the patients with SLE patients included in this study are check their kidney involvement, which may also influence the value of the results. Lupus nephritis is a major cause of morbidity and

mortality in SLE. The association between vitamin D deficiency and lupus nephritis was assessed in many studies, Kamen *et al.* [18] published a study in which was described an association between them.

SLE - by definition an autoimmune inflammatory disease – is correlate with high inflammation in patients suffering from this condition. In this study, a statistically significant increase in the values of ESR and C-reactive protein was observed in SLE patients compared with the healthy control ($p < 0.001$, $\alpha = 0.001$ for ESR and $p < 0.001$, $\alpha = 0.001$ PCR). There was an inverse association between low levels of 25 (OH) D and lupus disease activity index in this study ($p = 0.003$, $\alpha = 0.01$). These findings are in agree with many other observational studies which shown that vitamin D levels are correlate inversely with disease activity index in SLE [13,19,20]. This study has shown, similar with the study of Amezcua-Guerra *et al*. [21], a positive association between SLEDAI scores and ESR/PCR in patients with SLE. However, Firooz *et al*. [22] could not demonstrate an association between these inflammation markers and disease activity index.

The association between vitamin D levels and disease activity index in SLE patients is extremely important. Many clinical trials [23] reported an association between decreased values of vitamin D and increased activity of SLE, evaluated through SLEDAI. However, no study which examined the relationship between vitamin D and the of activity in SLE did not include all the main factors influencing the status of vitamin D. Recent studies suggested a role of vitamin D in the appearance and severity of fatigue found in SLE, but various descriptions of the symptoms from patient to patient make it difficult to determine.

This study has few limitations. First of all, this trial can not suggest any causality between the associations described and vitamin D deficiency in patients with SLE. The small number of patients included ($n = 20$) can influence the results, and also the low level of SLEDAI scores due to patients enrollment in remission.

Future studies with larger numbers of participants and a higher activity of the disease could provide more concrete results regarding the association between vitamin D deficiency, lupus nephritis, inflammation markers and disease activity index.

CONCLUSION

We concluded that low vitamin D levels are more prevalent in patients with SLE than in healthy controls. The SLEDAI score, serum creatinine, GFR, ESR and CPR values are significantly higher in patients with hypovitaminosis D than in patients with normal levels of vitamin D. These results suggest the possible involvement of low vitamin D levels in the disease severity in SLE patients and the importance of maintaining the normal levels of vitamin D to combat the activity of lupus.

Competing interests

The authors declare that they have no competing interests.

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REFERENCES

1. Ahmadpoor P, Dalili N, Rostami M. An update on pathogenesis of systemic lupus erythematosus. *Iran J Kidney Dis.* 2014; 8(3): 171-84.
2. de Souza VA, Bastos MG, Fernandes NM, Mansur HN, Raposo NR, de Souza DM, et al. Association of hypovitaminosis D with Systemic Lupus Erythematosus and inflammation. *J Bras Nefrol.* 2014; 36(4): 430-6.
3. Pons-Estel GJ, Alarcon GS, Scofield L, Reinlib L, Cooper GS. Understanding the epidemiology and progression of systemic lupus erythematosus. *Semin Arthritis Rheum.* 2010; 39(4): 257-68.
4. Lisnevskaia L, Murphy G, Isenberg D. Systemic lupus erythematosus. *Lancet.* 2014; 384(9957): 1878-88.
5. Kamen DL. Vitamin D in lupus - new kid on the block? *Bull NYU Hosp Jt Dis.* 2010; 68(3): 218-22.
6. Kusworini Handono1 LP, Achmad Rudijanto3, Singgih Wahono2 and Handono Kalim2. Vitamin D Serum Level And Disease Activity In Patients With Systemic Lupus Erythematosus. *International Journal of Pharmaceutical Science Invention.* 2013; 2(2): 6.
7. Marques CD, Dantas AT, Fragoso TS, Duarte AL. The importance of vitamin D levels in autoimmune diseases. *Rev Bras Reumatol.* 2010; 50(1): 67-80.
8. Luo J, Wen H, Guo H, Cai Q, Li S, Li X. 1,25-dihydroxyvitamin D3 inhibits the RANKL pathway and impacts on the production of pathway-associated cytokines in early rheumatoid arthritis. *Biomed Res Int.* 2013; 2013: 101805.
9. Bonelli M, Smolen JS, Scheinecker C. Treg and lupus. *Ann Rheum Dis.* 2010; 69 Suppl 1: i65-6.
10. Chen J, Bruce D, Cantorna MT. Vitamin D receptor expression controls proliferation of naive CD8+ T cells and development of CD8 mediated gastrointestinal inflammation. *BMC Immunol.* 2014; 15: 6.
11. Ruiz-Irastorza G, Egurbide MV, Olivares N, Martinez-Berriotxoa A, Aguirre C. Vitamin D deficiency in systemic lupus erythematosus: prevalence, predictors and clinical consequences. *Rheumatology (Oxford).* 2008; 47(6): 920-3.

12. Bonakdar ZS, Jahanshahifar L, Jahanshahifar F, Gholamrezaei A. Vitamin D deficiency and its association with disease activity in new cases of systemic lupus erythematosus. *Lupus.* 2011; 20(11): 1155-60.
13. Yap KS, Northcott M, Hoi AB, Morand EF, Nikpour M. Association of low vitamin D with high disease activity in an Australian systemic lupus erythematosus cohort. *Lupus Sci Med.* 2015; 2(1): e000064.
14. Petri M, Bello KJ, Fang H, Magder LS. Vitamin D in systemic lupus erythematosus: modest association with disease activity and the urine protein-to-creatinine ratio. *Arthritis Rheum.* 2013; 65(7): 1865-71.
15. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.* 1997; 40(9): 1725.
16. Holick MF. Vitamin D deficiency. *N Engl J Med.* 2007; 357(3): 266-81.
17. Fragoso TS, Dantas AT, Marques CD, Rocha Junior LF, Melo JH, Costa AJ, *et al*. 25-Hydroxyivitamin D3 levels in patients with systemic lupus erythematosus and its association with clinical parameters and laboratory tests. *Rev Bras Reumatol.* 2012; 52(1): 60-5.
18. Kamen DL, Cooper GS, Bouali H, Shaftman SR, Hollis BW, Gilkeson GS. Vitamin D deficiency in systemic lupus erythematosus. *Autoimmun Rev.* 2006; 5(2): 114-7.
19. Kamen D, Aranow C. Vitamin D in systemic lupus erythematosus. *Curr Opin Rheumatol.* 2008; 20(5): 532-7.
20. Schoindre Y, Jallouli M, Tanguy ML, Ghillani P, Galicier L, Aumaitre O, *et al*. Lower vitamin D levels are associated with higher systemic lupus erythematosus activity, but not predictive of disease flare-up. *Lupus Sci Med*. 2014; 1(1): e000027.
21. Amezcua-Guerra LM, Springall R, Arrieta-Alvarado AA, Rodriguez V, Rivera-Martinez E, Castillo-Martinez D, *et al*. C-reactive protein and complement components but not other acute-phase reactants discriminate between clinical subsets and organ damage in systemic lupus erythematosus. *Clin Lab*. 2011; 57(7-8): 607-13.
22. Firooz N, Albert DA, Wallace DJ, Ishimori M, Berel D, Weisman MH. High-sensitivity C-reactive protein and erythrocyte sedimentation rate in systemic lupus erythematosus. *Lupus.* 2011; 20(6): 588-97.
23. Breslin LC, Magee PJ, Wallace JM, McSorley EM. An evaluation of vitamin D status in individuals with systemic lupus erythematosus. *Proc Nutr Soc.* 2011; 70(4): 399-407.

RELAȚIA DINTRE VITAMINA D, INFLAMAȚIE ŞI INDEXUL DE ACTIVITATE AL LUPUSULUI ERITEMATOS SISTEMIC

REZUMAT

Obiectiv: Lupusul eritematos sistemic (LES) este o boală inflamatorie cronică autoimună. Rolul vitaminei D in preventia sau tratamentul LES este inca dezbatut. Obiectivul acestui studiu a fost evaluarea nivelul seric al vitaminei D la pacienții cu LES, relatia vitaminei D cu markerii inflamatori si cu indexul de activitate a lupusului eritematos sistemic (SLEDAI).

Metoda: Acest studiu prospectiv a cuprins 20 de femei diagnosticate cu LES, conform criteriilor Colegiului American de Reumatologie (ACR) și 12 pacienti sanatosi, de control. Am determinat nivelurile serice ale proteinei C reactive (CRP), vitezei de sedimentare a hematiilor (VSH), 25-hidroxi vitaminei D, calciului total, fosfatului seric si hormonului paratiroidian. Activitatea LES a fost evaluată cu ajutorul SLEDAI. Nivelurile sub 30 ng/mL ale vitaminei D au fost considerate anormale. Au fost măsurate nivelurile creatininei serice și rata de filtrare glomerulara (RFG) pentru a evalua afectarea renala la acesti pacienti.

Rezultate: Vârsta medie a pacientilor cu LES inclusi în acest studiu a fost 44,1 ± 11.65 ani. S-a observat ca nivelurile serice ale vitaminei D au fost semnificativ mai scăzute la pacientii cu LES comparativ cu lotul de control. Pacientii cu LES și cu niveluri scazute ale vitaminei D au avut valori mai crescute ale creatininei serice, RFG, VSH și PCR. Scorul SLEDAI a fost semnificativ mai mare pentru pacientii cu hipovitaminoza D, comparativ cu cei cu nivel normal al vitaminei D.

Concluzii: În acest studiu, deficitul vitaminei D a fost mai frecvent observat la pacientii cu LES. Parametrii inflamatori si scorul SLEDAI au fost semnificativ mai mari la pacientii cu hipovitaminoza D comparativ cu cei cu valori normale ale vitaminei D. Aceste rezultate sustin importanța menținerii nivelurilor normale ale vitaminei D pentru a combate activitatea LES.

Cuvinte cheie: vitamina D, LES, inflamație, scorul SLEDAI

ASSESSMENT OF A MURINE MELANOMA MODEL

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ABSTRACT

Malignant melanoma, the most aggressive type of skin cancer, still represents a hot topic for the researchers due to the lack of an effective treatment and finding new drugs with improved properties is a goal to achieve. The use of animal models in melanoma research makes this goal seem closer.

The aim of the present study was to develop a murine melanoma model using C57BL/6J mice by inoculation subcutaneously of B164A5 cell suspension.

In order accomplish the experiment, we used both female and male C57BL/6J mice (n=7 mice/group) with the age of 10-12 weeks. The day prior the experiment the mice were shaved on the back with a razor and B164A5 cell suspension ($1X10^6$ cells/100 µl PBS/mice) was subcutaneously inoculated to both females and male mice. The mice were examined daily.

The initiation of tumor had an early onset in the female group, but all the mice included in the study developed the tumor. These data sustain the fact that the model that we proposed is a reliable and a reproducible one.

Key words: melanoma, B164A5, C57Bl/6J.

INTRODUCTION

Malignant melanoma is considered the most deadly skin cancer type and is known to possess an increased metastatic potential and, in addition, is resistant to the conventional anticancer therapy [1].

It is not fully elucidated the mechanism involved in melanoma initiation and development, but are well established the histopathological stages of this disease: the presence of common and dysplastic nevi, radial growth phase melanoma (RGP), vertical growth phase melanoma (VGP) and the last stage and the most critical one, is metastatic malignant melanoma [1].

One of the reasons why melanoma still attracts a lot of interest among the researchers consists in the lack of an effective treatment, especially for the metastatic patients, and considerable efforts are made in this direction in order to find novel and efficient compounds or to find a better formulation with improved efficacy for the conventional therapy.

The animal models represent an useful tool in gathering information regarding the insights of cellular and molecular mechanisms involved in melanoma initiation and progression (cellular transformation, chromosomal mutations) [2-4]. Furthermore, the *in vivo* models play key roles in the testing process of new drugs [1, 5].

The melanoma studies regarding the genetic modifications that may occur during the progression of the disease or the evaluation of new drugs effectiveness are using the mouse model as a standard [1].

One of the most frequently used melanoma mouse model is the one developed on black mice, C57BL/6J by inoculation of different murine melanoma cells (B16F10, B16BL6, B164A5), this strain of mice being the most receptive host for this type of tumor cells [5 -7].

The objectives of this study were: (i) to develop a reproducible murine melanoma mouse model by inoculation subcutaneously of a B164A5 murine melanoma cell suspension and (ii) to verify if there are any differences related to mice gender in initiation of tumors.

MATERIALS AND METHODS

In this study we used B164A5 murine melanoma cells. The cell line was purchased from ECACC (European Collection of Cell Cultures) at passage 3. B164A5 cells were kept in liquid nitrogen and one week

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before the experiment started, we cultured in specific medium culture.

The cells (figure 1) were cultured in high glucose (4.5 g/l) Dulbecco's modified Eagle Medium (DMEM – Sigma Aldrich, Germany) supplemented with 15 mM Hepes, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (FBS). Cells were kept in a humidified atmosphere with 5% CO2 at 37°C and were passaged every two days. Cells number was assessed by using Neubauer chamber in the presence of Trypan Blue.

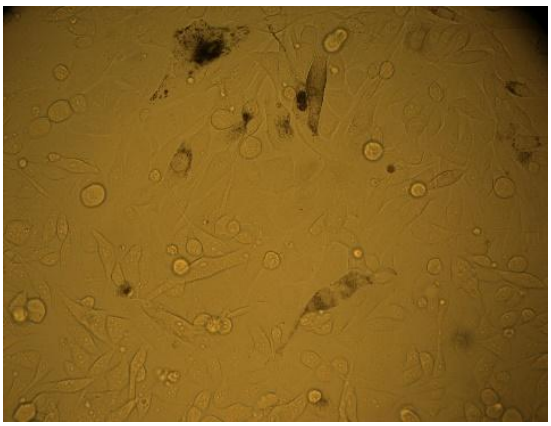

Fig. 1. B164A5 murine melanoma cells in culture

All the reagents used in the study (DMEM medium, phosphate saline buffer – PBS, penicillin, streptomycin, foetal bovine serum, Trypan Blue) were of analytical purity and were purchased from Sigma Aldrich, Germany.

The animals used in the present study were female and male C57BL/6J mice (10-12 weeks-old) which were obtained from Charles River Laboratories, Budapest, Hungary. All experimental procedures were conducted in accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes. The experimental protocol was approved by the Committee for Ethics Research of the University for Medicine and Pharmacy of Timisoara, Romania. Animals were fed *ad libitum* and kept under standard conditions: constant temperature of 22.5 ± 2° C, humidity 55 ± 5% and a 12-h light/dark cycle.

The murine melanoma model was obtained according to the following protocol: B164A5 cell suspension (1X10^6 cells/100 µl PBS/mice) was prepared and subcutaneously inoculated to both females and male mice which were shaved on their back a day prior the experiment. During the inoculation the mice were anesthetized with Isoflurane.

The evolution of the mice was followed daily and the tumors were measured using a caliper. The width (a) and length (b) of the tumors were measured by calipers for the evaluation of tumor volume according to the formula $V = ba^2/2$ [8].

RESULTS AND DISCUSSIONS

The aims of this present study were: (i) to obtain a reproducible murine melanoma model using B164A5 xenografts and (ii) to compare the moment of emergence of melanoma tumor in female *vs.* male mice.

In order to obtain the animal model of murine melanoma, we used B164A5 murine melanoma cells, which were counted and prepared as a suspension of 1X10^6 cells and a volume of 100 µl PBS/mice was injected to both groups of mice, females (n=7 mice/group) and males (n=7 mice/group).

The inoculation/ injection of the cells is a very important step in the initiation and development of the subcutaneous tumor, because if this step is done wrong then the tumor won't appear.

The suspension of cells must be carefully injected subcutaneously and a bubble must be observed at the place of inoculum, bubble that will be resorbed until the next day (Figure 2).

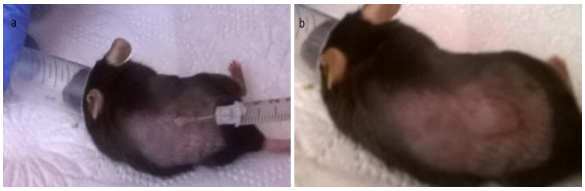

Fig. 2. Development of murine melanoma mouse model using C57BL/6J mice: a) inoculation of the cells suspension subcutaneously and b) the bubble aspect that should be seen at the inoculum site

The mice were examined daily and the first signs of tumor initiation (a small, round bubble at skin level in the same area were the cells were inoculated) were observed in the females group starting with the fourth day post-inoculation whereas in the males group no signs of tumor were detected at that time point.

At day 6 there were observed the first signs of tumor initiation in the group of male mice, too, since in the females group the tumor was small but well-defined (Figure 3).

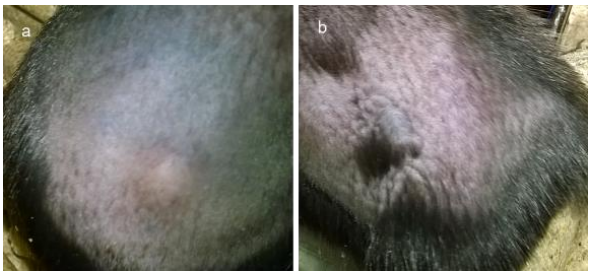

Fig. 3. Macroscopic aspect of the tumors at day 6 post-inoculation: a) C57BL/6J female and b) C57BL/6J male

At day 14 the tumors were well-defined in both groups and the mice were sacrificed between days 25 and 30 when the tumors reached the volume approved by the ethics protocol (1000 -1500 mm^2) and their condition became poor.

Our results indicated that we obtained a reproducible murine melanoma model since all the mice from the both group developed tumors post-inoculation of the cell suspension. Furthermore, we observed that the females were more susceptible to develop tumors, the time of appearance being more reduced as compared to the males group.

This model is a valuable source of information concerning the mechanisms involved in the pathogenesis of this mortal disease, but also offers opportunities for the test of new drugs that may cure melanoma.

B164A5 murine melanoma cells present fibroblast-like features and were described to have the capacity to produce melanin. These cells were derived from a melanoma tumor that developed in the skin of a C57BL/6J mouse [9].

The use of animal models offers multiple advantages, such as: to study and to understand better the insights of the pathogenesis of different diseases, to evaluate directly the toxicity and efficiency of new therapies, it can also be obtained mouse models with human tumor allografts this leading to the elucidation of the encoding genes involved in the initiation and progression of melanoma and other pathologies [1].

The C57BL/6J murine melanoma mouse model obtained by inoculation of B16 xenografts are known as syngeneic models and one of their advantages is the fact that they possess a functional immune system [9].

C57BL/6J mouse inbred line is frequently used in studies regarding mouse genetics and its genome has been incorporated into many genetic reference populations [10].

The novelty of the murine melanoma mouse model that we proposed in this study is represented by the number of the cells B164A5 ($1X10^6$ cells/100 µl PBS/ mouse). We chose this number of tumor cells after several attempts with a smaller number (10^5 cells/mouse or $0.5X10^5$ cells/mouse) [7, 11], but according to our last results this number induced tumors to all mice involved in the study.

C57BL/6 is the strain of mice that is used in all the studies concerning identification of biomarkers characteristic for murine melanoma or the development of new therapies with anticancer properties [5-7, 12-14].

Another advantage of this model was that the metastases did not occur so rapidly as in the model obtained by intraperitoneal injection of the B164A5 cells [15], and this offers the possibility to observe the evolution of tumor after the administration of a novel and improved treatment.

CONCLUSION

The animal model of murine melanoma that we developed in the present study proved to be a reliable and reproducible model and it represents the background for further studies focused on verifying the effect of some melanoma-targeted nanoparticles drugs.

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REFERENCES

1. Conde-Perez A, Larue L. Human relevance of NRAS/BRAF mouse melanoma models. *Eur J Cell Biol.* 2014; 93(1-2): 82-6.
2. Krauthammer M, Kong Y, Ha BH, Evans P, *et al*. Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. *Nature Genetics* 2012; 44: 1006-1014.
3. Kunz M, Dannemann M, Kelso J. High-throughput sequencing of themelanoma genome. *Experimental Dermatology* 2013; 22: 10-17.
4. Nikolaev SI, Rimoldi D, Iseli C, Valsesia A, *et al*. Exome sequencing identifies recurrent somatic MAP2K1and MAP2K2 mutations in melanoma. *Nature Genetics* 2015; 44: 133-139.
5. Zhang X, Teodoro JG, Nadeau JL. Intratumoral gold-doxorubicin is effective in treating melanoma in mice. *Nanomedicine.* 2015; 11(6): 1365-75.
6. Taylor P, Noriega R, Farah C, Abad MJ, Arsenak M, Apitz R. Ajoene inhibits both primary tumor growth and metastasis of B16/BL6 melanoma cells in C57BL/6 mice. *Cancer Lett*. 2006; 239(2): 298-304.
7. Soica C, Danciu C, Savoiu-Balint G, Borcan F, Ambrus R, Zupko I, Bojin F, Coricovac D, Ciurlea S, Avram S, Dehelean CA, Olariu T, Matusz P. Betulinic acid in complex with a gamma-cyclodextrin derivative decreases proliferation and in vivo tumor development of non-metastatic and metastatic B164A5 cells. *Int J Mol Sci.* 2014; 15(5): 8235-55.
8. Conway TF Jr, Sabel MS, Sugano M, Frelinger JG, Egilmez NK, Chen F and Bankert RB. Growth of human tumor xenografts in SCID mice quantified using an immunoassay fot tumor marker protein in serum. *J Immunol Methods* 2000; 233: 57-65.
9. McKinney AJ, Holmen SL. Animal models of melanoma: a somatic cell gene delivery mouse model allows rapid evaluation of genes implicated in human melanoma. *Chin J Cancer.* 2011; 30(3): 153-62.
10. Simon MM, Greenaway S, White JK, Fuchs H, Gailus-Durner V, Wells S, *et al*. A comparative phenotypic and genomic analysis of C57BL/6J and C57BL/6N mouse strains. *Genome Biol.* 2013; 14(7): R82.
11. Duicu O, Ciurlea S, Dehelean C, Ardelean S, Andrica F, Muntean D, Soica C, Antal D, Coricovac D, Pinzaru I: Analysis of a Betulinic Acid Formulation on Liver Mitochondria Isolated

from Mice with Induced Murine Melanoma. *Rev. Chim.* 2014, 65(8): 956-959.
12. Yu Z, Zhang T, Zhou F, Xiao X, Ding X, *et al*. Anticancer Activity of Saponins from Allium chinense against the B16 Melanoma and 4T1 Breast Carcinoma Cell. *Evid Based Complement Alternat Med.* 2015; 2015: 725023.
13. Dittz D, Figueiredo C, Lemos FO, Viana CT, Andrade SP, *et al*. Antiangiogenesis, loss of cell adhesion and apoptosis are involved in the antitumoral activity of Proteases from V. cundinamarcensis (C. candamarcensis) in murine melanoma B16F1. *Int J Mol Sci.* 2015; 16(4): 7027-44.
14. Li X, Sun J, Hu S, Liu J. Icariin Induced B16 Melanoma Tumor Cells Apoptosis, Suppressed Tumor Growth and Metastasis. *Iran J Public Health.* 2014; 43(6): 847-8.
15. Gheorgheosu D, Dehelean C, Cristea M, Muntean D. Development of the B16 Murine Melanoma Model. *Annals of the Romanian Society for Cell Biology*, 2011; XVI (2): 148-156.

REALIZAREA UNUI MODEL ANIMAL DE MELANOM MURINIC

REZUMAT

Melanomul, cel mai agresiv tip de cancer de piele, reprezintă încă un subiect de interes pentru cercetători datorită lipsei, la momentul actual al unui tratament curativ, iar găsirea unor noi medicamente cu eficacitate sporită este un obiectiv ce trebuie îndeplinit. Utilizarea modelelor animale în cercetarea referitoare la melanom face ca acest obiectiv să pară mai ușor de îndeplinit.

Scopul acestui studiu a fost acela de a realiza un model animal de melanom murinic la șoarecii C57Bl/6J prin injectarea subcutanată a unei suspensii de celule de melanom murinic B164A5.

Pentru realizarea experimentului s-au folosit șoareci C57BL/6J de sex feminin și masculin (n=7 șoareci/lot) cu vârsta cuprinsă între 10-12 săptămâni. Cu o zi înainte de injectare, șoarecii au fost epilați pe spate și apoi le-a fost injectată suspensia de celule $1x10^6$ celule/100 µl PBS/ per șoarece. Șoarecii au fost examinați zilnic post-injectare.

Primele semne de apariție a tumorii au fost observate mai întâi la lotul de femele, dar până la finalul experimentului toți șoarecii incluși în studiu au dezvoltat tumora. Aceste rezultate indică faptul că modelul animal propus este un model reproductibil.

Cuvinte cheie : melanom, celule B164A5, șoareci C57BL/6J.

LUPEOL A POTENT ANTI-INFLAMMTORY AGENT IN ACUTE INFLAMMATION MOUSE EAR MODEL

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ABSTRACT

Lupeol is a natural compound, member of the pentacyclic triterpene family, family known for its multiple pharmacological effects. This compound was described as a potent anti-inflammatory agent both in vitro and in vivo, and, also possess antitumoral effects. The main objectives of this study were to obtain an ear inflammation mouse model by topical application of 12-0-tetradecanoylphorbol acetate (TPA) in SKH1 hairless mice and to evaluate the effects of lupeol in this condition.

The mice were divided in 3 groups: control group – TPA treated, group treated with lupeol and group treated with indomethacin. There were evaluated parameters such as: transepidermal water loss (TEWL) by means of a non-invasive technique and ear oedema using Draize scoring.

Our results showed that lupeol treatment had a protective and anti-inflammatory effect, effect comparable with the one observed in the case of indomethacin. Moreover, the anti-inflammatory activity of lupeol becomes stronger in time (after 24 h).

Key words: inflammation, lupeol, indomethacin, TPA

INTRODUCTION

Lupeol is a compound of natural origin, member of the pentacyclic triterpenes family and is widely distributed in vegetables oils, cereals and fruits [1]. This plant-derived compound has a lupan-skeleton with four cyclohexane rings and one cyclopentane ring arranged in *trans* conformation what confers it a high liposolubility [2].

Lupeol is known to exhibit multiple biological properties, including: anti-inflammatory [3, 4], antitumoral [5], antimicrobial, cytoprotective [6], antidiabetic [7].

The pharmacological effects of lupeol were proved both *in vitro* and *in vivo* and another advantage associated to this product is that it has no toxicity on normal cells.

The ear inflammation mouse model obtained by topical application of 12-0-tetradecanoylphorbol acetate (TPA) is an easy model to develop and it might be considered a reliable source of information regarding the beneficial or toxic effects of some new compounds with potential anti-inflammatory effects.

The aims of the present study were to obtain an ear inflammation mouse model and to check the anti-inflammatory effect of lupeol after local administration in comparison with indomethacin.

MATERIALS AND METHODS

All the reagents used in the study (lupeol, acetone, TPA) were of analytical purity and were purchased from Sigma Aldrich, Germany and Chimopar, Bucharest.

The animals used in the present study were SKH1 hairless male mice (10-12 weeks-old) and were purchased from Charles River Laboratories, Budapest, Hungary. All experimental procedures were conducted in accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes. The experimental protocol was approved by the Committee for Ethics Research of the University for Medicine and Pharmacy of Timisoara, Romania. Animals were fed *ad libitum* and kept under standard conditions: constant

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temperature of 22.5 ± 2° C, humidity 55 ± 5% and a 12-h light/dark cycle.

The ear inflammation mouse model was obtained according to the following protocol: TPA acetone solution (2 µg/20µl acetone) was applied on the mice external ear and after 30 minutes, the test solutions were administered. After 24h post-administration of the solutions, the mice were sacrificed and the ears were cut and weighed.

The mice were divided in 3 groups: group 1 – control group that received only the TPA solution, group 2 – the group treated with lupeol solution and group 3 – the group that was treated with indomethacin.

A non-invasive technique was used in order to measure transepidermal waterloss (TEWL), a physiologic cutaneous parameter with important role in the diagnostic of different skin diseases that affect skin integrity. The measurements were executed by the means of Multiprobe Adapter System (MPA 5), Courage Khazaka, Germany.

All the measurements from this research were done in triplicate for each sample; the results were expressed as mean ± standard error. Paired Student's t tests or One-way Anova followed by Bonferroni's post-tests were used to determine the statistical difference between different experimental and control group. $p < 0.05$ was considered statistically significant; *, ** and *** indicate $p<0.05$, $p<0.01$ and <0.001

RESULTS AND DISCUSSIONS

The ear inflammation mouse model represents a useful tool in the evaluation of the anti-inflammatory potential of new compounds.

In the present study, we developed this model by using SKH1 hairless mice and acetone solution of 12-0-tetradecanoylphorbol acetate (TPA).

Another objective was to verify the anti-inflammatory effects of lupeol acetone solution after topical administration as compared to the group that was treated with indomethacin.

Regarding the macroscopic aspect of the mice ears, there were detected significant differences between the work groups (Figure 1).

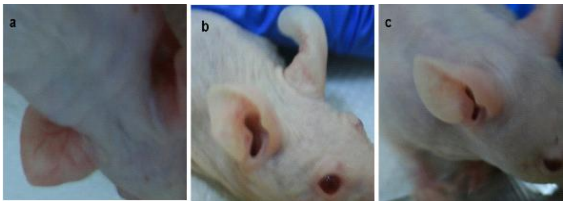

Fig. 1. Ear inflammation mouse model induced by topic application of TPA: a) control group - C, b) ears exposed to TPA solution and lupeol treatment (group L) and c) ears with TPA treated with Indomethacin (group I)

As it can be seen from the pictures, lupeol was able to reduce the ear oedema and redness as compared to control group.

More than half a century has passed since the importance of transepidermal water loss (known as TEWA or TEWL) was recognized as a procedure used for the evaluation of skin barrier function. G.E. Nilsson described for the first time this procedure which is based on the estimation of the vapour pressure gradient [8, 9].

This technique represents a well-established method in dermatology to assess the integrity of the skin barrier *in vivo*. When skin is damaged, its barrier function is unaccomplished resulting in higher water loss [10].

E. Ahaghotu *et al*. described ten years ago a corelation between different chemical compounds and the evolution of TEWA at hairless rats; they used a control group with TEWA around 5 g/m^2 h, and obtained an increase to 10-15 g/m^2 h for a 100 hours treatment with xylene or benzene and more than 15 units/100 hours in the case of tetramethyl-benzene isomers [11].

Moreover, the dependence between TEWA values and skin treated with cancer promoters such as 7,12-dimethylbenz[a]anthracene (DMBA) solution followed by UVB exposure was already proved by our research team [12].

In this study, there was observed that skin of mice from the control group (C) suffered of a continous and important degradation during the 24 hours. The most important degradation was recorded in the first 4 hours of exposure to TPA (around 2 units/hour). The protective effect of indomethacin, a well-known non-steroidal anti-inflammatory drug, leads to an aproximative constant trend for TEWA values during the experiment (between 3.8 and 5.5 g/m^2 h); once again the most important increase was recorded in the first four hours of evaluation of mice from group I.

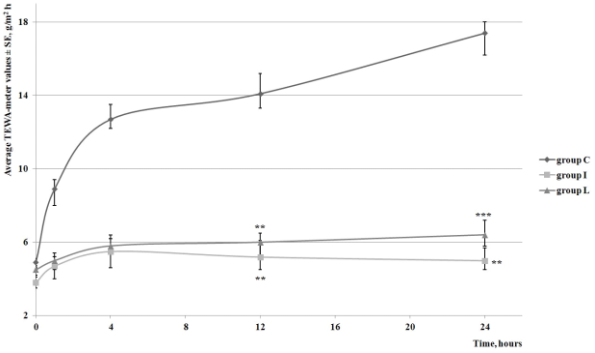

Fig. 2. Evolution of TEWA-meter values

In the case of mice trated with TPA and lupeol (group L) the difference between the first 4 hours and the others is not so important. The increase of average TEWA-values is more linear between 4.5 and 6.4 g/m^2 h. It is important to observe that lupeol presents a protective effect similar to indomethacin. A maximum value of

TEWA below 10 units, after 24 hours of treatment with TPA, an important inducer for endogenous superoxide production, used as a potent tumor promoter, evidenced one of the beneficial effects of this phytocompound.

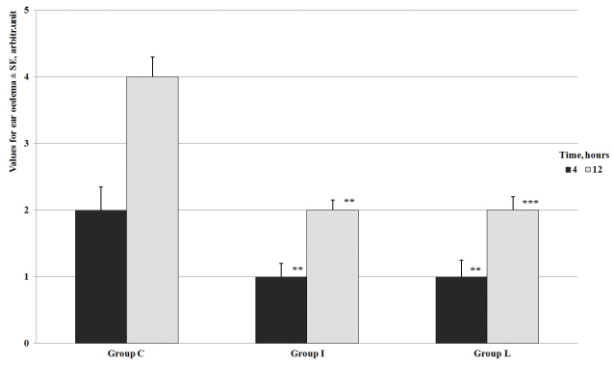

Fig. 3. Values for ear oedema after 4 and 12 hours, respectively

Dermal irritation has been assessed using the Draize test since 1944 [13]. Takashi Nishikawa *et al*. used the Draize scoring system to assess the effects of 10 polycyclic aromatic hydrocarbons on the dorsal skin of hairless mice; using this method, they concluded that these compounds caused the *in vivo* production of micronucleus which is correlated with the reported carcinogenicity of these chemical compounds [13].

In our study, increased values of Draize scoring system were obtained in the case of group C. The beneficial and similar effects of indomethacin and lupeol can be assumed to the results which indicate half scores for values of group I and group L than the values of control group.

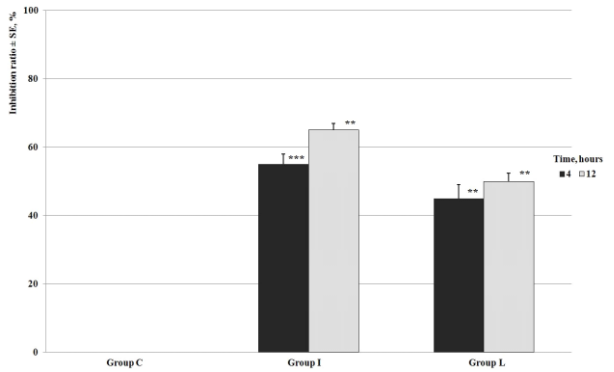

Fig. 4. Inhibition ratio after 4, and respectively 12 hours

The average value for oedema induced by TPA alone after 12 hours was 4.0 ± 0.3 with an inhibition ratio, IR = 0%, while the average value for oedema in case of treatment with indomethacin was 2.0 ± 0.15 and IR = 65.11%; for the lupeol, the Draize scoring system value was 2.0 ± 0.2 and IR = 49.83%. There were not obtained important differences between 4 and 12 hours in the evaluation of oedema and inhibition rate, but the results indicate a better effect of two tested compounds after a longer period.

CONCLUSION

These results allow drawing the conclusion that lupeol has an anti-inflammatory activity which becomes stronger after a longer period, but the values are smaller than in case of mice treated with indomethacin.

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REFERENCES

1. Siddique HR, Saleem M. Beneficial health effects of lupeol triterpene: A review of preclinical studies. *Life Sci*. 2011; 88: 285-293
2. Broniatowski M, Flasiński M, Zięba K, Miśkowiec P. Langmuir monolayer studies of the interaction of monoamphiphilic pentacyclic triterpenes with anionic mithcondrial and bacterial membrane phospholipids – Searching for the most active terpene. *Biochim Biophys Acta* 2014; 1838: 2460-2472
3. Chaturvedi PK, Bhui K, Shukla Y. Lupeol: Connotations for chemoprevention. *Cancer Lett*. 2008; 263: 1-13
4. Chatterjee I, Chakravarty AK, Gomes A. Daboia russellii and Naja kaouthia venom neutralization by lupeol acetate isolated from the root extract of Indian sarsaparilla Hemidesmus indicus R. *J Ethnopharmacol.* 2006; 106: 38-43
5. Saleem M. Lupeol, a novel anti-inflammatory and anti-cancer dietary triterpene. *Cancer Lett.* 2009; 285: 109-115
6. Sudhahar V, Kumar SA, Mythili Y, Varalakshmi P. Remedial effect of lupeol and its ester derivative on hypercholesterolemia-induced oxidative and inflammatory stresses. *Nutrition Res*. 2007; 27: 778-787.
7. Ramu R, Shirahati PS, Zameer F, Ranganatha LV, Nagendra Prasad MN. Inhibitor Ry effect of banana (Musa sp. Var. Nanjangud rasa bale) flower extract and its constituents Umbelliferone and Lupeol on α-glucosidase, aldose reductase and glycation at multiple stages. *South African Journal of Botany* 2014; 95: 54-63.
8. Wilson DR, Maibach HI. A review of transepidermal water loss. In: Neonatal skin - structure and function. Maibach HI and Boisits EK Eds., Marcel Dekker Inc, New York 1982.
9. Nilsson GE. Measurement of Water Exchange through Skin. *Med Biol Comput.* 1977; 15: 209-18.
10. Pinnagoda J, Tupker RA, Agner T, Serup J. Occupational, guidelines for transepidermal water loss (TEWL) measurement. A report from the standardization group of the European society of contact dermatitis. *Contact Dermatitis* 1990; 22(3): 164–178.
11. Ahaghotu E, Babu RJ, Chatterjee A, Singh M. Effect of methyl substitution of benzene on the percutaneous absorption and skin irritation in hairless rats. *Toxicol Lett.* 2005; 159: 261–271.
12. Coricovac D, Danciu C, Ionescu D, Trandafirescu C,

Simu G, Soica C, Kemeny L, Ardelean S, Dehelean C. The influence of betulinic acid formulated as nanoemulsion on UVB activity as a tumor promoter in a mouse model of skin carcinoma. *Toxicol Lett.* 2014; 229: S50-S51

13. Miles A, Berthet A, Hopf NB, Gilliet M, Raffoul W, Vernez D, Spring P. A new alternative method for testing skin irritation using a human skin model: A pilot study. *Toxicol In Vitro*. 2014; 28(2): 240-247.

LUPEOLUL – UN AGENT ANTI-INFLAMATOR PUTERNIC ÎN INFLMAȚIA ACUTĂ PE UN MODEL EXPERIMENTAL DE URECHE MURINĂ

REZUMAT

Lupeolul este un compus natural, membru al familiei triterpenelor pentaciclice, familie recunoscută de-alungul timpului pentru multiplele sale efecte farmacologice. Acest compus a fost descris drept un potent agent antiinflamator atât *in vivo,* cât și *in vitro*, și mai posedă și activitate antitumorală. Obiectivele acestui studiu au constat în obținerea modelului de inflamație a urechii la șoarece după aplicarea locală a forbolului 12-O-tetradecanoil acetat (TPA) la șoarecii fără păr SKH1 și verificarea efectului lupeolului asupra inflamației induse.

Șoarecii au fost împărțiți în 3 loturi: lotul control – TPA, lotul tratat cu lupeol și lotul tratat cu indometacin. Au fost evaluați parametrii precum pierderea transepisermică a apei (TEWL) printr-o tehnică non-invazivă și gradul edemului urechii cu ajutorul scorului Draize.

Rezultatele obținute au indicat faptul că tratamentul cu lupeol a avut un efect protector și antiinflamator, efect comparabil cu cel al indometacinului. Mai mult decât atât s-a observat că tratamentul cu lupeol este mai eficace în timp (după 24 ore de la aplicare).

Cuvinte cheie: inflamație, lupeol, indometacin, TPA.

MOLECULAR MARKERS IN BREAST CANCER AND THEIR CLINICAL SIGNIFICANCE

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ABSTRACT
Breast cancer management has experienced incremental improvements in the past decades due to the discovery of new biomarkers very useful for early detection, prognosis and prediction of treatment responses. This review aims to highlight all the biomarkers that can be used in order to obtain individualized therapies in breast cancer treatment. Established molecular markers such as estrogen receptor and progesterone receptor have played a significant role in the selection of patients benefiting from endocrine therapy for many years. More recently, a new panel of biomarkers has emerged, but additional studies on large patient cohorts and using consistent methodologies are needed to define their precise value for the diagnosis and prognosis of breast cancer and for their predictive usefulness. Taking into account the quick development of this molecular biomarkers and the effectiveness of targeted molecular therapies, there is a continuous need for searching and identifying new markers.

INTRODUCTION

Cancer is the second cause of death worldwide. More conclusively, breast cancer is one of the most prevalent forms of cancer in women, and among the leading causes of cancer mortalities [1]. However, due to recent advances in the field, there has been a sustained decline in the mortality rates over the last decades. For example, the recurrence rate after surgery in patients detected with early breast cancer is nowadays relatively low. Improved imaging methods and screening programs assure in many cases an early diagnosis, emphasizing the need for new factors and sets of biomarkers to assess the individual risk of patients and to pinpoint the inherent value of additional treatment methods. Thus, the focus has shifted to individualized therapeutic approaches and personalized medicine. The estimation of the net benefit of systemic chemotherapy has to be taken in consideration when making therapeutic decisions for each patient individually. It becomes important to avoid unnecessary overtreatment in patients who only receive a modest benefit in the prolonging and quality of life, while at the same time suffering from significant side effects. On the other hand, physicians must also avoid decisions leading to incomplete, or incorrect treatments. It is therefore necessary to define the specific characteristics, molecular or clinical, which provide the future option for individual, patient-by-patient, treatment optimization [2].

The morphopathological development of breast cancer involves a progression through series of stages, beginning with ductal hyperproliferation, followed by subsequent evolution to carcinoma in situ, invasive carcinoma, and finally towards metastatic disease [1]. Given the variability in clinical progression of breast cancer, the identification of markers that could predict tumor behavior has become significantly important. Furthermore, the determination of tumor markers from tests is a useful tool for the clinical management of cancer patients, assisting in overall diagnostic procedures, tumor staging, evaluation of response to treatment, detection of relapses and prognosis. Not least important, studying the emerging field of breast cancer molecular markers aids in the development of new personalized treatment courses. Classical clinicopathological features of breast cancer, that indicate patient prognosis, include tumor size, histological subtype and grade, lymph node metastases, and lymphovascular invasion are all derived from careful histological analysis of primary breast cancer samples. The TNM (tumor size, nodes, and metastasis) system integrates these into tumor stages that are important when establishing the prognosis of each patient. However, with recent advancements in high-throughput methods, an array of novel bio-markers have been reported with prognostic and predictive purposes. Out of these, only a few have made their way into clinical routine due to the lack of sufficient

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validation to reach a Level of Evidence I or II according to the American Society of Clinical Oncology's Tumor Marker Utility Grading System [3]. Using this system, only a few biomarkers, including hormone receptors such as the estrogen receptor (ER) and progesterone receptor (PR), as well as the human epidermal growth factor receptor 2 (HER2), have been established and are assessed routinely in every breast cancer. Nevertheless, the discovery of new markers has led to more in depth insight into tumor biology and has underlined the importance of the existing biomarkers.

The purpose of this review is to aggregate all established diagnostic and predictive factors, as well as some emerging biomarkers that are currently undergoing testing for technical validity and clinical utility, in one single resource (Table I). This will help pathologists and clinicians have a clear glance at the actual state of research so to provide more efficient, personalized medicine.

PROGNOSIS AND PREDICTION IN BREAST CANCER

Prognostic and predictive markers have distinct roles in the establishment of personalized therapies, but both are of high relevance for therapeutic decisions. These factors are derived from either the individual characteristics of the patient or the tumor type. Prognostic factors intend to objectively predict patient clinical outcome independent of treatment, while predictive factors aim to foretell the response of a patient to a specific therapeutic intervention and are associated with tumor sensitivity or resistance to a specific therapy. For example, predictive markers can be targeted by specific therapies, in of themselves. The monoclonal antibody trastuzumab targets the oncogene HER2, giving HER2 positive status mixed prognostic/predictive significance. In the same manner, Ki67, has an important prognostic value as a marker of tumor proliferation, while also predicting a good response to systemic chemotherapy. In general, prognostic markers help determine whether a patient is suitable for treatment, while predictive factors are useful in deciding which therapeutic course will be the most efficient and have the best outcome. Nowadays, there has been a surge in the application of multiple marker sets to establish treatment-specific prognosis. This is particularly important when defining the residual recurrence risk of individualized treatments, and to evaluate the potential benefits of further therapeutic options. Significant efforts have been made recently to discern which ER-positive early breast cancer patients would actually benefit from additional chemotherapy and which ones would not, thus being able to avoid the side effects [4].

Table I. Synthesis of established and emerging biomarkers used in breast cancer, their area of research interest, a score of clinical significance and most relevant references to support the findings

Biomarker	Field of importance	Level of clinical significance	References*
ER	Diagnosis/Prognosis/ Prediction of drug response	+ + + +	[5]
PR	Diagnosis/Prognosis/ Prediction of drug response	+ + +	[6], [7]
HER-2	Diagnosis/Prognosis/ Prediction of drug response	+ + + +	[8], [9]
Ki67	Prognosis/ Prediction of drug response	+ +	[10]
p53	Prognosis	+	[11], [12]
CA 15-3, CEA	Diagnosis/Prognosis	+ + +	[13], [14]
BRCA1, BRCA2	Diagnosis/Prognosis	+ + +	[15], [16]
Cyclin D1	Prognosis/ Prediction of drug response	+	[17], [18]
Cyclin E	Prognosis/ Prediction of drug response	+	[19], [20]
ERβ	Prognosis/ Prediction of drug response	+	[21], [22]

PCNA	Prognosis		+	[23]
Caveolin	Prognosis		+	[24]
CXCR4	Prognosis		+	[25], [26]
CCL2, CCL5	Prognosis		+	[27], [28]
Growth Factors**	Prognosis		+	[31]
FOXP3	Prognosis		+	[32], [33]
MYC	Prognosis		#	[34]
microRNAs	Prognosis/ Prediction of drug response		+	[35], [36], [37]
uPA+PAI+TF	Diagnosis		+	[38], [39]
h-MAM	Diagnosis/ Prognosis		+	[40], [41], [42]
Osteopontin	Prognosis		+	[43], [44], [45]
PTEN	Prognosis		+	[46]
FGFR2	Prognosis		+	[47], [48]
Snail 1	Prognosis		+	[49], [50]
Sirtuins	Prognosis		+	[51], [52]
Twist	Prognosis		+	[50]
Zeb-1	Prognosis		+	[53]
CYP2D6	Prediction of drug response	+		[54]
3PIK3CA	Prediction of drug response	+		[55], [53]
RARA	Prediction of drug response	+		[54]
STAT3	Prediction of drug response	+		[55]
TIMP-1	Prediction of drug response	+		[59], [60]
Lin28	Prediction of drug response	+		[61]
RS/DJ-1	Diagnosis		+	[62]
HSP60, HSP90	Prognosis		+	[63]
Mucin-related	Prognosis		+	[64], [65]
α-2-HS-glycoprotein	Diagnosis		^	[66]
Lipophilin B	Diagnosis		^	[66]
Beta-globin	Diagnosis		^	[66]
Hemopexin	Diagnosis		^	[66]
Vitamin D-binding protein	Diagnosis		^	[66]

* most relevant

** EGF, HGF, IGF, VEGF, and TGF-β

Legend:

+ + + + = established therapeutic target

+ + + = diagnosis, prognosis and predictive factors used in everyday clinical practice

+ + = could be considered as a prognostic factor for therapeutic decision; however, standardization of techniques and scoring methods are needed for integration in everyday practice

+ = different level of evidence for clinical significance, bur further investigations are needed before any kind of implementation

= lack of evidence for the prognostic significance; could represent a clinically useful predictive parameter in metastatic breast cancer

^ = authors only identified abundant proteins in the nipple aspirate fluid whose over- or underexpression was somewhat modest; because, there is no analyze from patients with inflammatory breast disease, conclusions cannot be drawn regarding breast cancer specificity of protein expression.

MULTIGENE TESTS IN BREAST CANCER

Gene expression profiling of tumors measures thousands of mRNA transcripts in one single test using DNA microarrays. These signatures (Table II) are composed of different gene sets with few overlaying genes, mostly those associated with proliferation. Clinical context (e.g age, pure prognosis, endocrine treatment) and available biopsy material (e.g. MammaPrint and RS need fresh tissue, the others can use FFPE) should dictate the oncologist choice.

Table II. Multigene parameters in breast cancer, the number of genes used in each test, their area of clinical importance and most relevant references that support

Test	Number of genes	Field of importance	References*
MammaPrint	70	Prognostic: 5-years prognosis of recurrence for all node-negative and node-positive patients	[67]
Oncotype DX	21	Predictive: Residual risk of DR in ER+ patients treated with tamoxifen or AIs; predictive of chemotherapy benefit in node-negative ER+ patients	[68], [69]
Genomic-grade index	97	Prognostic/Predictive: Relapse in endocrine- treated ER+ breast cancer	[70], [71]
Molecular grade index	5	Predictive: Poor outcome despite endocrine therapy in ER+ breast cancer	[72]
Rotterdam signature	76	Prognostic: Development of distant metastases within 5 years	[73], [74]

*most relevant

Routine use of multigene assays such Oncotype Dx and MammaPrint to guide adjuvant therapy decisions in breast cancer has been shown to provide good cost-effectiveness, since it allows a correct choice of chemotherapy and reduction in chemotherapy utilization [75, 76]. The St Gallen consensus clearly states that the use of a validated multigene profiling assay is justified as a supplement to high-quality phenotyping of breast cancer in cases in which the indication for adjuvant chemotherapy remains unclear [77], but this tests are far from being widespread regarding clinical use.

CONCLUSION

There is an intense interest in breast biomarkers, as they can offer precious information about disease progression, patient's prognostic and response to specific treatment. Knowing and dealing with this complicated therapeutic schemes, where each patient's management should be individualized, is definitely a challenge for the clinicians. Some of the markers discussed in this review provide strong evidence about their effectiveness and clinical applicability, whereas others are still under study, with clear signs they will play a significant role in future therapies. Unfortunately, at present, there have not been identified any biomarkers that are capable of an early detection of breast cancer, and many women are still diagnosed at advanced stages with poor outcome. Very few of this biomarkers are capable on their own to be predictive and have a veritable clinical use, so a lot of randomized prospective trials are conducted in order to have some relevant results. Taking into account all the development done in this field, there still is a need for future molecular discoveries, which eventually will lead to new markers and of course to better targeted therapies. The future will provide us with unique case-specific patterns of biomarkers that will be able to predict specific responses.

ABBREVIATIONS

BRCA1 and BRCA2: Breast Cancer Susceptibility Genes; CA 15-3: Carbohydrate 15-3; CEA: Carcinoembryonic Antigens; CCL2 and CCL5: Chemokine (C-C Motif) Ligands 2 and 5; CXCR4: C-X-C Chemokine Receptor Type 4CYP2D6: Cytochrome P450 2D6; ER: Estrogen Receptor; EGF: Epidermal Growth Factor; FFPE: Formalin-Fixed Paraffin-Embedded; FGFR2: Fibroblast Growth Factor Receptors 2; FOXP3: Forkhead Box Protein 3; GCDFP-15: Gross Cystic Disease Fluid Protein-15; h-MAM: Human Mammaglobin; HER2: Human Epidermal Receptor 2; HGF: Hepatocyte Growth Factor, HSP: Heat Shock Protein; IGF: Insulin-like Growth Factor, ILC: Infiltrating Lobular Carcinomas; MGBA: Mammaglobin A; MMPs: Matrix Metalloproteinases; MYC: V-Myc Myelocytomatosis Viral Oncogene Homolog (Avian); PAI: Plasminogen Activator Inhibitor; PARP: Poly ADP Ribose Polymerase; PCNA: Proliferating Cell Nuclear Antigen; PIK3CA: Phosphatidylinositol-4,5-bipho-3-kinase; PR: Progeteron Receptor; PTEN: Phosphatase and Tensin Homolog; RARA: Retinoic Acid Receptor Alpha; SERM: Selective Estrogen Receptor Modulator; SIRT: Sirtuins; SNPs: Single Nucleotide Polymorphisms; STAT3: Signal Transducer and Activator of Transcription 3; TF: Thomsen-Friedenreich; TGFβ: Transforming Growth Factor Beta; TICs: Tumor-Initiating Cells; TIMP-1: Tisue Inhibitor of Metalloproteinase 1; TGF-β: Transforming Growth Factor-β; uPA: Urokinase-Dependent Plasminogen Activator System; VEGF: Vascular Endothelial Growth Factor.

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REFERNCES

1. Banin Hirata BK, Oda JMM, Losi Guembarovski R, Ariza CB, de Oliveira CE. De, Watanabe MA. Molecular markers for breast cancer: Prediction on tumor behavior. *Dis Markers.* 2014; 2014: 513158.
2. Weigel MT, Dowsett M. Current and emerging biomarkers in breast cancer: Prognosis and prediction. *Endocr Relat Cancer.* 2010; 17(4): R245-62.
3. Harris L, Fritsche H, Mennel R, Norton L, Ravdin P, Taube S, Somerfield MR, Hayes DF, Bast RC Jr. American Society of Clinical Oncology 2007 update of recommen- dations for the use of tumor markers in breast cancer. *J Clin Oncol.* 2007; 25: 5287-5312.
4. Dowsett M, Goldhirsch A, Hayes DF, Senn HJ, Wood W, Viale. International web-based consultation on priorities for translational breast cancer research. *Breast Cancer Research* 2007; 9: R81.
5. Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. *Clinical Cancer Research*, 2003; 9(6): 1980-89.
6. Dowsett M, Houghton J, Iden C, Salter J, Farndon J, A'Hern R, Sainsbury R, Baum M. Benefit from adjuvant tamoxifen therapy in primary breast cancer patients according oestrogen receptor, progesterone receptor, EGF receptor and HER2 status. *Annals of Oncology* 2006; 17: 818-26.
7. Viale G, Regan MM, Maiorano E, Mastropasqua MG, Dell'Orto P, Rasmussen BB, Raffoul J, Neven P, Orosz Z, Braye S, *et al*. Prognostic and predictive value of centrally reviewed expression of estrogen and pro- gesterone receptors in a randomized trial comparing letrozole and tamoxifen adjuvant therapy for postmeno- pausal early breast cancer: BIG 1-98. *Journal of Clinical Oncology* 2007; 25: 3846-52.
8. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*. 1987; 235: 177-182.
9. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE Jr, Davidson NE, Tan-Chiu E, Martino S, Paik S, Kaufman PA. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *New England Journal of Medicine* 2005; 353: 1673-84.
10. Luporsi E, Andre F, Spyratos F, *et al*. Ki-67: level of evidence and methodological considerations for its role in the clinical management of breast cancer: analytical and critical review. *Breast Cancer Research and Treatment*, 2012; 132(3): 895- 915.
11. Allred DC, Clark GM, Elledge R, *et al*. Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. *Journal of the National Cancer Institute*, 1993; 85(3): 200-206.
12. Dumay A, Feugeas JP, Wittmer E, Lehmann-Che J, Bertheau P, Espie M, *et al*. Distinct tumor protein p53 mutants in breast cancer subgroups. *International Journal of Cancer*, 2013; 132(5): 1227-31.
13. Ebeling FG, Stieber P, Untch M, *et al*. Serum CEA and CA 15- 3 as prognostic factors in primary breast cancer. *British Journal of Cancer*, 2002; 86(8): 1217-22.
14. Uehara M, Kinoshita T, Hojo T, Akashi-Tanaka S, Iwamoto E, Fukutomi T. Long-term prognostic study of carcinoembryonic antigen (CEA) and carbohydrate antigen 15-3 (CA 15-3) in breast cancer. *International Journal of Clinical Oncology*, 2008; 13(5): 447-51.
15. Venkitaraman AR. Cancer susceptibility and thefunctions of BRCA1 and BRCA2. *Cell*, 2002; 108(2): 171-182.
16. Senst N, Llacuachaqui M, Lubinski J, Lynch H, Armel S, Neuhausen S, et al. Parental origin of mutation and the risk of breast cancer in a prospective study of women with a BRCA1 or BRCA2 mutation. *Clinical Genetics*, 2013; 84(1): 43-46.
17. Bieche I, Olivi M, Nogues C, Vidaud M, Lidereau R. Prognostic value of CCND1 gene status in sporadic breast tumours, as determined by real-time quantitative PCR assays. *British Journal of Cancer* 2002; 86: 580-586.
18. Stendahl M, Kronblad A, Ryde n L, Emdin S, Bengtsson NO, Landberg G. Cyclin D1 overexpression is a negative predictive factor for tamoxifen response in postmenopausal breast cancer patients. *British Journal of Cancer* 2004; 90: 1942-48.
19. Keyomarsi K, Tucker SL, Buchholz TA, Callister M, Ding Y, Hortobagyi GN, Bedrosian I, Knickerbocker C, Toyofuku W, Lowe M, *et al*. Cyclin E and survival in patients with breast cancer. *New England Journal of Medicine* 2002; 347: 1566-75.
20. Smith ML, Seo YR. Sensitivity of cyclin E-overexpressing cells to cisplatin/taxol combinations. *Anticancer Research* 2000; 20: 2537-39.
21. Park BW, Kim KS, Heo MK, Ko SS, Hong SW, Yang WI, Kim JH, Kim GE, Lee KS. Expression of estrogen receptor-beta in normal mammary and tumor tissues: is it protective in breast carcinogenesis? *Breast Cancer Research and Treatment* 2003; 80: 79-85.
22. Hopp TA, Weiss HL, Parra IS, Cui Y, Osborne CK, Fuqua SA. Low levels of estrogen receptor beta protein predict resistance to tamoxifen therapy in breast cancer. *Clinical Cancer Research* 2004; 10: 7490-99.
23. Zhao H, Ho PC, Lo YH, et al. Interaction of proliferation cell nuclear antigen (PCNA) with c-Abl in cell proliferation and response to DNA damages in breast cancer. *PLoS ONE*, 2012; 7(1): e29416.
24. Aoki MN, Amarante MK, Oda JMM, Watanabe MAE. Caveolin involvement and modulation in breast cancer. *Mini-Reviews in Medicinal Chemistry*, 2011; 11(13): 1143-52.
25. Mukherjee D, Zhao J. The Role of chemokine receptor CXCR4 in breast cancer metastasis. *American Journal of Cancer Research*, 2013; 3(1): 46-57.
26. Chen HW, Du CW, Wei XL, Khoo US, Zhang GJ. Cytoplasmic CXCR4 high-expression exhibits distinct poor clinicopathological characteristics and predicts poor prognosis in triple-negative breast cancer. *Current Molecular Medicine*, 2013; 13(3): 410-416.
27. Steiner L, Murphy EA. Importance of chemokine (CC- motif) ligand 2 in breast cancer. *The International Journal of Biological Markers*, 2012; 27(3): e179–e185.
28. Velasco-Velazquez M, Pestell RG. The CCL5/CCR5 axis promotes metastasis in basal breast cancer. *Oncoimmunology*, 2013; 2(4): e23660.
29. Kucera R, Cerna M, Narsanska A, *et al*. Growth factors and

breast tumors, comparison of selected growth factors with traditional tumor markers. *Anticancer Research*, 2011; 31(12): 4653-56.
30. Mezi S, Todi L, Orsi E, Angeloni A, Mancini P. Involvement of the Src-cortactin pathway in migration induced by IGF-1 and EGF in human breast cancer cells. *International Journal of Oncology*, 2012; 41(6): 2128-38.
31. Ahmed HH, Metwally FM, Mahdy ES, Shosha WG, Ramadan SS. Clinical value of serum hepatocyte growth factor, B-cell lymphoma-2 and nitric oxide in primary breast cancer patients. *European Review for Medical and Pharmacological Sciences*, 2012; 16; 958.
32. Zuo T, Wang L, Morrison C, *et al*. FOXP3 is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene. *Cell*, 2007; 129(7): 1275-86.
33. Overbeck-Zubrzycka D, Ali S, Kirby J, Lennard T. FOXP3 transcription factor regulates metastatic spread of breast cancer via control of expression of CXCR4 chemokine receptor. *British Journal of Surgery*, 2011; 98(1): 84-85.
34. Todorovic-Rakovic N, Neskovic-Konstantinovic Z, Nikolic-Vukosavljevic D. C-myc as a predictive marker for chemotherapy in metastatic breast cancer. *Clinical and Experimental Medicine*, 2012; 12(4): 217-23.
35. Wu X, Somlo G, Yuetal Y. *De novo* sequencing of circulating miRNAs identifies novel markers predicting clinical outcome of locally advanced breast cancer. *Journal of Translational Medicine*, 2012; 10(1): 42.
36. Wang B, Wang H, Yang Z. MiR-122 inhibits cell proliferation and tumorigenesis of breast cancer by targeting IGF1R. *PloS ONE*, 2012; 7(10): e47053.
37. Shen L, Li J, Xu L, *et al*. miR-497 induces apoptosis of breast cancer cells by targeting Bcl-w. *Experimental and Therapeutic Medicine*, 2012; 3(3): 475-480.
38. Qin W, Gui G, Zhang K, Twelves D, Kliethermes B, Sauter ER. Proteins and carbohydrates in nipple aspirate fluid predict the presence of atypia and cancer in women requiring diagnostic breast biopsy. *BMC cancer*. 2012; 12: 52.
39. Huang Y, Zhang HQ, Wang J, Song XG, Wang GH, Guan Q, et al. Cloning expression, monoclonal antibody preparation and serologic study of mammaglobin in breast cancer. *Neoplasma*. 2011; 58: 436-40.
40. Galvis-Jimenez JM, Curtidor H, Patarroyo MA, Monterrey P, Ramirez-Clavijo SR. Mammaglobin peptide as a novel biomarker for breast cancer detection. *Cancer biology & therapy*. 2013; 14: 327-32.
41. Lee GW, Kim JY, Koh EH, Kang D, Choi DS, Maeng KY, et al. Plasma human mammaglobin mRNA associated with poor outcome in patients with breast cancer. Genetics and molecular research: GMR. 2012; 11: 4034-42.
42. Liu Y, Ma L, Liu X, Wang L. Expression of human mammaglobin as a marker of bone marrow micrometastasis in breast cancer. *Experimental and therapeutic medicine*. 2012; 3: 550-4.
43. Mi Z, Bhattacharya SD, Kim VM, Guo H, Talbot LJ, Kuo PC. Osteopontin promotes CCL5-mesenchymal stromal cell-mediated breast cancer metastasis. *Carcinogenesis*. 2011; 32: 477-87.
44. Li NY, Weber CE, Mi Z, Wai PY, Cuevas BD, Kuo PC. Osteopontin up-regulates critical epithelial-mesenchymal transition transcription factors to induce an aggressive breast cancer phenotype. *Journal of the American College of Surgeons*. 2013; 217: 17-26;
45. Bramwell VH, Tuck AB, Chapman JA, Anborgh PH, Postenka CO, Al-Katib W, et al. Assessment of osteopontin in early breast cancer: correlative study in a randomised clinical trial. *Breast cancer research: BCR*. 2014; 16: R8.
46. Zhang HY, Liang F, Jia ZL, Song ST, Jiang ZF. PTEN mutation, methylation and expression in breast cancer patients. *Oncol Lett.* 2013; 6: 161-8.
47. KimS, Dubrovska A , Salamone RJ, Walker JR, Grandinetti KB, Bonamy GM,et al. FGFR2 promotes breast tumorigenicity through maintenance of breast tumor-initiating cells. *PloS one*. 2013; 8: e51671.
48. Zhu X, Asa SL, Ezzat S. Histone-acetylated control of fibroblast growth factor receptor 2 intron 2 polymorphisms and isoform splicing in breast cancer. *Mol Endocrinol*. 2009; 23: 1397-405.
49. Blanco MJ, Moreno-Bueno G, Sarrio D, Locascio A, Cano A, Palacios J, et al. Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. *Oncogene*. 2002; 21: 3241-6.
50. Tran DD, Corsa CA, Biswas H, Aft RL, Longmore GD. Temporal and spatial cooperation of Snail1 and Twist1 during epithelial-mesenchymal transition predicts for human breast cancer recurrence. *Mol Cancer Res*. 2011; 9: 1644-57.
51. Finley LW, Carracedo A, Lee J, Souza A, Egia A, Zhang J, et al. SIRT3 opposes reprogramming of cancer cell metabolism through HIF1alpha destabilization. *Cancer cell*. 2011; 19: 416-28.
52. Kim HS, Patel K, Muldoon-Jacobs K, Bisht KS, Aykin-Burns N, Pennington JD, et al. SIRT3 is a mitochondria-localized tumor suppressor required for maintenance of mitochondrial integrity and metabolism during stress. *Cancer cell*. 2010; 17: 41-52.
53. Chaffer CL, Marjanovic ND, Lee T, Bell G, Kleer CG, Reinhardt F, et al. Poised chromatin at the ZEB1 promoter enables breast cancer cell plasticity and enhances tumorigenicity. *Cell*. 2013; 154: 61-74.
54. Zafra-Ceres M, de Haro T, Farez-Vidal E, Blancas I, Bandres F, de Duenas EM, et al. Influence of CYP2D6 polymorphisms on serum levels of tamoxifen metabolites in Spanish women with breast cancer. *International journal of medical sciences*. 2013; 10: 932-7.
55. Hanker AB, Pfefferle AD, Balko JM, Kuba MG, Young CD, Sanchez V, et al. Mutant PIK3CA accelerates HER2-driven transgenic mammary tumors and induces resistance to combinations of anti-HER2 therapies. *Proc Natl Acad Sci* U S A. 2013; 110: 14372-7.
56. Cizkova M, Dujaric ME, Lehmann-Che J, Scott V, Tembo O, Asselain B, et al. Outcome impact of PIK3CA mutations in HER2-positive breast cancer patients treated with trastuzumab. *British journal of cancer*. 2013; 108: 1807-9.
57. Johansson HJ, Sanchez BC, Mundt F, Forshed J, Kovacs A, Panizza E, et al. Retinoic acid receptor alpha is associated with tamoxifen resistance in breast cancer. *Nature communications*. 2013; 4: 2175.
58. Yi EH, Lee CS, Lee JK, Lee YJ, Shin MK, Cho CH, et al. STAT3-RANTES autocrine signaling is essential for tamoxifen resistance in human breast cancer cells. *Mol Cancer Res*. 2013; 11: 31-42.
59. Hekmat O, Munk S, Fogh L, Yadav R, Francavilla C, Horn H, et al. TIMP-1 Increases Expression and Phosphorylation of Proteins Associated with Drug Resistance in Breast Cancer Cells. *Journal of proteome research*. 2013; 12: 4136-51.
60. Deng X, Fogh L, Lademann U, Jensen V, Stenvang J, Yang H, et al. TIMP-1 overexpression does not affect sensitivity to HER2-targeting drugs in the HER2-gene-amplified SK-BR-3

human breast cancer cell line. *Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine*. 2013; 34: 1161-70.
61. Wang L, Yuan C, Lv K, Xie S, Fu P, Liu X, *et al*. Lin28 mediates radiation resistance of breast cancer cells via regulation of caspase, H2A.X and Let-7 signaling. *PloS one*. 2013; 8: e67373.
62. Le Naour F, Misek DE, Krause MC, *et al*. Proteomics-based identification of RS/DJ-1 as a novel circulating tumor antigen in breast cancer. *Clinical Cancer Research*, 2001; 7(11): 3328-35.
63. Conroy SE, Sasieni PD, Fentiman I, Latchman DS. Autoantibodies to the 90 kDa heat shock protein and poor survival in breast cancer patients. *European Journal of Cancer*, 1998; 34(6): 942-943.
64. Von Mensdorff-Pouilly S, Verstraeten AA, Kenemans P, *et al*. Survival in early breast cancer patients is favorably influenced by a natural humoral immune response to polymorphic epithelial mucin. *Journal of Clinical Oncology*, 2000; 18(3): 574-583.
65. Blixt O, Bueti D, Burford B, *et al*. Autoantibodies to aberrantly glycosylated MUC1 in early stage breast cancer are associated with a better prognosis," *Breast Cancer Research*, 2011; 13(2): R25.
66. Pawlik TM, Hawke DH, Liu Y, *et al*. Proteomic analysis of nipple aspirate fluid from women with early-stage breast cancer using isotope-coded affinity tags and tandem mass spectrometry reveals differential expression of vitamin D binding protein. *BMC Cancer*, 2006; 6: 68.
67. van de Vijver MJ, He YD, van 't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, *et al*. A gene-expression signature as a predictor of survival in breast. *New England Journal of Medicine* 2002; 347: 1999-2009.
68. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, Baehner FL, Walker MG, Watson D, Park T, *et al*. A multigene assay to predict recurrence of tamoxifen- treated, node-negative breast cancer. *New England Journal of Medicine* 2004; 351: 2817-26.
69. Sparano JA, Paik S. Development of the 21-gene assay and its application in clinical practice and clinical trials. *Journal of Clinical Oncology* 2008; 26: 721-728.
70. Loi S, Haibe-Kains B, Desmedt C, Lallemand F, Tutt AM, Gillet C, Ellis P, Harris A, Bergh J, Foekens JA, *et al*. Definition of clinically distinct molecular subtypes in estrogen receptor-positive breast carcinomas through genomic grade. *Journal of Clinical Oncology* 2007; 25: 1239-46.
71. Desmedt C, Giobbie-Hurder A, Neven P, Paridaens R, Christiaens MR, Smeets A, Lallemand F, Haibe-Kains B, Viale G, Gelber RD, *et al*. The gene expression grade index: a potential predictor of relapse for endocrine-treated breast cancer patients in the BIG 1-98 trial. *BMC Medical Genomics* 2009; 2: 40.
72. Ma XJ, Salunga R, Dahiya S, Wang W, Carney E, Durbecq V, Harris A, Goss P, Sotiriou C, Erlander M, *et al*. A five- gene molecular grade index and HOXB13:IL17BR are complementary prognostic factors in early stage breast cancer. *Clinical Cancer Research* 2008; 14: 2601-8.
73. Wang Y, Klijn JG, Zhang Y, Sieuwerts AM, Look MP, Yang F, Talantov D, Timmermans M, Meijer-van Gelder ME, Yu J. Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet* 2005; 365: 671-679.
74. Loi S, Haibe-Kains B, Desmedt C, Lallemand F, Tutt AM, Gillet C, Ellis P, Harris A, Bergh J, Foekens JA, *et al*. Definition of clinically distinct molecular subtypes in estrogen receptor-positive breast carcinomas through genomic grade. *Journal of Clinical Oncology*, 2007; 25: 1239-46.
75. Rouzier R, Pronzato P, Chereau E, Carlson J, Hunt B, Valentine WJ. Multigene assays and molecular markers in breast cancer: systematic review of health economic analyses. Breast cancer research and treatment. 2013; 139: 621-37.
76. Cosler LE, Lyman GH. Economic analysis of gene expression profile data to guide adjuvant treatment in women with early-stage breast cancer. *Cancer Investigation.* 2009; 27: 953-9.
77. Goldhirsch A, Ingle JN, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer 2009. *Annals of Oncology* 2009; 20: 1319-29.

MARKERI MOLECULARI ÎN CANCERUL MAMAR ŞI SEMNIFICAȚIA LOR CLINICĂ

REZUMAT

Managementul cancerului mamar a cunoscut îmbunătățiri incrementale în ultimele decenii, ca urmare a descoperirii de noi biomarkeri foarte utili în depistarea precoce, prognosticul și predicția răspunsului la terapie. Acest review își propune să evidențieze toți biomarkerii ce pot fi utilizați pentru a obține terapii individualizate în tratamentul cancerului de sân. Markeri moleculari consacrați, cum ar fi receptorul de estrogen și receptorul de progesteron, au jucat de mai mulți ani un rol important în selectarea pacienților ce pot beneficia de terapie endocrină. Mai recent, s-a conturat un nou panou de biomarkeri, dar studii suplimentare pe loturi mari de paciente și folosind metodologii consistente sunt necesare pentru a defini valoarea lor exactă în diagnosticul și prognosticul cancerului de sân și capacitatea lor predictivă. Luând în considerare dezvoltarea rapidă a acestor biomarkeri și eficacitatea terapiilor moleculare specifice, există o nevoie continuă pentru identificarea de noi markeri.

IN VITRO MORPHOLOGICAL CHARACTERIZATION OF ENDOTHELIAL CELLS DERIVED FROM BOVINE CORNEA

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ABSTRACT
The monolayer of cells called the corneal endothelium that lines the posterior corneal surface is derived from the neural crest during embryologic development.
In this paper we describe a simple procedure for growing bovine cornea1 endothelial cells (BCEC) in culture, followed by morphological and ultrastructural analysis of cells. Also, we present data regarding the laser confocal microscopic images of epithelium, stroma and endothelium of *ex vivo* bovine cornea. The cultured cells demonstrate the roughly hexagonal shape and close apposition to one another. That is a distinguishing characteristic of this endothelial type. There are small nits or depressions on surface caused bv cvtoplasmic processes of endothelial cell that extend into the membrane. The cells presented a uniform distribution of cytoskeletal fibers stained for Vimentin, which were present even in the cytoplasmic processes extending from one cell to the others.
The present study demonstrates the possibility of obtaining primary endothelial cell cultures, and shows an extensive characterization of the cells, with the possibility of using these cells in tissue-engineering strategies.
Key words: corneal endothelial cells, SEM, TEM, confocal microscopy

INTRODUCTION

The cornea1 endothelium is a neural crest-derived, simple squamous epithelium that covers the posterior surface of the cornea [l]. The endothelial layer maintains the clarity of the cornea by pumping salts and water out of the connective tissue stroma and into the anterior chamber of the eye [2]. Comeal endothelial cells also elaborate a thick basement membrane termed Descemet's membrane [3]. Descemet's membrane increases in thickness throughout life, as a result of the ongoing synthesis and deposition of membranous elements by the corneal endothelium [4]. The membrane possesses a distinctive internal substructure which consists of a series of hexagonally arranged nodes interconnected by fine filaments. This substructure is present to a variable extent in amphibians, birds, and mammals [5].

Successful primary outgrowths of cornea1 endothelium have been observed from explants of a Descemet's membrane-endothelial cell complex that had been manually dissected from the comeal stroma. Perlman et al. [10] were the first to describe the synthesis and deposition of a collagen-containing extracellular matrix by cultured (rabbit) cornea1 endothelial cells. Subsequent studies by Tseng et al. [11] and Gospodarowicz et al. [12, 13] using bovine cornea1 endothelial cells grown in the presence of an exogenously supplied mitogen, fibroblast growth factor (FGF), demonstrated that endothelial cells from this species also synthesize collagen types III, I, IV and V, fibronectin and laminin.

In this paper we describe a simple procedure for growing bovine cornea1 endothelial cells in culture, followed by morphological and ultrastructural analysis of cells. Also, we present data regarding the laser confocal microscopic images of epithelium, stroma and endothelium of *ex vivo* bovine cornea.

MATERIALS AND METHODS

Corneal endothelial cells harvest and culture

Adult bovine eyes were obtained from slaughtered cattle within 2-5 h after death. Adhering extraocular tissues were removed from the globes by dissection. The eyes were then vigorously rinsed in room temperature, running tap water and subsequently covered for 15 mm

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with-gauze wetted with an aqueous solution 2% Penicillin/Streptomycin mixture (Pen/Strep, 10,000 IU/ml; PromoCell, Heidelberg, Germany). The cornea was excised with an attached l-2 mm wide scleral ring and placed with the endothelial side up in a Petri dish. The endothelial layer was peeled off together with Descemet membrane and further submitted to enzymatic treatment using Collagenase I AS for 2 hours (Sigma-Aldrich Company, Ayrshire, UK). The next step in obtaining endothelial cells suspension was another enzymatic digestion with 0.25% Trypsin-EDTA solution (Sigma-Aldrich Company) for 5 minutes. The cells were then rinsed with PBS (Sigma-Aldrich Company), centrifuged 300 x g for 10 minutes and plated in fibronectin-coated culture flasks. Endothelial cell culture and expansion was performed using F99 culture medium (DMEM F12 + M199 2:1) supplemented with 5% fetal calf serum (FCS; PromoCell, Heidelberg, Germany), 20 µg / ml ascorbic acid, 1 x ITS, and 10 ng/ml FGF. All culture media and supplements were purchased from Sigma-Aldrich Company, unless otherwise specified.

Confocal microscopy of the bovine cornea

The entire eye balls of the bovine specimens were investigated *ex vivo* by laser corneal confocal microscope (Heidelberg Retina Tomograph-HRT3, Rostock Corneal Module) (Heidelberg, Germany).

Immunocytochemical/immunofluorescence analyses

Bovine corneal endothelial cells (BCEC) prepared for these analyses were fixed with methanol and investigated for expression of interest markers, employing antibodies for vimentin (clone V9) (DakoCytomation, Glostrup, Denmark). Vimentin expression was revealed by fluorescence method after coupling the primary antibody with specific fluorochrome-conjugated secondary antibody (AlexaFluor 488, Invitrogen™, Carlsbad, CA, USA).

Cells scanning electron microscopy (SEM)

Scanning electron microscopy was performed for identification of morphological features of bovine corneal endothelial cells. Cells were cultured at density of 7000 cells/cm^2 in 24-well format cell culture inserts (BD Labware Europe, Le Pont De Claix, France). 24 hours after plating, cells were pre-fixed for 1 hour with 2.5 % buffered glutaraldehyde (in PBS), rinsed three times in PBS, and the 0.4 µm pore-sized membranes were detached from the culture inserts. For better image quality, cells fixed on the membranes were sputter-coated with platinum-palladium and examined with a FEI Quanta 3D FEG electron microscope (FEI Company, Eindhoven, NL) generating digital electron micrographs.

Cells transmission electron microscopy (TEM)

Bovine corneal endothelial cells were employed for investigation of ultrastructural details. Cells were prefixed for 1 h with glutaraldehyde (2.5 % in PBS), rinsed three times in PBS, and postfixed for 1 h in osmium acid (2 % in PBS). Dehydration was done in graded acetone in distilled water dilutions, followed by infiltration with Epon resin. Sections of about 100 nm, obtained on a diamond knife (Diatome) with Leica UC6 ultramicrotome were post-stained with lead citrate and uranyl acetate. The grids were examined with a FEI Tecnai 12 transmission electron microscope (FEI Company).

RESULTS

Cultured bovine corneal endothelial cells, upon reaching confluence form a highly ordered monolayer that closely resembles the arrangement of the native endothelium. The cells demonstrate the roughly hexagonal shape (Figure 1).

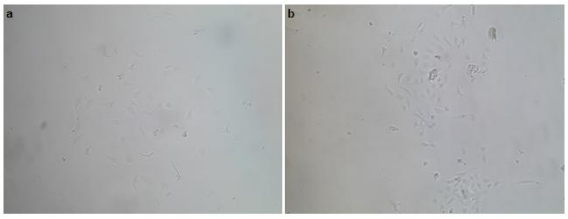

Fig. 1. Optic microscopy of BCEC. a. 5 days after harvesting and plating; **b.** 10 days in culture (Magnification 40x).

When immunocytochemically stained for Vimentin, BCEC revealed a uniform distribution of cytoskeletal fibers, which were present even in the cytoplasmic processes extending from one cell to the others. These cellular connections makes the endothelial cells to function together and send signals even at a larger distance (Figure 2).

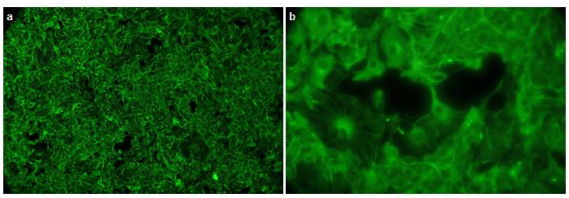

Fig. 2. Immunocytochemical staining for Vimentin of BCEC after 3 weeks in culture (Magnification 40x and 100x).

The Heidelberg Retina Tomograph (HRT) is a confocal scanning laser ophthalmoscope. The instrument can be converted into a confocal corneal microscope using an optional add-on kit. The kit is composed of an additional microscope lens which attaches to the standard lens. Along with corneal analysis software, the HRT is able to image cells and cell layers within the cornea.

To create an image, a beam of light scans the cornea, creating a 384 x 384 point image in a 400 micron square at a magnification of 63X. Because the system is confocal, it has two unique properties: the first is that the instrument can be focused at varying depths through the full thickness of the cornea, and second, stray light is blocked, enabling crisp, clear images not available from specular microscopes using white light sources.

Laser scanning with confocal imaging represents one of the most significant advances in ophthalmic imaging because it enables visualization deep within living tissue. Confocal imaging has several advantages over white light photography including the ability to image within tissue at sequential depths, capturing sharply defined optical sections. This is accomplished through a "pinhole" concept, which only allows light from the targeted focal plane to reach the sensor.

Layer-by-Layer Corneal Imaging: most anatomical layers and cell types may be viewed easily including epithelial cells (superficial, intermediate and basal), nerve plexi, stromal layers with keratocytes, Descemet's membrane, endothelial cells, and immune response cells (Figure 3).

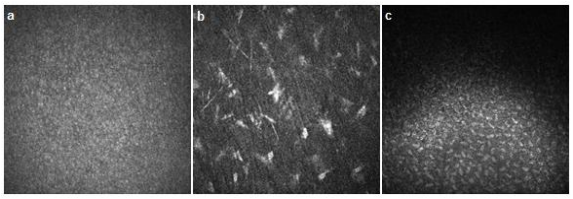

Fig. 3. Confocal microscopic corneal images. **a. 215 µm -** superficial epithelium; **b**. 841 µm - posterior stroma; **c. 1049 µm -** endothelium.

Corneal confocal microscopy is a novel clinical technique for the study of corneal cellular structure. It provides images which are comparable to in-vitro histochemical techniques delineating corneal epithelium, Bowman's layer, stroma, Descemet's membrane and the corneal endothelium. Because, corneal confocal microscopy is a non-invasive technique for *in vivo* imaging of the living cornea it has huge clinical potential to investigate numerous corneal diseases.

When viewed with the scanning electron microscope (SEM), the cultured cells demonstrate the roughly hexagonal shape and close apposition to one another. That is a distinguishing characteristic of this endothelial type. There are small nits or depressions on surface caused bv cvtoplasmic processes of endothelial cell that extend into the membrane. The smooth surfaced ridges occasionally deposited beneath or around individual cells or groups of cells (Figure 4).

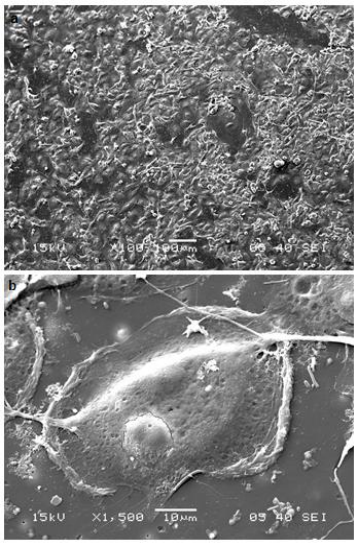

Fig. 4. SEM of bovine corneal endothelial cells (BCEC) 3 weeks after plating and culture.

Ultrastructurally, cultured endothelial cells possess an abundant complement of organelles required for the synthesis of secretory proteins and glycoproteins. There are numerous profiles of rough endoplasmic reticulum, the cistemae of which are filled with a moderately electron-opaque, granular material. An extensive juxtanuclear Golgi complex composed of multiple arrays of flattened lamellae and a wide variety of vesicles is also present. Occasional large vesicles filled with a granular or flocculent material together with segments of trilaminar membrane are also present in the cytoplasm. These latter structures are thought to be secondary lysosomes containing sequestered secretory products. Typical secondary lysosomes filled with membranous elements increase in number with prolonged culture. These structures could be the result of an intense vesicular traffic (Figure 5).

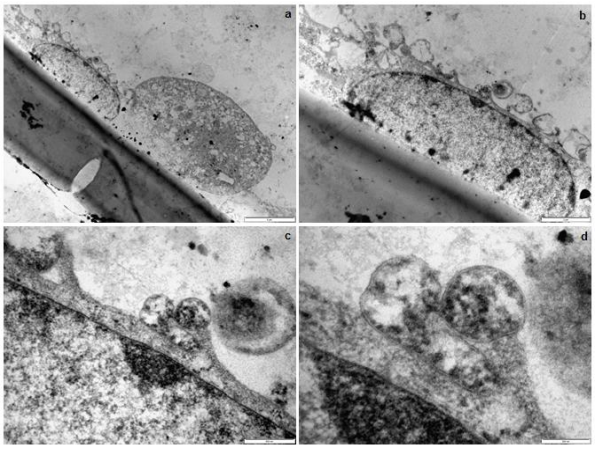

Fig. 5. TEM of bovine corneal endothelial cells (BCEC) 3 weeks after plating and culture.

DISCUSSION

Bovine corneal endothelial cells can be easily grown in culture using conventional techniques. The resiliency and thickness of Descemet's membrane in the bovine eye interposes an effective barrier between the endothelium and the underlying stromal fibroblasts (keratocytes) - a barrier that is not breached by gentle rubbing of the endothelial surface with a rubber spatula. Therefore, if the separating medium is kept away from the cut stromal surface, contamination of the cultures by stromal fibroblasts rarely occurs.

The monolayer of cells called the corneal endothelium that lines the posterior corneal surface is derived from the neural crest during embryologic development [14]. Human endothelial cell density is approximately 6000 cells/mm^2 during the first month of life [15] but decreases to about 3500 cells/mm2 by age 5 years [16]. Growth of the cornea accounts for some of this decrease in density, but a decrease in the number of cells also occurs.2 There is no evidence that human endothelial cells divide under normal circumstances, although they can be induced to divide in cultured corneas [17,18].

The cornea is an exquisite example of natural engineering; the requirement for a living, optically clear lens on the surface of the eye was solved by packing collagen and cells in an orderly lamellar arrangement without blood vessels. The crystalline organization and critical spacing of collagen fibrils makes this tissue optically transparent. Any accumulation of fluid would disrupt this spacing and degrade the transparency. The endothelium must serve two functions to maintain the health and clarity of the stroma: it must control hydration (maintain stromal deturgescence) and it must be permeable to nutrients and other molecules.

The present study demonstrates the possibility of obtaining primary endothelial cell cultures, as well as characterization of the BCEC, with the possibility of using these cells in tissue-engineering strategies.

ACKNOWLEDGEMENT

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REFERENCES

1. Johnson MC, Noden DM, Hazelton RD, Coulombre JL, Couiombre AJ. *Exp Eye Res* 1972; 29: 27.
2. Stocker FW. The endotheiium of the cornea and its clinical implications 2nd edn. Ch. C. Thomas, Springfield, Ill. (1971).
3. Hay ED, Revel JP. Fine structure of the developing avian cornea. S. Karger, New York (1969).
4. Cogan DG, Kuwabara T. *Trans Ophthalmo* 1971; 191: 875.
5. Jakus M. *J Cell Bio1*. 1956; 2: 243.
6. Matsui, J. *Arch Exp Zellforsch.* 1929; 8: 553.
7. Stocker FW, Eiring A, Georgiade R, Georgiade N. *Am J Ophthalmol*. 1958; 146: 294.
8. Newsome D, Takasugi M, Kenyon K, Stark W, Opelz G. *Invest Ophthalmol Vis Sci*. 1974; 13: 23.
9. Yanoff M. *Dot Ophthalmol.* 1976; 141: 157.
10. Perlman M, Baum J, Kaye G. *J Cell Biol.* 1974; 63: 306.
11. Tseng S, Savion N, Gospodarowicz D, Stern R. *J Biol Them.* 1981; 256: 3361.
12. Gospodarowicz D, Greenburg G, Vlodavsky I, Alvarado J, Johnson LK. *Exp Eye Res.* 1979; 29: 485.
13. Gospodarowicz D, Greenburg D, Foidart JM, Savion N. *J Cell Physiol.* 1981; 107: 171.
14. Johnson DH, Bourne WM, Campbell RJ. The ultrastructure of Descemet's membrane. I. Changes with age in normal corneas. *Arch Ophthalmol* 1982; 100: 1942-1947.
15. Bahn CF, Glassman RM, MacCallum DK, Lillie JH, Meyer RF, Robinson BJ et al. Postnatal development of corneal endothelium. *Invest Ophthalmol Vis Sci* 1986; 27: 44-51.
16. Nucci P, Brancato R, Mets MB, Shevell SK. Normal endothelial cell density range in childhood. *Arch Ophthalmol* 1990; 108: 247-248.
17. Senoo T, Joyce NC. Cell cycle kinetics in corneal endothelium from old and young donors. *Invest Ophthalmol Vis Sci* 2000; 41: 660-667.
18. Senoo T, Obara Y, Joyce NC. EDTA: a promoter of proliferation in human corneal endothelium. *Invest Ophthalmol Vis Sci* 2000; 41: 2930-2935.

CARACTERIZAREA MORFOLOGICĂ *IN VITRO* A CELULELOR ENDOTELIALE OBȚINUTE DIN CORNEEA BOVINĂ

REZUMAT

Celulele organizate în monstrat numite endoteliu cornean, care sunt localizate pe partea posterioară a suprafeței corneei sunt derivate din creasta neurală în timpul dezvoltării embrionare.

În acest articol descriem o procedură simplă de cultivare a celulelor endoteliale corneene bovine (BCEC), urmată de analiza morfologică și ultrastructurală a celulelor. De asemenea, sunt prezentate date referitoare la imaginile obținute prin tehnici de microscopie confocală *ex vivo* a corneei bovine, cu 3 dintre straturile corneene – epiteliu, stroma și endoteliu. Celulele obținute și crescute în condiții de cultură demonstrează forme hexagonale și apoziția celulelor, aceasta fiind o caracteristică a celulelor endoteliale. Celulele au prezentat o distribuție uniformă a fibrelor citoscheletice marcate pentru Vimentină, fiind prezente și la nivelul proceselor citoplasmatice care se extind de la o celulă la alta.

Studiul prezent demonstrează posibilitatea de a obține culturi celulare primare endoteliale și prezintă o caracterizare extensivă a celulelor obținute, cu posibilitatea de a utiliza aceste celule în strategii de inginerie tisulară

Cuvinte cheie: celule endoteliale corneene, SEM, TEM, microscopie confocală

ANTHROPOMETRIC BASIS FOR THE PHYSIOLOGICAL DEMAND OF ANAEROBIC POWER AND AGILITY IN YOUNG INDIAN NATIONAL LEVEL FIELD HOCKEY PLAYERS

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ABSTRACT

The study was conducted to assess selected anthropometric correlates & predictors of anaerobic power and agility variables in young Indian field hockey players. 13 female (16.000±2.160 years) & 18 male (15.000±1.815 years) trainees in NERC, SAI, Imphal were the volunteers. The variables taken for the study were: demographic and anthropometric [age, gender, height, body weight (BW), lean body mass (LBM), %body fat (%BF) and body mass index (BMI)]; anaerobic [Running-based Anaerobic Sprint Test (RAST) & vertical jump test (VJ) related power variables; bicycle ergometer based anaerobic test: maximum revolution per minute (RPMmax) and anaerobic power index (API)]; and agility [505 agility test timing]. The result showed that male players had better body composition, more anaerobic power, and were more agile. BW was the single major determinant of anaerobic power among all the anthropometric variables. LBM was the next major contributor. %BF & BMI were also found to have significant positive correlation with anaerobic power. However, it was found that those who had higher %BF & BMI also had higher LBM & BW. The power variables of RAST & VJ were positively correlated, indicating similar underlying energy systems, & were not affected by gender. API was found to be a better correlate of anaerobic power than RPMmax. The data also indicated that agility might improve with better body composition & more anaerobic power. The study could be used as a monitoring guide for field hockey training, and might also serve as a platform for future research.

Key words: Running-based Anaerobic Sprint Test, Maximum revolution per minute, Anaerobic power index, Vertical jump and 505 agility test.

INTRODUCTION

In field hockey, not only there is importance of physique and body composition [1, 2], but also are aerobic-anaerobic fitness [1, 3], power and strength [1, 4]. Agility and technical skills [5] are other major areas which the elite players need to concentrate.

As quick acceleration and deceleration [6], and repeated back-to-back sprints [6, 7] are essential parts of the game, a high anaerobic power is very much needed [8]. This makes sprint training regimens very beneficial to field hockey players [6, 7]. Running-based Anaerobic Sprint Test (RAST) is an anaerobic test for those athletes where running is the primary method of movement [9, 10]. Though not so sports specific in hockey [11], anaerobic capability is commonly assessed by bicycle based tests like Wingate Anaerobic Test (WANT) [12] and its varieties.

Strength training is an important training component of field hockey [6, 7]. Power output by a muscle is related to its strength. In field hockey, many activities are forceful and explosive, measuring strength and power of the concerned muscles will thus guide training programme. Strength training is therefore, beneficial, not only for explosive activities, but also for injury prevention [8, 13]. Vertical jump test (VJ) or Sargent Jump Test is a good test for knee and hip extensor muscles' strength [14]. Explosive or anaerobic power of the lower limbs and hips can be measured by vertical jump and jump tests [15].

The agility or the ability to rapidly change directions without losing balance and speed is another important factor for successful performance. 505 agility test [16] is good and reliable in assessing the ability of changing direction rapidly [17].

The present study tried to assess the selected anthropometric correlates & predictors of anaerobic

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power and agility test variables in young Indian national level female and male Manipuri field hockey players.

METHODOLOGY

Participants & Testing:

Sports Sciences & Fitness Centre, North-East Regional Centre (NERC), Sports Authority of India (SAI), Imphal conducted the study. The subjects who participated voluntarily in the study were thirteen female (mean age: 16.000±2.160years) & eighteen male (mean age: 15.000±1.815years) healthy Indian national level field Hockey players from Manipur, who were trainees in NERC, SAI, Imphal. The age of the subjects were taken from legal certificates submitted to the authority. A well informed and written consent was taken, after explaining the purpose and the procedures of every test with merit & a possible chance of injury. All the tests were done in the morning about the same time (±1hours). The players were instructed to come after a sound night sleep, and not to have any physical exertion before 12 hours of the test. They were not allowed to drink any caffeinated drinks or eat solid food 4 hours before the test. They were encouraged and motivated throughout to give their best. The study was approved by the Ethical Committee of the Institute.

Anthropometric variables measurement:

The heights (HT) of all the subjects were measured to nearest 0.1cm using a stadiometer (Seca220, UK). Weight (BW) and body composition variables were measured using TANITA Body Composition Analyzer (TBF310 Model, Japan). A patented foot to foot pressure contact electrode Bioelectrical Impedance Analysis technique is the basis of the instrument [18].

Anaerobic variables measurements:

RAST was conducted following the standard methodology [9, 10, 19]. PRmax (maximum power), PRmin (minimum power), PRavg (average power) were calculated as per the standard technique [9, 10, 19].

For the bicycle based anaerobic test, electronically operated computerized bicycle ergo-meter (Jaeger, LE900, Germany) was used. The subject remained seated with toe clips on the bicycle ergometer for the whole duration of the test. Warm up cycling for 5minutes at 60-70 rpm was done at a fixed power of 1 times the body weight in kg. After that the subject was instructed to pedal as fast as possible till exhaustion with constant verbal encouragement at a fixed power (5 times body weight in kg for females, 6 times body weight for males). Delay time (DT) was noted as the time taken in second to reach 100 rpm. The time duration during which 100 rpm was maintained, was noted as total time (TT) in second. Anaerobic power index (API) was calculated as TT by DT. The maximum rpm reached (RPMmax) was also noted.

Vertical jump test was done following the standard methodology [20]. Three trials were attempted with a minimum of 30sec between attempts. The best score was used to estimate PVpeak (peak power) and PVavg (average power) [21]. MPP (mean peak power) and MAP (mean average power) were calculated as arithmetic mean of PRmax & PVpeak, and PRavg & PVavg respectively.

Agility variables measurements:

505 agility test was conducted as per the standard methodology [16, 22]. The minimum timing each for left (Tl) and right (Tr) foot was recorded after three attempts for each foot.

Statistical Analysis

SPSS (Statistical Package for Social Science) version 20 software was used for data analysis. Standard descriptive statistics were determined. One way ANOVA was used for comparison between female and male players. In case, there was significant violation of homogeneity of variances, Welch's test was applied instead of one way ANOVA. To study relationships among variables measured, Pearson's correlation coefficients (zero-order) were measured. Partial correlation was used to study effect of gender & other interested variables on the associations among various variables. Various regression models were generated for predicting MPP & MAP using linear regression analysis. Durbin-Watson statistics were used to detect serial correlation. Hierarchical multiple regression analysis (including semi-partial correlation R^2 calculation) was done to assess the effect of a particular independent variable above & beyond others on predicting MPP & MAP. In each case, statistical significance was chosen at α value of $\leq 5\%$.

RESULTS AND DISCUSSION

Among the anthropometric parameters, both the female and male players were comparable in case of BW, BMI (body mass index) & age (Table I). The male players had significantly higher HT, LBM (lean body mass) & lower %BF (%body fat) as compared to their female counterparts (Table I). As both the groups were given similar training of field hockey for similar duration (DOT) (Table I), and had similar dietary and physical activity habits, the difference in their body composition parameters were due to gender specific difference in their physiology.

Table I. Comparison of various parameters between the female and male players

Parameters	Females (n=13) Mean±SD	Males (n=18) Mean±SD	F value (df=1,29)	p-value (2-tailed)
Age (years)	16.000±2.160	15.000±1.815	1.954	.173
DOT (years)	3.308±1.535	4.056±1.381	2.017	.166
HT (cm)	155.138±5.320	162.928±6.661	12.141	.002**
BW (kg)	51.169±7.687	53.528±7.286	.756	.392
LBM (kg)	38.154±3.818	43.367±4.628	11.034	.002**
%BF (%)	24.923±3.909	18.472±5.181	14.241	.001**
BMI (kg/m^2)	21.138±1.820	20.072±1.758	2.697	.111
PRmax (W)	329.923±73.526	514.889±116.439	25.356	.000**
PRmin (W)	205.000±39.147	332.000±90.078	28.363!	.000**
PRavg (W)	264.769±55.682	425.500±94.686	29.824	.000**
RPMmax.	126.846±11.172	141.167±16.982	7.014	.013*
API	2.723±.821	3.290±1.454	1.602	.216
VJ (cm)	34.385±3.501	41.556±4.617	22.092	.000**
PVpeak (W)	2106.615±559.729	2693.278±620.027	7.318	.011*
PVavg (W)	1003.923±270.615	1264.389±297.156	6.240	.018*
MPP (W)	1218.269±313.305	1604.083±359.918	9.640	.004**
MAP (W)	634.346±159.399	844.944±188.648	10.670	.003**
Tl (sec)	3.162±.176	3.050±.092	5.280	.029*
Tr (sec)	3.092±.119	2.911±.083	25.041	.000**

*p -value≤0.05: significant;** p-value≤0.01: highly significant (One way ANOVA).!Welch statistic at df (1, 24.664).

PRmax, PRmin, PRavg, PVpeak, PVavg, MPP & MAP were significantly higher among the male players, indicating more anaerobic power (Table I). The male players had higher lower limb explosive power as they had higher vertical jump scores, its associated power variables & also more RPMmax (Table I). There was significant positive correlation between anaerobic power variables (PRmax, PRmin, PRavg, PVpeak, PVavg, MPP & MAP) and VJ with BW, LBM and HT in both the groups (Table II, III & IV, except for VJ among the female players, Table II). The relationships remain still significant when the effect of gender was controlled for (Table V & VI), and when controlling was done for %BF & BMI in addition to gender (Table VI. 1 & 2 (g)).

Table II. Zero-order correlation among various variables of female players (n=13)

Variables	Significantly correlated with (R-value)		
	Anthropometric-demographic	Anaerobic	Agility
PRmax	HT (.745**), BW (.818**), LBM (.756**), %BF (.836**) & BMI (.818**)	PRmin (.840**), PRavg (.955**), VJ (.670*), PVpeak (.898**) & PVavg (.894**)	–
PRmin	HT (.782**), BW(.818**), LBM (.834**), %BF (.702**) & BMI (.802**)	PRmax (.840**), PRavg (.932**), RPMmax (.608*), PVpeak (.808**) & PVavg (.783**)	–
PRavg	HT (.775**), BW (.851**), LBM (.816**), %BF (.811**) & BMI (.849**)	RPMmax (.564*), PRmax (.955**), PRmin (.932**), PVpeak (.856**) & PVavg (.839**)	–
RPMmax.	–	PRmin (.608*) & PRavg (.564*)	Tr (-.579*)
API	LBM (.575*)	–	–
VJ	–	PRmax (.670*), PVpeak (.775**) & PVavg (.820**)	–
PVpeak	HT (.831**), BW (.893**), LBM (.830**), %BF (.899**) & BMI (.878**)	PRmax (.898**), PRmin (.808**), PRavg (.856**), VJ (.775**) & PVavg (.995**)	–
PVavg	HT (.772**), BW (.850**), LBM (.777**), %BF (.879**) & BMI (.849**)	PRmax (.894**), PRmin (.783**), PRavg(.839**), VJ (.820**) & PVpeak (.995**)	–
MPP	HT (.830**), BW (.894**), LBM (.831**), %BF (.902**) & BMI (.880**)	–	–
MAP	HT (.790**), BW (.870**), LBM (.802**), %BF (.888**) & BMI (.869**)	–	–
Tl	–	–	–
Tr	–	RPMmax (-.579*)	–

*p -value≤0.05: significant;** p-value≤0.01: highly significant (Pearson's Correlation). Positive correlations of anaerobic power variables with MPP & MAP, and among MPP, MAP & VJ not shown.

Table III. Zero-order correlation among various variables of male players (n=18)

Variables	Significantly correlated with (R-value)		
	Anthropometric-demographic	Anaerobic	Agility
PRmax	Age (.585*), HT (.704**), BW (.868**), LBM (.767**), %BF (.668**) & BMI (.713**)	PRmin (.849**), PRavg (.982**), VJ(.570*), PVpeak (.832**) & PVavg (.818**)	–
PRmin	HT (.679**), BW (.761**), LBM (.659**), %BF (.593**) & BMI (.572*)	PRmax (.849**), PRavg (.898**), VJ (.513*), PVpeak (.728**) & PVavg (.703**)	–
PRavg	Age (.545*), HT (.715**), BW (.864**), LBM (.732**), %BF (.718**) & BMI (.707**)	PRmax (.982**), PRmin (.898**), VJ (.555*), PVpeak (.820**) & PVavg (.801**)	–
RPMmax.	–	API (.512*)	–
API	–	RPMmax (.512*)	–
VJ	HT (.681**), BW(.539*) & LBM (.545*)	PRmax (.570*), PRmin (.513*), PRavg (.555*), PVpeak (.855**) & PVavg (.857**)	–
PVpeak	Age (.652**), HT (.806**), BW (.892**), LBM (.829**), %BF (.607**) & BMI (.641**)	PRmax (.832**), PRmin (.728**), PRavg (.820**), VJ (.855**) & PVavg (.994**)	–
PVavg	Age (.669**), HT (.734**), BW (.865**), LBM (.794**), %BF (.605**) & BMI (.665**)	PRmax (.818**), PRmin (.703**), PRavg (.801**), VJ (.857**) & PVpeak (.994**)	–
MPP	Age (.656**), HT(.808**), BW(.908**), LBM (.838**), %BF (.631**) & BMI (.668**)	–	–
MAP	Age (.664**), HT (.757**), BW (.898**), LBM (.809**), %BF (.656**) & BMI (.701**)	–	–
Tl	–	–	–
Tr	–	–	–

*p -value≤0.05: significant;** p-value≤0.01: highly significant (Pearson's Correlation). Positive correlations of anaerobic power variables with MPP & MAP, and among MPP, MAP & VJ not shown.

Table IV. Zero-order correlation among various variables of both female & male players as a whole (n=31)

Variables	Significantly correlated with (R-value)		
	Anthropometric-demographic	Anaerobic	Agility
PRmax	HT (.807**), BW (.706**) & LBM (.830**)	PRmin (.912**), PRavg (.987**), RPMmax (.500**), API (.372*), VJ (.775**), PVpeak (.855**) & PVavg (.836**)	TI(-.364*)& Tr(-.463**)
PRmin	HT (.787**), BW (.639**) & LBM (.776**)	PRmax (.912**), PRavg (.944**), RPMmax (.450*), API (.367*), VJ (.716**), PVpeak (.774**) & PVavg (.746**)	TI(-.444*)& Tr(-.471**)
PRavg	HT (.813**), BW (.689**) & LBM (.819**)	PRmax (.987**), PRmin (.944**), RPMmax (.484**), VJ (.758**), PVpeak (.831**) & PVavg (.807**)	TI(-.420*)& Tr(-.482**)
RPMmax.	LBM (.388*)	PRmax (.500**), PRmin (.450*), PRavg (.484**), API (.497**), VJ (.419*), PVpeak (.422*), PVavg (.426*), MPP (.445*) & MAP (.459**)	Tr(-.499**)
API	HT (.418*), BW (.423*) & LBM (.478**)	PRmax (.372*), PRmin (.367*), RPMmax (.497**), VJ (.406*), PVpeak (.485**), PVavg (.481**), MPP (.475**) & MAP (.461**).	–
VJ	HT (.733**), BW (.467**) & LBM (.652**)	PRmax (.775**), PRmin (.716**), PRavg (.758**), RPMmax (.419*), API (.406*), PVpeak (.852**) & PVavg (.852**)	Tr (-.547**)
PVpeak	HT (.854**), BW (.856**), LBM (.866**) & BMI (.496**)	PRmax (.855**), PRmin (.774**), PRavg (.831**), RPMmax (.422*), API (.485**), VJ (.852**) & PVavg (.995**)	Tr (-.407*)
PVavg	HT (.796**), BW(.834**), LBM (.829**) & BMI (.517**)	PRmax (.836**), PRmin (.746**), PRavg (.807**), RPMmax (.426*), API (.481**), VJ (.852**) & PVpeak (.995**)	Tr (-.397*)
MPP	HT (.864**), BW (.848**), LBM (.878**) & BMI (.474**)	RPMmax (.445*) & API (.475**)	TI (-.363*)& Tr (-.426*)
MAP	HT (.833**), BW (.827**), LBM (.859**) & BMI (.472**)	RPMmax (.459**) & API (.461**)	TI (-.380*)& Tr (-.436*)
TI	LBM (-.394*)	PRmax (-.364*), PRmin (-.444*), PRavg (-.420*), MPP(-.363*) & MAP (-.380*)	Tr (.558**)
Tr	HT (-.413*), LBM (-.395*) & %BF (.361*)	PRmax (-.463**), PRmin (-.471**), PRavg(-.482**), RPMmax (-.499**), VJ (-.547**), PVpeak (-.407*), PVavg (-.397*), MPP (-.426*) & MAP (-.436*)	TI (.558**)

*p -value≤0.05: significant;** p-value≤0.01: highly significant (Pearson's Correlation).
Positive correlations of anaerobic power variables with MPP & MAP, and among MPP, MAP & VJ not shown.

The combined effect of these anthropometric variables in the form of M, where M= (BW+LBM+HT)/3, uniquely accounted for 16.16% & 11.97% of the total variability in MPP & MAP respectively, which were statistically significant, after controlling for any overlapped or shared variances accounted for by other statistically significant, zero-order correlated predictors & gender (Table VII & VIII model 1). To assess the unique contributions of BW, LBM & HT on MPP & MAP, hierarchical multiple regression analysis was done. BW & LBM uniquely accounted for 11.49% & 5.48% of the total variance in MPP, and 13.84% & 6.25% of the total variance in MAP respectively over & beyond those explained by HT & gender, which were statistically significant (Table VII & 8 model 3 (b) & (c)). Whereas BW contributed a statistically significant unique variance of 8.29% & 9.3% out of the total variance in MPP & MAP respectively, after controlling the overlapping effect of LBM & gender (Table VII & VIII model 4 (ii)). The relative importance of BW over & above HT & LBM also became

clear from Table VI. 1 & 2 (d). Apart from the above variables, when BW & gender were controlled, there no longer was any significant correlation of MPP & MAP with any other variables (Table VI. 1 & 2 (b)). Therefore, the extent of the unique contributions of the above anthropometric variables on MPP & MAP in decreasing order was: BM>LBM>HT.

Table V. Partial correlation among various variables of both female & male players as a whole (n=31)

Sl. No.	Variables	Covariate(s) (controlling for)	Significantly correlated with (R-value)
1.	PRmax	Gender	Age (.407*),HT(.711**), BW (.828**), LBM (.758**), %BF (.708**), BMI (.727**), PRmin (.839**), PRavg (.976**), VJ (.593**), PVpeak (.840**) & PVavg (.828**)
2.	PRmin	Gender	HT (.679**), BW (.721**), LBM (.672**), %BF (.599**), BMI (.586**), PRmax (.839**), VJ (.497**), PVpeak (.712**) & PVavg (.688**)
3.	PRavg	Gender	Age (.439*), HT(.723**), BW(.830**), LBM (.745**), %BF (.736**), BMI (.725**), PRmax (.976**), PRmin (.898**), VJ (.547**), PVpeak (.815**) & PVavg (.796**)
4.	RPMmax	Gender	API (.453*)
5.	API	Gender	BW (.402*), BM (.432*), RPMmax (.453*), PVpeak (439*), PVavg (.435*), MPP (.428*) & MAP (.411*)
6.	VJ	Gender	HT (.594**), BW (.487**), LBM (.478**), PRmax (.593**), PRmin (.497**), PRavg (.547**), PVpeak (.827**) & PVavg (.842**)
7.	PVpeak	Gender	Age (.394*), HT (.813**), BW (.890**), LBM (.829**), %BF (.700**), BMI (.734**), PRmax (.840**), PRmin (.712**), PRavg (.815**), API (.439*), VJ (.827**) & PVavg (.994**)
8.	PVavg	Gender	Age (.387*), HT (.745**), BW (.857**), LBM (.788**), %BF (.691**), BMI (.737**), PRmax (.828**), PRmin (.688**), PRavg (.796**), API (.435*), VJ (.842**) & PVpeak (.994**)
9.	TI	Gender	Tr (.432*)
10.	Tr	Gender	TI .432*

*p -value≤0.05: significant;** p-value≤0.01: highly significant (Partial Correlation). Positive correlations of anaerobic power variables with MPP & MAP, and among MPP, MAP & VJ not shown.

Table VI. Partial correlation of MPP & MAP with various variables of both female & male players as a whole (n=31)

Sl. No.	Variables	Covariate(s) (controlling for)		Significantly correlated with (R-value)
1	MPP	(a)	Gender	Age (.404*), HT (.815**), BW (.899**), LBM (.835**), %BF (.715**), BMI (.748**) & API (.428*)
		(b)	Gender & BW	Nil
		(c)	Gender & LBM	BW (.603**) & %BF (.612**)
		(d)	Gender, HT&LBM	BW (.553**), %BF (.566**) & BMI (.517**)
		(e)	Gender&%BF	Age (.402*), HT (.710**), BW (.779**), LBM (.783**), BMI (.416*) & API (.416*)
		(f)	Gender & BMI	HT (.750**), BW (.770**) & LBM (.617**)
		(g)	Gender, %BF&BMI	HT (.727**), BW (.750**) & LBM (.753**)
		(h)	Gender, LBM & %BF	Nil
2	MAP	(a)	Gender	Age (.413*), HT (.768**), BW (.882**), LBM (.807**), %BF (.727**), BMI (.762**) & API (.411*)
		(b)	Gender & BW	Nil
		(c)	Gender & LBM	BW (.605**), %BF (.622**) & BMI (.382*)
		(d)	Gender, HT & LBM	BW (.570**), %BF (.590**) & BMI (.537**)
		(e)	Gender & %BF	Age (.420*), HT (.627**), BW (.731**), LBM (.739**), BMI (.434**) & API (.396*)
		(f)	Gender & BMI	HT (.670*), BW (.691**) & LBM (.539**)
		(g)	Gender, %BF & BMI	HT (.638**), BW (.664**) & LBM (.674**)
		(h)	Gender, LBM & %BF	Nil

*p -value≤0.05: significant;** p-value≤0.01: highly significant (Partial Correlation). Positive correlations of anaerobic power variables with MPP & MAP, and among MPP, MAP & VJ not shown.

Table VII. Regression models for predicting MPP

Sl. No.		Regression equations [Durbin-Watson statistics]	Significant predictors (standardized ß-weight; p-value)	Adjusted R^2 (Semi-Partial correlation R^2 for significant predictors)	R^2Change (p-value for F change)	F value (df)	p-value (2-tailed)
1.		MPP=-2768.707+45.204 ($M^!$)-133.481 ($G^\#$)+ 27.250 (BMI)-179.715(Tr)+1.334 (RPMmax)+ 10.539 (API)+69.177 (TI) [2.429]	$M^!$ (.721;<.001**)	82% (16.16%)	–	20.553 (7,23)	<.001**
2.		MPP=-3006.917+53.240 ($M^!$)-113.212 ($G^\#$) [2.067]	$M^!$ (.849;<.001**)	83.6% (59.75%,)	–	77.233 (2,28)	<.001**
3.	(a)	^MPP=-5774.963+45.290 (HT)-33.034 ($G^\#$) [1.863]	HT (.841;<.001**)	73% (49.84%)	–	41.494 (2,28)	<.001**
	(b)	^MPP=-1859.340+34.106 (BW)-227.073 ($G^\#$)+10.052 (HT) [2.416]	BW & $G^\#$ (.654,-.294; <.001**,.007**)	84.7% (11.49%, 4.37%)	11.5% (<.001**) [from 3(a)]	56.377 (3,27)	<.001**
	(c)	^MPP=-3577.579+40.705 (LBM)+20.969 (HT)-10.294 ($G^\#$) [1.798]	LBM (.523;.011*)	78% (5.48%)	5.5% (.011*) [from 3(a)]	36.540 (3,27)	<.001**
4.	(i)	^^MPP=-1263.679+66.128 (LBM)-41.099 ($G^\#$) [1.994]	LBM (.850;<.001**)	75.7% (52.42%)	–	47.658 (2,28)	<.001**
	(ii)	^^MPP=-626.057+40.168 (BW)-281.454 ($G^\#$)+1.846 (LBM) [2.490]	BW&$G^\#$ (.770,-.364; .001**,.004**)	84% (8.29%, 5.2%)	8.3% (.001**) [from 4(i)]	53.343 (3,27)	<.001**

*p -value≤0.05: significant;** p-value≤0.01: highly significant. G# (Gender) = 0 for male player &1 for female player. $M^!$ = (HT+BW+LBM)/3. ^&^^Hierarchical Multiple Regression Analysis. HT, BW &LBM couldn't be used in one equation due to multicollinearity.

Table VIII. Regression models for predicting MAP

Sl. No.		Regression equations [Durbin-Watson statistics]	Significant predictors (standardized ß-weight; p-value)	Adjusted R^2 (Semi-Partial correlation R^2 for significant predictors)	R^2Change (p-value for F change)	F value (df)	p-value (2-tailed)
1.		MAP=-1338.234+20.458 ($M^!$)-103.070 ($G^\#$)+22.227 (BMI)+.892 (RPMmax)-67.235 (Tr)+4.085 (API)+7.096 (TI) [2.434]	$M^!$ (.621,<.001**)	78.7% (11.97%)	–	16.874 (7,23)	<.001**
2.		MAP=-1463.908+26.659 ($M^!$)-74.099 ($G^\#$) [1.935]	$M^!$ (.809;<.001**)	79.7% (54.17%)	–	59.935 (2,28)	<.001**
3.	(a)	^MAP=-2762.548+22.142 (HT)-38.130 ($G^\#$) [1.795]	HT (.782;<.001**)	67.8% (43.03%)	–	32.644 (2,28)	<.001**
	(b)	^MAP=-498.495+19.720 (BW)-150.325 ($G^\#$)+1.767 (HT) [2.386]	BW & $G^\#$ (.719,-.370; <.001**,.002**)	82.1% (13.84%, 6.97%)	13.9% (<.001**) [from 3(a)]	46.731 (3,27)	<.001**
	(c)	^MAP=-1527.464+22.879 (LBM)+8.471 (HT)-25.348 ($G^\#$) [1.747]	LBM (.559;.013*)	73.6% (6.25%)	6.2% (.013*) [from 3(a)]	28.868 (3,27)	<.001**
4.	(i)	^^MAP=-592.657+33.150 (LBM)-37.794 ($G^\#$) [1.916]	LBM (.811;<.001**)	72.7% (47.61%)	–	40.891 (2,28)	<.001**
	(ii)	^^MAP=-236.664+22.426 (BW)-171.987 ($G^\#$)-2.740 (LBM) [2.477]	BW & $G^\#$ (.817,-.424; .001**,.002**)	82% (9.3%, 7.02%)	9.3% (.001**) [from 4(i)]	46.631 (3,27)	<.001**

*p -value≤0.05: significant;** p-value≤0.01: highly significant. G# (Gender)=0 for male player &1 for female player. $M^!$ = (HT+BW+LBM)/3. ^&^^Hierarchical Multiple Regression Analysis. HT, BW & LBM couldn't be used in one equation due to multicollinearity.

It was found that gender controlled BW had significant positive correlation with MPP & MAP after additionally controlling for either %BF & %BF+BMI, or

LBM (Table VI. 1 & 2 (e), (g) & (c)). But the significant positive correlation disappeared when both %BF & LBM were controlled (Table VI. 1 & 2 (h)). This may due to that fact that BW has both fat component (%BF) & LBM component, & both the components might have contributed uniquely to the overall gender controlled significant positive correlations of BW with MPP & MAP seen in our result. Since the gender controlled positive correlations of LBM with MPP & MAP were stronger than those of %BF (larger R values, Table VI. 1 & 2 (a)), the contribution of LBM might be more.

The positive correlation of anaerobic power with BW has been reported earlier [21, 23, 24]. This is understandable since BW is one of the components of anaerobic power computation [21, 25]. Also, in repeated sprints, the player with more BW requires to produce relatively more power to reach a fixed distance in same duration, producing same average acceleration or velocity. To jump same vertical height with more BW also, more power is needed [21]. However, an earlier study reported that increase in BW due to increase in LBM alone didn't adversely affect anaerobic power, whereas those associated with increase body fat might decrease anaerobic power [23]. Although the study was done on cycling, a non weight bearing exercise. In our study, the anaerobic power output analyzed was based on weight bearing exercise.

The contribution by LBM component is understandable since LBM reflects muscle mass, where ATP, phosphocreatine (PC) & glycogen is reserved for quick energy supply. Earlier study also reported performance dependent strength of correlation between lean body mass and anaerobic power [26]. Besides gender difference in muscle mass, relatively more type II muscle fibers, & hence more ATP, PC reserves plus more activity of ATP enzymes, creatine phosphokinase, phosphorylase and M subunit of lactate dehydrogenase & phosphofructokinase in males [27] might be responsible for gender difference in anaerobic power (Table I).

The reason behind the discrepancy of significant zero-order positive correlations of anaerobic power variables including MPP & MAP with %BF in female data, male data, and significant partial correlation controlled for gender as found in our study (Table II, III, V & VI) was not fully clear. However, when the combined data was analyzed, %BF was found not to be a significant correlate of anaerobic power variables including MPP & MAP (Table IV). Some earlier studies also reported more anaerobic power output by obese subjects [28]. Body fat is an inert extra load which needs to be carried while running or jumping vertically, hence higher power output may be needed for rapid movement [28]. Also, among the studied players, those with higher %BF also had more LBM & BW (LBM: zero-order correlation R=.840** in female, R=.365 in male & R=.054 in combined data, and partial correlation R, controlling for gender, =.509**; BW: zero-order correlation R=.937** in female, R=.722** in male & R=.547** in combined data, and partial correlation R, controlling for gender, =.789**, not given in Tables). Earlier study also reported a positive & linear relationship between fat mass and fat-free mass over the range of body fat extending from 10 to 90 kg [29].

Just like %BF, BMI also had significant positive correlation with anaerobic power variables including MPP & MAP in our study (Table II, III, IV, V, VI). The positive correlation of BMI with anaerobic power was reported earlier, although the study was done on obese adult population [30]. BMI presents both LBM & fat mass, hence it should be used with much caution among sports populations as a surrogate for %BF for indicating or classifying fatness or obesity [31]. A player with high LBM & low %BF may have high BMI, & may be even falsely classified as being obese [31, 32]. Among the studied players, there were significant positive correlations of BMI with LBM & BW (LBM: zero-order R=.938** in female, R=.665** in male & R=.468** in combined data, and partial correlation R, controlling for gender, =.763**; BW: zero-order correlation R=.975** in female, R=.824** in male & R=.794**in combined data, and partial correlation R, controlling for gender=.890**, not given in Tables). The gender controlled significant positive correlations of BMI with MPP & MAP were still maintained after additionally controlling for %BF (Table VI. 1 & 2 (e)), but not after LBM (Table VI. 1 & 2 (c)). Also, after additionally controlling for BMI, the gender controlled significant positive correlations of %BF with MPP & MAP were lost (Table VI. 1 & 2 (f)). Hence, the significant positive correlations of BMI with MPP & MAP could be contributed by two components, one associated with LBM & other with %BF, & out of these, the major share was by that associated with LBM.

Generalization of the above finding of significant positive correlations of %BF & BMI with anaerobic power variables, including MPP & MAP should be avoided, as the finding was valid only for the present studied group which was selected non randomly & didn't represent the population of adolescent hockey players. Besides, many earlier studies reported inverse relation of vertical jump & anaerobic power with %BF (33), and inverse or inverted J shaped relationship with BMI [33, 34].

The anaerobic power variables of both RAST & VJ had highly significant positive zero-order & partial correlation with one another (Table II, III & IV) indicating both involved similar energy systems & mechanism, which were also not affected by gender (Table V).

RPMmax was shown to have significant positive correlation with PRmin (in females), PRavg (in females) and API (in males) (Table II & III). However, when both the groups were considered as whole, RPMmax was found to have significant positive correlation with LBM,

anaerobic power variables, API, VJ, MPP & MAP (Table IV). The relationships became non significant when the effect of gender was controlled (Table V & VI). Thus RPMmax could only give a rough idea of anaerobic power of a player.

API did not differ significantly between the two groups (Table I). The low API values in both the groups indicated that there was need for improvement of speed endurance. API was found to have significant positive correlation with LBM (in females) and RPMmax (in males) (Table II & III). When the two groups were considered as a whole, there was significant positive correlation of API with HT, BW, LBM, anaerobic power variables (except PRavg), RPMmax, VJ, MPP & MAP (Table IV). Even after controlling for gender, API continued to maintain the above significant positive relationships (except for HT, RAST power variables & VJ) (Table V & VI), showing clearly that API could be used for rough monitoring of anaerobic power of a player, better than RPMmax.

Male players had significantly lower time scores for both the feet in 505 agility test, indicating they were more agile than their female counterparts (Table I). There was significant negative correlation between Tr and RPMmax in female players (Table II). However, when the two groups were considered as a whole, there were significant negative correlations of both Tl & Tr with LBM & RAST power variables; Tr with HT, RPMmax, VJ & it's power variables (Table IV). Tr had significant positive correlation with %BF (Table IV). Hence, the agility seems to improve with improving body composition & anaerobic power. However, the effect of gender on agility was very profound & when its effect was controlled, all the above relationships of agility became non-significant (Table V).

CONCLUSION

The male players were found to have better body composition; more anaerobic power, RPM max and API. They were also more agile than their female counterparts. Out of all the anthropometric variables measured, BW was the single major determinant of anaerobic power. LBM was the next major contributor. %BF & BMI were also found to have significant positive correlation with anaerobic power. However, it was found that those who had higher %BF & BMI also had higher LBM & BW. The power variables of RAST & VJ were positively correlated, indicating similar underlying energy systems, & were not affected by gender. API was found to be a better indicator of anaerobic power than RPMmax. Our result indicated that agility might improve with better body composition & anaerobic power. The study could not only be used as a monitoring guide for training & fitness of the players, but also might serve as a platform for future research in the related area.

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REFERENCES

1. Montgomery DL. Physiological profile of professional hockey players - a longitudinal comparison. *Appl Physiol Nutr Metab*. 2006; 31(3): 181-5.
2. Quinney HA, Dewart R, Game A, Snydmiller G, Warburton D, Bell G. A 26 year physiological description of a National Hockey League team. *Appl Physiol Nutr Metab*. 2008; 33(4): 753-60.
3. Elferink-Gemser MT, Visscher C, van Duijn MA, Lemmink KA. Development of the interval endurance capacity in elite and sub-elite youth field hockey players. *Br J Sports Med.* 2006; 40(4): 340-5.
4. Bloomfield J, Polman R, O'Donoghue P, McNaughton L. Effective speed and agility conditioning methodology for random intermittent dynamic type sports. *J Strength Cond Res.* 2007; 21(4): 1093-100.
5. Lemmink KA, Elferink-Gemser MT, Visscher C. Evaluation of the reliability of two field hockey specific sprint and dribble tests in young field hockey players. *Br J Sports Med.* 2004; 38(2): 138-42.
6. Spencer M, Bishop D, Lawrence S. Longitudinal assessment of the effects of field-hockey training on repeated sprint ability. *J Sci Med Sport.* 2004; 7(3): 323-34.
7. Ebben WP, Carroll RM, Simenz CJ. Strength and conditioning practices of National Hockey League strength and conditioning coaches. *J Strength Cond Res.* 2004; 18(4): 889-97.
8. Reilly T, Borrie A. Physiology applied to field hockey. *Sports Med.* 1992; 14(1): 10-26.
9. Adamczyk JG. The estimation of the RAST test usefulness in monitoring the anaerobic capacity of sprinters in athletics.The usefulness of the RAST test in athletics. *Polish Journal of Sport and Tourism.* 2011; 18: 214-23.
10. Draper N, Whyte G. Here's a new running based test of anaerobic performance for which you need only a stopwatch and a calculator. Peak performance 1997; 97.
11. Abbasian S, Golzar S, Onvani V, Sargazi L. The predict of RAST test from WANT test in elite athletes. *Research Journal of Recent Sciences.* 2012; 1(3): 72-5.
12. Zupan MF, Arata AW, Dawson LH, Wile AL, Payn TL, Hannon ME. Wingate Anaerobic Test peak power and anaerobic capacity classifications for men and women intercollegiate athletes. *J Strength Cond Res.* 2009 ; 23(9): 2598-604.
13. Gröger A, Oettl G, Tusker F. Anthropometry and muscle force measurement of German male national junior hockey players. Sportverletzung Sportschaden: Organ der Gesellschaft fur Orthopadisch-Traumatologische *Sportmedizin*. 2001; 15(4): 87-91.

14. Tambalis KD, Panagiotakos DB, Arnaoutis G, Sidossis LS. Endurance, explosive power, and muscle strength in relation to body mass index and physical fitness in greek children aged 7-10 years. *Pediatr Exerc Sci.* 2013; 25(3): 394-406.
15. Ostojic SM, Stojanovic M, Ahmetovic Z. Vertical jump as a tool in assessment of muscular power and anaerobic performance. *Med Pregl.* 2010; 63(5-6): 371-5.
16. Draper JA, Lancaster MG. The 505 test: A test for agility in the horizontal plane. *Aust J Sci Med Sport.* 1985; 17(1): 8-15.
17. Tarter BC, Kirisci L, Tarter RE, Weatherbee S, Jamnik V, McGuire E, *et al*. Use of aggregate fitness indicators to predict transition into the National Hockey League. *The Journal of Strength & Conditioning Research.* 2009; 23(6): 1828-32.
18. Nunez C, Gallagher D, Visser M, Pi-Sunyer FX, Wang Z, Heymsfield SB. Bioimpedance analysis: evaluation of leg-to-leg system based on pressure contact footpad electrodes. *Med Sci Sports Exerc.* 1997; 29(4): 524-31.
19. Zacharogiannis E, Paradisis G, Tziortzis S. An evaluation of tests of anaerobic power and capacity. *Medicine & Science in Sports & Exercise*. 2004; 36(5): S116.
20. Sargent DA. The Physical Test of a Man. *American Physical Education Review.* 1921; 26: 188-94.
21. Johnson DL, Bahamonde R. Power output estimate in university athletes. *The Journal of Strength & Conditioning Research.* 1996; 10(3): 161-6.
22. Tarter BC, Kirisci L, Tarter RE, Weatherbee S, Jamnik V, McGuire EJ, *et al*. Use of aggregate fitness indicators to predict transition into the National Hockey League. *J Strength Cond Res.* 2009; 23(6): 1828-32.
23. Maciejczyk M, Wiecek M, Szymura J, Szygula Z, Brown LE. Influence of increased body mass and body composition on cycling anaerobic power. *J Strength Cond Res.* 2001; 29(1): 58-65.
24. Kalinski M, Norkowski H, Kerner M, Tkaczuk W. Anaerobic power characteristics of elite athletes in national level team-sport games. *European Journal of Sport Science*. 2002; 2(3): 1-21.
25. Maud PJ, Shultz BB. Gender comparisons in anaerobic power and anaerobic capacity tests. *Br J Sports Med.* 1986; 20(2): 51-4.
26. Kim J, Cho HC, Jung HS, Yoon JD. Influence of performance level on anaerobic power and body composition in elite male judoists. *J Strength Cond Res.* 2005; 25(5): 1346-54.
27. Esbjornsson M, Sylven C, Holm I, Jansson E. Fast twitch fibres may predict anaerobic performance in both females and males. *Int J Sports Med.* 1993; 14(5): 257-63.
28. Kitagawa K, Suzuki M, Miyashita M. Anaerobic power output of young obese men: comparison with non-obese men and the role of excess fat. *Eur J Appl Physiol Occup Physiol.* 1980; 43(3): 229-34.
29. Gray DS, Bauer M. The relationship between body fat mass and fat-free mass. *J Am Coll Nutr.* 1991; 10(1): 63-8.
30. Sartorio A, Proietti M, Marinone PG, Agosti F, Adorni F, Lafortuna CL. Influence of gender, age and BMI on lower limb muscular power output in a large population of obese men and women. *Int J Obes Relat Metab Disord.* 2004; 28(1): 91-8.
31. Ode JJ, Pivarnik JM, Reeves MJ, Knous JL. Body mass index as a predictor of percent fat in college athletes and nonathletes. *Med Sci Sports Exerc.* 2007; 39(3): 403-9.
32. Witt KA, Bush EA. College athletes with an elevated body mass index often have a high upper arm muscle area, but not elevated triceps and subscapular skinfolds. *J Am Diet Assoc.* 2005; 105(4): 599-602.
33. Nikolaidis PT. Elevated body mass index and body fat percentage are associated with decreased physical fitness in soccer players aged 12-14 years. *Asian J Sports Med.* 2002; 3(3): 168-74.
34. Bovet P, Auguste R, Burdette H. Strong inverse association between physical fitness and overweight in adolescents: a large school-based survey. *Int J Behav Nutr Phys Act.* 2007; 4: 24.